Studies in Plant Science, 5

Plant Tissue Culture:
Theory and Practice, a Revised Edition

S.S. Bhojwani
M.K. Razdan

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Preface

Since the publication of this book, in 1983, several new and exciting developments have taken place in the field of Plant Tissue Culture, and it now forms a major component of what is popularly called Plant Biotechnology. Many of the important crop plants which were then regarded as recalcitrant are now amenable to regeneration from cultured protoplasts, cells, and calli, enabling subjection of these crops to improvement by biotechnological methods of cell manipulation. Embryogenic cultures can be established for most of the important crop plants, including many hardwood and softwood tree species.

During the last decade the emphasis of research in tissue culture has been on its industrial and agricultural applications. Chief among the proven applications of plant tissue culture are the routine use of androgenesis in plant breeding programmes (Chapter 7), development of new varieties through somaclonal and gametoclonal variant selection (Chapter 9), production of industrial compounds (Chapter 17), regeneration of transgenic plants from genetically manipulated cells (Chapter 15), clonal propagation of horticultural and forest species (Chapter 16), and conservation of germplasm of crop plants and endangered species (Chapter 18).

In the process of translating the laboratory protocols into commercial protocols several problems were identified and research was focused on finding solutions thereof. Until the early 1980s, for example, most of the contributions on somatic embryogenesis concerned the differentiation of structures that resembled embryos but when the protocols were critically examined for application to commercial plant propagation it was soon realized that the somatic embryos showed an extremely low degree of germination owing to their physiological and biochemical immaturity. This necessitated introduction of an additional stage of embryo maturation to ensure an acceptably high rate of conversion of somatic embryos into plantlets. Concurrently, mass production of somatic embryos in bioreactors has been studied and synthetic seed technology has been developed to facilitate their mechanized field planting. Fermentor technology has also been developed for large scale plant cell culture (Chapter 4) required in industrial production of secondary plant products.

These developments and the gratifying world-wide response the earlier edition of this book received, provided the impetus to update it under the earlier title. All the chapters in the first edition have been thoroughly revised without disturbing the original character. Two new chapters, one

on 'Production of Industrial Compounds' (Chapter 17) and another on 'Genetic Engineering' (Chapter 14), have been added. The chapter on 'Cytogenetic Studies' has been revised with emphasis on applied aspects and retitled as 'Variant Selection' (Chapter 9).

When the revision of the book was contemplated, I did not realize the magnitude of the task. The proliferation of literature has been such that each chapter or, in some instances, even a section of it can be and indeed has been developed as a book. The last decade has witnessed movement of many tissue culture scientists from public sector institutions to private commercial laboratories which are making notable contributions. However, due to this shift from the 'open research system' of universities and government institutes to the 'closely guarded research system' of industry, the scientific information often remains unknown until the process and/or the product are patented.

I hope that our earnest endeavour will have a greater reception by students, teachers and plant scientists interested in both theoretical and applied aspects of plant tissue culture.

I am indebted to my co-author, Dr M.K. Razdan, for his help and co-operation in completing the manuscript. I am highly obliged to Dr Arlette Reynaerte for valuable suggestions on the manuscript of Chapter 14. I am grateful to several of my colleagues and students, particularly Professor S.P. Bhatnagar, Dr W. Marubashi, Mr A.P. Raste, Dr P.K. Dantu, Himani Pande, Pradeep Kumar, Ashwani Kumar, Dennis Thomas, Deepali Saxena and Sushma Arora for their help in various ways. I thank Mr S.K. Das, Mr J.P. Narayan and Mr Manwar Singh for their constant cooperation in photography and preparation of the illustrations and the manuscript, respectively.

The task of completing this book could not have been accomplished without the patience and understanding of my wife, Shaku. I lovingly dedicate this book to her.

Sant Saran Bhojwani
Delhi, India
February 29, 1996

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ERRATA

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The lines below should read as follows.

Page 184 - line 10
buds centrifuged at 400 g for 4, 8 or 12 min or at 280 g for 5 or 10 min. All

Page 193 - line 5
A 12 h pulse treatment of pollen grains with 25 mg l^{-1} of colchicine, an

Page 425 - line 36
rice (Christou et al., 1991) and maize (Gordon Kamm et al., 1990), thus

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Chapter 1

Introductory History

One of the most important biological events in the life cycle of an organism is fertilization, which involves the fusion of two gametes of opposite sex or strain resulting in the formation of a zygote. From this single-celled zygote originates the entire multicellular and multiorganed body of a higher organism; may it be a flowering plant or a human body. In a flowering plant, for example, structures as morphologically and functionally diverse as underground roots, green photosynthesizing leaves, and beautiful flowers all arise from the single-celled zygote through millions of mitoses. The latter process is a type of cell division characterized by identical products. Theoretically, therefore, all the cells in a plant body, whether residing in the flowers, conducting tissues or root tips, should have received the same genetic material as originally present in the zygote. All this would then suggest that there must be some other factor(s) superimposed on the genetic characteristics of cells which bring about this vast variation expressed by the genetically identical cells. The process involved in the manifestation of these variations is called differentiation. The morphological differentiation is actually preceded by certain cellular and subcellular changes. A pertinent question that arises at this stage is: whether the cellular changes underlying differentiation of various types of cells are permanent and, consequently, irreversible, or whether it is merely a social feature in which a cell undergoes an adaptive change to suit the functional need of the organism in general and the organ in particular. The fact that during the normal life cycle of a plant a cell which has differentiated into a palisade cell dies as a palisade cell and an epidermal cell does not revert to meristemic state may suggest that the events leading to differentiation are of a permanent nature. However, the classic experiments of Vochting on polarity in cuttings, carried out in 1878, suggest otherwise. He observed that all cells along the stem length are capable of forming roots as well as shoots, but their destiny is decided by their relative position in the cutting. The best way to answer this question and understand more about the inter-relationship between different cells of an organ and different organs of an organism would, however, be to remove them from the influence of their neighbouring cells and tissues and grow them in isolation on nutrient media. To put it in the words of the great German botanist Gottlieb Haberlandt (1854–1945), now aptly regarded as the father of plant tissue culture, 'To

GOTTLIEB HABERLANDT
(1854-1945)

PHILIP R. WHITE
(1901-1968)

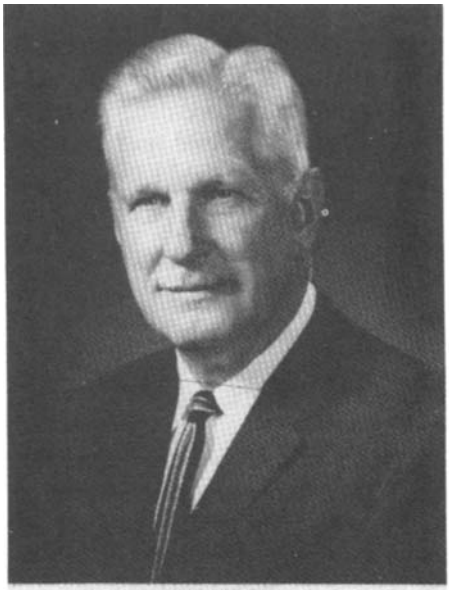
PANCHANAN MAHESHWARI
(1904-1966)

ROGER J. GAUTHERET
(Born 1910)

my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet the results of such culture experiments should give some interesting insight to the properties and potentialities which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within a multicellular whole organism are exposed'. Haberlandt was the first person to culture isolated, fully differentiated cells as early as 1898 and the above lines are cited from the English translation of his classic paper presented in 1902 in which he described the results of his pioneering experiments (Krikorian and Berquam, 1969).

For his experiments Haberlandt (1902) chose single isolated cells from leaves. He used tissue of *Lamium purpureum* and *Eichhornia crassipes*, the epidermis of *Ornithogalum* and epidermal hairs of *Pulmonaria mollissima*. He grew them on Knop's (1865) salt solution with sucrose, and observed obvious growth in the palisade cells. In the first place they remained alive for up to 1 month. They grew in size from an initial length/width of 50 μm/27 μm to up to 180 μm/62 μm, changed shape, thickening of cell walls occurred, and starch appeared in the chloroplasts which initially lacked it. However, none of the cells divided. Some of the reasons for this failure were that he was handling highly differentiated cells and the present-day growth hormones, necessary for inducing division in mature cells, were not available to him. Charles Darwin once remarked 'I am a firm believer that without speculation there is no good or original research'. Despite the failure to achieve his goal, Haberlandt made several predictions in his paper of 1902. With the passage of time most of these ideas were confirmed experimentally, proving Haberlandt's broad vision and foresight. It was unfortunate that Haberlandt did not test his postulates experimentally or else several discoveries could have been made much earlier. Instead, he devoted his time to 'sensory physiological investigations'.

It would be worthwhile mentioning here some of the postulates of Haberlandt (1902). Despite the fact that he could not demonstrate the ability of mature cells to divide, he was clear in his mind that in the intact plant body the growth of a cell simply stops after acquiring the features required to meet the need of the whole organism. To this effect he states: 'This happens not because the cells lose their potential capacity for further growth, but because a stimulus is released from the whole organism or from particular parts of it'. 'The isolated cell is capable then of resuming uninterrupted growth'. Haberlandt had also perceived the concept of growth hormones, which he called 'growth enzymes', and felt these are released from one type of cells and stimulate growth and devel-



FOLKE SKOOG
(Born 1908)

GEORGES MOREL
(1916-1973)

HERBERT E. STREET
(1913-1977)

JEAN P. NITSCH
(1921-1971)

opment in other cells. Based on the observations of Winkler (1901) that pollen tubes stimulate growth in ovules and ovary, Haberlandt suggested '... it would be worthwhile to culture together in hanging drops vegetative cells and pollen tubes; perhaps the latter would induce the former to divide'. He continues, 'One could also add to the nutrient solutions used an extract from vegetative apices or else culture the cells from vegetative apices. One might also consider utilization of embryo sac fluids'. Haberlandt finally states 'Without permitting myself to pose further questions, I believe, in conclusion, that I am not making too bold a prediction if I point to the possibility that, in this way, one should successfully cultivate artificial embryos from vegetative cells. In any case, the technique of cultivating isolated plant cells in nutrient solutions permits the investigation of important problems from a new experimental approach.'

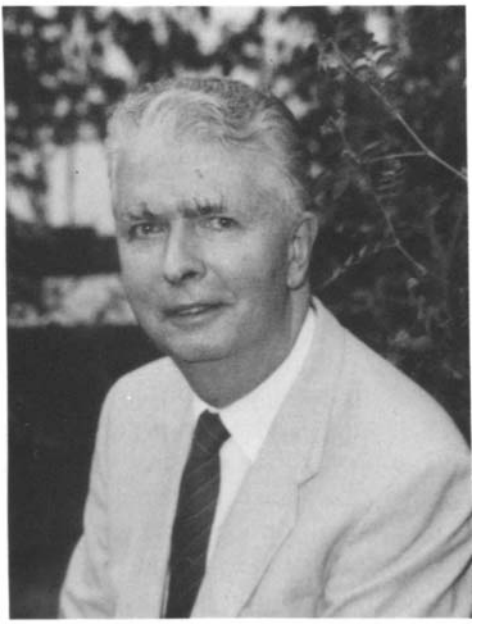
From the time Haberlandt presented his paper in 1902 until about 1934 hardly any progress was made in the field of plant tissue culture as conceived by Haberlandt. In 1904, however, Hannig had initiated a new line of investigation which later developed into an important applied area of in vitro techniques. Hannig excised nearly mature embryos of some crucifers (*Raphanus sativus*, *R. landra*, *R. candatus*, *Cochlearia danica*) and successfully grew them to maturity on mineral salts and sugar solution. He also tested, although unsuccessfully, the embryo sac fluid to support the growth of excised embryos. Proving one of the predictions of Haberlandt true, in 1941 Van Overbeek and co-workers demonstrated for the first time the stimulatory effect of coconut milk (embryo sac fluid) on embryo development and callus formation in *Datura* (Van Overbeek et al., 1941). Actually, this work proved a turning point in the field of embryo culture, for it enabled the culture of young embryos which failed to grow on a mixture of mineral salts, vitamins, amino acids and sugar. Subsequent detailed work by Raghavan and Torrey (1963), Norstog (1965) and others led to the development of synthetic media for the culture of younger embryos (see Raghavan, 1976a). However, until recently only post-globular embryos could be cultured ex-ovulo. Younger embryos either did not survive or exhibited callusing. Recently, Liu et al. (1993a) described a double layer culture system and a complex nutrient medium which supported embryogenic development of excised early globular (35 μm) embryos of *Brassica juncea*. Even more spectacular is the development of germinable embryos from naked 'zygote' formed by in vitro fusion of male and female gametes (Kranz and Lorz, 1993).

Fairly early in the history of embryo culture, Laibach (1925, 1929) demonstrated the practical application of embryo culture in the field of plant breeding. He isolated embryos from non-viable seeds of the cross *Linum perenne* × *L. austriacum* and reared them to maturity on a nutri-

FREDERICK C. STEWARD
(1904-1993)

GEORGE MELCHERS
(BORN 1906)

ARMIN C. BRAUN
(BORN 1911)



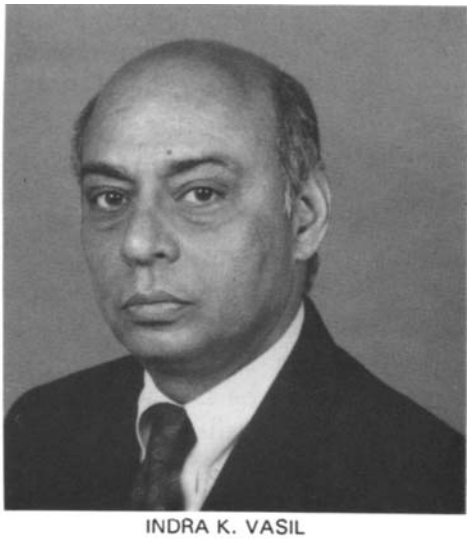
EDWARD C. COCKING
(BORN 1931)

ent medium. He also provided special impetus for further work in the area by stating, 'In any case, I deem it advisable to be cautious in declaring combination between higher plants to be inviable after fertilization has taken place and after they have begun to develop. Experiments to bring the aborted seed to development should always be undertaken if it is desirable for theoretical or practical reasons. The experiments will not always be successful, but many a result might be obtained by studying the conditions of ripeness of the embryo and by finding out the right time for the preparing out of the seed.' It should be mentioned here that to date several hybrids have been reared through embryo culture which would otherwise have failed due to embryo abortion (see Raghavan, 1976a).

As mentioned earlier, for a considerable time after Haberlandt's classic paper, work continued on organized structures. Pioneering work on root culture appeared during this period. In 1922, working independently, Robbins (USA) and Kotte (a student of Haberlandt in Germany) reported some success with growing isolated root tips. Further work by Robbins and Maneval (1924) enabled them to improve root growth, but the first successful report of continuously growing cultures of tomato root tips was made by White in 1934. Initially White used a medium containing inorganic salts, yeast extract and sucrose, but later yeast extract was replaced by three B-vitamins, namely pyridoxine, thiamine and nicotinic acid (White, 1937). On this synthetic medium, which has proved to be one of the basic media for a variety of cell and tissue cultures, White maintained some of the root cultures initiated in 1934 until shortly before his death in 1968. During 1939–1950 extensive work on root culture was undertaken by Street and his students to understand the role of vitamins in plant growth and shoot–root relationship.

The two important discoveries made in the mid-1930s which gave a big push to the development of plant tissue culture technique were: (a) identification of auxin as a natural growth regulator, and (b) recognition of the importance of B-vitamins in plant growth. In 1934, Gautheret had cultured cambium cells of some tree species (*Salix capraea*, *Populus nigra*) on Knop's solution containing glucose and cysteine hydrochloride and recorded that they proliferated for a few months. The addition of B-vitamins and IAA considerably enhanced the growth of *Salix* cambium. However, the first continuously growing tissue cultures from carrot root cambium were established by Gautheret in 1939. In the same year White (1939a) reported the establishment of similar cultures from tumour tissue of the hybrid *Nicotiana glauca* × *N. langsdorffii*. Gautheret and White, together with Nobecourt, who had independently reported the establishment of continuously growing cultures of carrot in the

TOSHIO MURASHIGE
(BORN 1930)



INDRA K. VASIL
(BORN 1932)

same year, are credited for laying the foundation for further work in the field of plant tissue culture. The methods and media now used are, in principle, modifications of those established by the three pioneers in 1939. Although continuously growing cultures could be established in 1939, the tissues used by all the three workers included meristematic cells.

The induction of divisions in isolated mature and differentiated cells had to wait the discovery of another growth regulator. Skoog (1944) and Skoog and Tsui (1951) had demonstrated that in tobacco pith tissue cultures the addition of adenine and high levels of phosphate increased callus growth and bud formation even in the presence of IAA which otherwise acted as bud-inhibitor. However, the division of cells occurred only if vascular tissue was present; pith cells alone did not show any division (Jablonski and Skoog, 1954). Actually, the importance of the association of vascular tissue for inducing cell divisions in mature parenchyma cells of potato tuber was demonstrated by Haberlandt as early as 1913. In their search to replace the need for vascular tissue, Jablonski and Skoog tested several plant extracts by either adding them to the nutrient medium or injecting them into the tissue. One of the substances most effective in this respect was yeast extract (YE), which had enabled White (1934) to establish the first continuously growing root cultures. However, for cell division the active component of YE was not B-vitamins, but

something with properties common to purine. Based on this observation, when DNA was tested in place of YE it proved to be an enormously richer source of activity than any other substance tested before for cell division in pith tissue. Initially the activity was noticed in old samples of DNA, but it could also be produced by autoclaving weakly acid slurries of freshly isolated DNA (Miller et al., 1955b). Miller et al. (1955a) separated the first known cytokinin from the DNA of herring sperm and named it kinetin. At present, many synthetic as well as natural compounds with kinetin-like activity are known. The availability of these substances, collectively called cytokinins, has made it possible to induce divisions in cells of highly mature and differentiated tissue, such as mesophyll and endosperm from dried seeds.

At this stage, the dream of Haberlandt was realized only partially, for he foresaw the possibility of cultivating isolated single cells. Only small pieces of tissue could be grown in cultures. Further progress in this respect was made by Muir (1953). He demonstrated that by transferring callus tissues of *Tagetes erecta* and *Nicotiana tabacum* to liquid medium and agitating the cultures on a shaking machine it was possible to break the tissue into single cells and small cell aggregates. Muir et al. (1954) also succeeded in mechanically picking single cells from these shake cultures (suspension cultures) as well as soft callus tissues, and making them divide by placing them individually on separate filter papers resting on the top of a well-established callus culture. Apparently the callus tissue, which was separated from the cell only by thin filter paper, supplied the necessary factor(s) for cell division. This nurse culture method was very similar to the untested idea of Haberlandt wherein he suggested growing single cells along with pollen tubes so that the former may receive cell division stimulus from the latter. In 1960 Jones et al. designed a microculture method for growing single cells in hanging drops in a conditioned medium (medium in which tissue has been grown for some time). The advantage of this technique was that it allowed continuous observation of the cultured cells. Using this technique but replacing the conditioned medium by a fresh medium, enriched with coconut milk, Vasil and Hildebrandt (1965) raised whole plants starting from single cells of tobacco. An important biological technique of cloning large number of single cells of higher plants was, however, developed in 1960 by Bergmann. He filtered the suspension cultures of *Nicotiana tabacum* and *Phaseolus vulgaris* and obtained a population containing about 90% free cells. These were incorporated into a 1 mm layer of solidified medium containing 0.6% agar. In this experiment some of the single cells divided and formed visible colonies. This technique is now widely used for cloning cells, and in protoplast culture experiments.

The free cells thus far cultured successfully were derived from actively growing tissues in cultures. It was indeed the work of Kohlenbach in 1966 that came closest to Haberlandt's experimental material and objectives. Kohlenbach successfully cultured mature mesophyll cells from *Macleaya cordata*. The tissue obtained from these cells subsequently differentiated somatic embryos.

In 1957, Skoog and Miller put forth the concept of hormonal control of organ formation (Fig. 5.6). In this classic paper, they showed that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin-cytokinin ratio, and that organ differentiation could be regulated by changing the relative concentrations of the two substances in the medium; high concentrations of auxin promoted rooting, whereas high levels of cytokinin supported shoot formation. At equal concentrations of auxin and cytokinin the tissue tended to grow in an unorganized fashion. This concept of hormonal regulation of organogenesis is now applicable to a large number of plant species. However, the exogenous requirement of growth regulators for a particular type of morphogenesis varies, depending on the endogenous levels of these substances in the tissue in question.

The differentiation of whole plants in tissue cultures may occur via shoot and root differentiation or, alternatively, the cells may undergo embryogenic development to give rise to bipolar embryos, referred to as 'somatic embryos' in this book to distinguish them from zygotic embryos. The first reports of somatic embryo formation from carrot tissue appeared in 1958–1959 by Reinert (Germany) and Steward (USA). To date, numerous plant species have been reported to form somatic embryos. In some plants, like carrot and buttercup, embryos can be obtained from virtually any part of the plant body.

Until the mid-1970s hormonal manipulation in the culture medium remained the main approach to achieve plant regeneration from cultured cells and it proved very successful with many species. However, some very important crop plants, such as cereals and legumes, did not respond favourably to this strategy and were, therefore, declared recalcitrant (Bhojwani et al., 1977a). In 1972, Saunders and Bingham reported that different cultivars of alfalfa varied considerably in their regeneration potential under a culture regime. More detailed studies by Bingham and his associates (Bingham et al., 1975; Reisch and Bingham, 1980) demonstrated that regeneration in tissue cultures is a genetically controlled phenomenon. Genotypic variation has been since observed in several plant species; it occurs between varieties and, in outbreeding crops, within varieties. The success in obtaining regeneration in tissue cultures of forage legumes has been mainly due to a shift in the emphasis from

medium selection to genotype selection. Similar success with cereals became possible only after the physiological state of the explant was recognized as another important factor affecting regeneration. In this group of plants the regeneration potential is largely restricted to immature embryos (Green and Phillips, 1975; Vasil and Vasil, 1980). Vasil and his associates, at the University of Florida, demonstrated that embryogenic cultures of most cereals can be established using immature embryos as the explant, and such cultures are suitable for protoplast isolation and culture as well as genetic manipulation of these plants (Vasil and Vasil, 1986; Vasil, 1988; Vasil et al., 1992). Immature embryos have also proved to be an ideal explant to raise embryogenic cultures of numerous other herbaceous and woody species, including Gymnosperms.

Establishment of suspension cultures of plant cells in liquid medium, similar to microbes, in the mid-1950s prompted scientists to apply this system for the production of natural plant products as an alternative to whole plant. The first attempt for the industrial production of secondary metabolites in vitro was made during 1950–1960 by the Pfizer Company (see Gautheret, 1985) and the first patent was obtained in 1956 by Routien and Nickell. However, not much progress in this area was made for many years. Apparently, the industrial production of secondary metabolites required large scale culture of cells. In 1959, Tulecke and Nickell published the first report of plant cell culture in a 134 l reactor. Noguchi et al. (1977) used 20 000 l reactor for the culture of tobacco cells. Since plant cells are different from microbes in many respects the reactors traditionally used in microbiology had to be modified to suit plant cell culture. Several different kinds of bioreactors have been designed for large scale cultivation of plant cells (see Chapters 4 and 17). The technology for mass culture of plant cells is now available but slow growth of plant cells, genetic instability of cultured cells, intracellular accumulation of secondary products and organ-specific synthesis of secondary products are some of the problems making tissue culture production of industrial compounds uneconomical. Despite these problems in several cases cell cultures have been shown to produce certain metabolites in quantities equal to (first reported by Kaul and Staba, 1967) or many fold greater than (first reported by Zenk, 1978) the parent plant. In 1979, Brodelius et al. developed the technique of immobilization of plant cells so that the biomass could be utilized for longer periods, besides its other advantages. Culture of 'hairy roots', produced by transformation with *Agrobacterium rhizogenes*, has been shown to be a more efficient system than cell cultures for the production of compounds which are normally synthesized in roots of intact plants. The first tissue culture product to be commercialized, by Mitsui Petrochemical Co. of Japan, is Shikonin from

cell cultures of *Lithospermum erythrorhizon* (Curtin, 1983). In 1988, another Japanese company (Nitto Denko) started marketing ginseng cell mass produced in culture (Misawa, 1994).

Differentiation of plants from callus cultures has been suggested as a potential method for rapid propagation of selected plant species because hundreds and thousands of plants can be raised from a small amount of tissue and in a continuous process. But this method suffers from one serious drawback that cells in long-term cultures are genetically unstable. A more important technique, which was later to become a viable horticultural practice, was developed by Ball in 1946. He successfully raised transplantable whole plants of *Lupinus* and *Tropaeolum* by culturing their shoot tips with a couple of leaf primordia. However, the demonstration of the practical usefulness of this important technique must be credited to Morel who, with Martin (Morel and Martin, 1952), for the first time recovered virus-free *Dahlia* plants from infected individuals by excising and culturing their shoot tips in vitro. The basis of this approach is that even in a virus-infected plant the cells of the shoot tip are either free of virus or carry a negligible amount of the pathogen. This technique of shoot tip culture, alone or in combination with chemotherapy or thermotherapy, has since then been widely used with a variety of plant species of horticultural and agronomic importance and has become a standard practice to raise virus-free plants from infected stocks (see Chapter 15).

While applying the technique of shoot-tip culture for raising virus-free individuals of an orchid, Morel (1960) also realized the potential of this method for the rapid propagation of these plants. The technique allowed the production of an estimated 4 million genetically identical plants from a single bud in a period of 1 year. Until this time orchid propagation was done by seeds. A serious problem inherent in this method is the appearance of a great variation in the progeny. Seeing a tremendous advantage in the technique, the commercial orchidologists soon adopted this novel technique as a standard method for propagation. This contribution of Morel not only revolutionized the orchid industry, but also gave impetus to the utilization of shoot-bud culture for rapid cloning of other plant species.

Murashige was instrumental in giving the techniques of in vitro culture a status of a viable practical approach to propagation of horticultural species. He worked extensively for the popularization of the technique by developing standard methods for in vitro propagation of several species ranging from ferns, to foliage, flower and fruit plants. Indeed, Murashige's name became intimately associated with the technique. Incidentally, the principle of the technique being used for in vitro propagation of most flowering plants is very different from that used for orchids.

It is based on another important finding made in 1958 by Wickson and Thimann. They showed that the growth of axillary buds, which remain dormant in the presence of terminal buds, can be initiated by the exogenous application of cytokinins. The implication of this is that one could induce the release of lateral buds on a growing shoot with an intact terminal bud by growing the shoot in a medium containing cytokinin. This would release buds from apical dominance not only on the initial stem segment, but also those on the lateral branches developed from it in cultures, giving rise to a bushy witch's broom-like structure with numerous shoots. Individual branches from this cluster can be made to repeat the process of shoot multiplication to build up innumerable shoots in a rather short period. Routinely, a portion of the total shoots may be rooted in another medium to get full plantlets ready for transfer to soil through careful handling.

Axillary bud proliferation is widely practised for in vitro propagation of plants because it ensures maximum genetic uniformity of the resulting plants but from economic considerations this method is not very attractive as it is slow and labour intensive. Therefore, attention is being given to developing somatic embryogenic systems for mass propagation of plants as it offers the possibility of rapid multiplication in automated bioreactors, with low inputs. Since the first attempt of Backs-Husemann and Reinert (1970) to scale-up somatic embryogenesis in carrot using a 20 l carboy, different types of bioreactors have been tested (see Chapter 6). For poinsettia embryo production, Preil (1991) used a round bottom 2 l bioreactor in which stirring was achieved by vibrating plates and bubble-free O_2 was supplied through a silicon tubing which was inserted as a spiral of 140 cm total tube length. For mechanical planting of somatic embryos in the field the concept of synthetic seeds has been proposed. Currently, two types of synthetic seeds, viz. desiccated and hydrated, are being developed in which somatic embryos are individually encapsulated in suitable compounds (see Chapter 6).

Regeneration of plants from carrot cells frozen at the temperature (–196°C) of liquid nitrogen was first reported by Nag and Street in 1973. Seibert (1976) demonstrated that even shoot tips of carnation survived exposure to the super-low temperature of liquid nitrogen. This and subsequent success with freeze preservation of cells, shoot tips and embryos gave birth to a new applied area of tissue culture, called germplasm storage (Chapter 18). Cultured shoots/plantlets can also be stored at 4°C for 1–3 years. These methods are being applied at several laboratories to establish in vitro repository of valuable germplasm.

The spontaneous occurrence of variation in tissue cultures with regard to the ploidy, morphology, pigmentation and growth rates had been ob-

served for quite some time. Changes to auxin habituation was reported by Gautheret (1955). However, for long these variations were ignored as mere abnormalities. The first formal report of morphological variation induced in tissue cultures was published from the Hawaiin Sugar Planter's Association Experimental Station. Heinz and Mee (1971) reported variation in sugarcane hybrids regenerated from cell cultures. The agronomic importance of such variability was immediately recognized and the regenerants were screened for useful variation. During the next few years, *Saccharum* clones with resistance to various fungal and viral diseases as well as variation in yield, growth habit and sugar content were isolated (Krishnamurthi and Tlaskal, 1974; Heinz et al., 1977). In the following 5–6 years useful variants of crops, such as geranium (Skirvin and Janick, 1976a,b) and potato (Shepard et al., 1980), were obtained from tissue culture derived plants. However, it was the article by Larkin and Scowcroft (1981) which drew the attention of tissue culturists and plant breeders to tissue culture as a novel source of useful genetic variation. They proposed the term 'somaclonal variation' for the variation detected in plants regenerated from any form of culture and termed the regenerated plants as somaclones. Evans et al. (1984a) introduced the term 'gametoclones' for the plants regenerated from gametic cells. During the past decade scientists have examined their tissue cultures and the plants regenerated from them more critically and confirmed that tissue culture can serve as a novel source of variation suitable for crop improvement. Several somaclones and gametoclones have already been released as new improved cultivars (see Chapter 9).

By the early 1960s, methods of in vitro culture were reasonably well developed and the emphasis was shifting towards applied aspects of the technique. Around this time the Botany School at the University of Delhi, led by P. Maheshwari, became actively engaged with in vitro culture of reproductive organs of flowering plants (see Maheshwari and Rangaswamy, 1963). Prompted by her success with 'intra-ovarian pollination' (Kanta, 1960), Kanta developed the technique of 'test-tube fertilization' (Kanta et al., 1962). In essence, it involves culturing excised ovules and pollen grains together on the same medium; the pollen germinates and fertilizes the ovules. In theory, this technique could be applied to overcome any sexual incompatibility for which reaction occurs in the stigma and/or style. Using this approach, Zenkteler and co-workers (Zenkteler, 1967, 1970; Zenkteler et al., 1975) developed interspecific (*Melandrium album* × *M. rubrum*) and intergeneric (*M. album* × *Silene schafta*) hybrids unknown in nature. Similarly, self-incompatibility in *Petunia axillaris* could be overcome following this method. Therefore, for almost a decade this simple technique to overcome sexual incompatibility barriers

remained overshadowed by more sophisticated techniques of somatic hybridization and genetic engineering which were gaining popularity with the scientists during this period. A renewed interest in the technique of in vitro pollination occurred in the mid-1980s, when a number of laboratories used this technique to produced some rare hybrids (see Chapter 10). A major breakthrough in this area was made at the beginning of 1990s when Kranz et al. (1990) reported electrofusion of isolated male and female gametes of maize and, 3 years later (Kranz and Lorz, 1993), plant regeneration from the fusion product. The naked 'zygote' formed embryo and eventually fertile plants (see Chapter 10). This is the first and so far the only demonstration of in vitro fertilization in higher plants.

The role of haploids in breeding and genetics of higher plants had been emphasized for a considerable time but the restricted availability of such individuals, with the gametic number of chromosomes (half of that present in body cells), did not allow their full exploitation. In 1966, Guha and Maheshwari demonstrated the possibility of raising large numbers of androgenic haploid plantlets from pollen grains of *Datura innoxia* by culturing immature anthers. Later work by Bourgin and Nitsch (1967) confirmed the totipotency of pollen grains. They raised full haploid plants of tobacco. By the use of this technique, several promising new varieties of tobacco, rice and wheat have been introduced.

In 1970, Kameya and Hinata reported callus formation in isolated pollen cultures of *Brassica* sp. A couple of years later C. Nitsch and her associates, at the CNRS, France, succeeded in raising haploid plants from isolated microspore cultures of *Nicotiana* and *Datura* (Nitsch and Norreel, 1973; Nitsch, 1974). Initially, a nurse tissue was used to culture isolated microspores (Pelletier and Durran, 1972; Sharp et al., 1972) but soon it was possible to culture them on synthetic media. With the refinement of culture techniques and media it has become possible to raise androgenic plants by isolated microspore culture on synthetic media for a large number of species. So far pollen plants have been obtained by anther/pollen culture for over 134 species and the techniques are being used in plant breeding programmes (see Chapter 7). Isolated microspore culture of *B. napus* has emerged as a model system to study cellular basis of androgenesis (see Chapter 7).

Although the number of haploid cells in an ovule are very limited, it is possible to produce parthenogenetic or apogamous haploids by unfertilized ovary/ovule culture. It was first reported in barley by San Noeum (1976). To date gynogenetic haploids have been reported for about 19 species (see Chapter 7).

Although the isolation of protoplasts (Klercker, 1892) and their fusion (Kuster, 1909) were reported almost 100 years ago it was to the credit of

Cocking (1960) whose work introduced the concept of enzymatic isolation of plant protoplasts. He had used culture filtrate of the fungus *Myrothecium verrucaria*, but in 1968 cellulase and macerozyme became commercially available and isolation of large quantities of viable protoplasts by enzymatic degradation of cell wall soon became a routine technique (see Chapter 12). By 1970 it was demonstrated that isolated protoplasts are capable of regenerating a new wall (Pojnar et al., 1967) and the reconstituted cell is capable of sustained divisions (Kao et al., 1970a; Nagata and Takebe, 1970). In 1971 the totipotency of isolated protoplasts was demonstrated (Nagata and Takebe, 1971; Takebe et al., 1971). At almost the same time, Cocking's group at the University of Nottingham achieved fusion of isolated protoplasts using $NaNO_3$ (Power et al., 1970). These two observations, totipotency of protoplasts and induced fusion of protoplasts, gave birth to a new field of plant tissue culture, viz. somatic hybridization. This was one of the most active areas of research from 1970 to the mid-1980s because of its potential application in crop improvement by genetic manipulation of somatic cells. During this period, more efficient methods of protoplast fusion, using as high pH–high Ca^{+2} (Keller and Melchers, 1973), polyethylene glycol (Wallin et al., 1974; Kao et al., 1974) and electrofusion (Zimmermann and Vienka, 1982), and improved culture methods and media were developed. Also, regeneration of plants from protoplasts of a large number of species was achieved.

The first somatic hybrids between *Nicotiana glauca* and *N. langsdorffii* was produced in 1972 by Carlson and his co-workers. However, these two species could be crossed sexually. In 1978, Melchers et al. produced an intergeneric hybrid between sexually incompatible parents, potato and tomato, but the somatic hybrid was sexually sterile. It was soon realized that although somatic hybrids could be produced between highly unrelated parents but such wide hybrids would not be agronomically useful. The technique of protoplast fusion is now being used to produce asymmetric hybrids, wherein only a part of the nuclear genome of the donor parent is transferred to the recipient parent. A novel application of protoplast fusion is in the production of cybrids with novel nuclear-cytoplasmic combinations. This technique has already been utilized to transfer male sterility inter- and intra-specifically (see Chapter 13).

The property of isolated protoplasts to take-up organelles and macromolecules prompted several scientists to employ this system for genetic transformation of plants by feeding them with purified DNA but it did not meet with much success (Bhojwani and Razdan, 1983). The field of genetic engineering, which refers to insertion of selected gene(s) for genetic modification of plants, became reality with the development of *Agrobacterium tumefaciens* based vectors. Smith and Townsend (1907)

had shown that this gram negative soil bacteria causes crown gall disease in some plants. Based on his observation that crown gall tissue displayed the tumorigenic character for autonomous growth on salt-sugar medium, even in the absence of the bacterium, Braun (1947) suggested that probably during infection the bacterium introduces a tumour-inducing principle in the plant genome. Transfer of bacterial genetic material into the crown gall cells was also proposed by Morel (1971) based on his observation that the crown gall cells acquired the new trait for the synthesis of opines, some novel amino acids. The elusive DNA was identified as a large plasmid (Ti-plasmid) found only in a virulent strain of the *A. tumefaciens* (Zaenen et al., 1974). The utility of the bacteria as a gene transfer system in plants was first recognized when Chilton et al. (1977) demonstrated that the crown galls were actually produced as a result of the transfer and integration of genes from the bacteria into the genome of plants. Barton et al. (1983) demonstrated that heterologous DNA inserted into the T-DNA of Ti-plasmid could be transferred to plants along with the existing T-DNA genes. With refinement of the *A. tumefaciens* system in the early 1980s, research to produce genetically engineered plant varieties blossomed. Efficient plant transformation vectors were constructed by removing the phytohormone biosynthesis genes from the T-DNA region and thereby eliminating the ability of the bacteria to induce aberrant cell proliferation (Fraley et al., 1985). The first transgenic tobacco plants expressing engineered foreign genes were produced with the aid of *A. tumefaciens* (Horsch et al., 1984). Since then derivatives of this bacteria have proved to be an efficient and highly versatile vehicle for the introduction of genes into plants and plant cells. Most of the transgenic plants produced to date were created through the use of this system. However, this transformation system is species-specific; it does not work with most monocotyledons which include the major cereals. Therefore, during the last decade the arsenal of the transformation system has been expanded to include free DNA delivery techniques, such as electroporation, particle gun and microinjection, which are not species limited and can be used with cells, tissues and organized structures. Of these, particle gun, also called microprojectile bombardment or biolistic, is the most promising DNA delivery system for plants. In 1986, the first plants were genetically engineered for a useful agronomic trait (Abel et al., 1986). During the last decade, the list of genetically improved varieties produced by this molecular breeding method has considerably enlarged (see Chapter 14).

These, in brief, are some of the milestones in the development of the techniques of plant tissue culture. Like any other area of science, it started as an academic exercise to answer some questions related to

plant growth and development, but proved to be of immense practical value, as an aid to plant propagation, raising and maintenance of high health-status plants, germplasm storage, and a valuable adjunct to the conventional methods of plant improvement.

Chapter 2

Laboratory Requirements And General Techniques

2.1. INTRODUCTION

The size of a tissue culture set-up and the extent to which it is equipped are governed by the nature of the project undertaken and the funds available. However, a standard tissue-culture laboratory should provide facilities for: (a) washing and storage of glassware, plasticware and other labwares, (b) preparation, sterilization and storage of nutrient media, (c) aseptic manipulation of plant material, (d) maintenance of cultures under controlled conditions of temperature, light and, if possible, humidity, (e) observation of cultures, and (f) acclimatization of in vitro developed plants. For research work at least two separate laboratories or rooms should be available; one for glassware washing and storage, and media preparation (media room), and a second (growth/culture room) to store cultures. The culture room should contain a culture observation table provided with binoculars and an adequate light source. Depending on the local conditions, the sterile transfer cabinets may be housed in the culture room, in a quiet corner of an ordinary research laboratory, or a specially designed transfer room. A separate balance room may be shared with other laboratories. For a commercial set-up, a more elaborate set-up is required.

For other reviews on the subject, see De Fossard (1976), Biondi and Thorpe (1981), Bridgen and Bartok (1987), Pierik (1987), Torres (1989) and Mageau (1991).

2.2. REQUIREMENTS

2.2.1. Structures and utilities

Very often, a research or commercial laboratory is required to be set up in already existing structures; few can construct facilities from the ground up. In either case certain basic guidelines should be followed. If a new laboratory is being constructed, its location should preferably be away from the city or otherwise adequate precautions should be taken to

protect the facility from heavy pollution and vehicular vibrations. Care should also be taken to locate it away from fields where combines or threshers are used in order to cut down contamination spurts during the harvest season. Preferably, the facility should be protected from any onslaught of heavy winds and rain which are carriers of spores, mites and thrips. The growth room and the transfer room should be adequately insulated to conserve energy. This has been achieved in some cases by trapping air between a double wall construction. During a hot season, advantage could be had by venting the air between the two walls.

A tissue culture facility requires large quantities of good quality water and provision for waste water disposal. This aspect requires special consideration where public water and sewer facilities are not available. Disposal of any waste is also governed by local municipal codes for health and the environment.

A generator back up should be provided, at least to the transfer room, growth room and other essential equipment to prevent shut-down of transfer hoods during the operation and an abrupt change in temperature in the growth room due to power failures, which could happen even where a reliable source of electricity is available.

The organization for a commercial tissue culture set-up has been described, with diagrams, by several authors (Torres, 1989; Mageau, 1991). These should be treated as guidelines because the size and design of a facility would be governed by the shape and size of the land available and the proposed capacity of the company.

A single level structure, providing easy access to various work areas, is preferable to facilitate the frequent movement of materials between areas. The layout of the rooms, their pass-through windows, doors and hallways must allow a work flow pattern that maintains maximum cleanliness and promotes minimal backtracking. A clearcut demarcation of the unclean (washing room, medium preparation room, autoclaving room, general store, offices) and clean area (transfer room(s) and growth room(s)) should be made. Entry into the clean area should be restricted and generally through a passage where the workers must take-off shoes, wash hands and feet, change outer clothes, and wear headgear and slippers provided inside. Commercial laboratories should, as a rule, maintain a positive air pressure, if not in the whole building, at least in the clean area. These precautions are mandatory to deter the introduction of microorganisms into the culture vessels. Movement of material in (sterilized medium, instruments, water, etc.) and out (glassware and other things for washing and sterilization, tissue culture produced plants for hardening, etc.) of the clean areas through double door hatches should help in maintaining higher asepsis in the clean area.

2.2.2. Media room

The washing area in the media room should be provided with brushes of various sizes and shapes, a washing machine (if possible), a large sink (preferably lead-lined to resist acids and alkalis) and running hot and cold water. It should also have steel or plastic buckets to soak the labware to be washed, ovens or a hot-air cabinet to dry the washed labware and a dust-proof cupboard to store them. When the preparation of the medium and washing of the labware are done in the same room, as in many research laboratories, a temporary partition can be erected between the two areas to guard against the danger of soap solution splashing into the medium and any other interference in the two activities. If this is not possible, the washing time should be so arranged that it does not overlap with media preparation. An industrial dishwasher may be useful for a commercial set-up.

A good supply of water is a must for media preparation and final washing of glassware. Since tap water cannot be used for preparing medium, provision must be made to purify water. De-ionized water may be used for teaching laboratories but for research and commercial purposes, water distillation apparatus, a reverse osmosis unit or milli-Q water purification systems need to be installed. For a research laboratory, a glass distillation unit with a handling capacity of 1.5–2 l h^{-1} of water should be sufficient. For commercial houses, a Milli-Q purification system (Millipore Co., USA), which can provide 90 l h^{-1} of purified water, free of organic impurities, ionic contaminants, colloids, pyrogens, and traces of particles and micro-organism, may be used. Proper storage tanks should be installed to store purified water. For further details on water purification and storage refer to Gabler et al. (1983) and Callaghan (1988).

The usual facilities required for the preparation of culture medium include: (a) benches at a height suitable to work while standing, (b) a deep freeze for storing the stock solutions, enzyme solutions, coconut milk, etc., (c) a refrigerator to store various chemicals, plant materials, short-term storage of stock solutions, etc. (d) plastic carboys for storing distilled water, (e) weighing balance(s), (f) a hot plate-cum-magnetic stirrer for dissolving chemicals, (g) a pH meter, (h) an aspirator or vacuum pump to facilitate filter-sterilization, (i) a steamer for melting agar, and (j) an autoclave or a domestic pressure cooker for media sterilization. Of these items, a refrigerator and deep freeze may be kept in a corridor or another laboratory close to the media room. Use of weighing balances in the media room should be avoided. Alternatively, a small weighing chamber may be provided in a comparatively dry corner of the media room.

2.2.3. Culture vessels

Different types of vessels have been used to culture plant materials. While in some cases the choice of culture vials is dictated by the nature of the experiment, in others it has been guided mainly by the convenience and preference of the worker. For standard tissue and organ culture work, glass test tubes have been widely employed. Wide-mouth glass bottles of different sizes and sometimes even milk bottles have been used, especially for micropropagation. In tissue culture work only borosilicate or Pyrex glassware should be used. Soda glass may be toxic to some tissues, especially with repeated use (De Fossard, 1976).

In many laboratories the glass culture vials and other labware required for media preparation have been largely replaced by suitable plasticware. Some of the plastics are autoclavable. A wide range of presterilized, disposable culture vials (made of clear plastic), especially designed for protoplast, cell, tissue and organ culture work are now available in the market under different brands. These are becoming increasingly popular with those who can afford them.

Disposable plastic culture vials (petri-dishes, jars, bottles, various cell culture plates) and screw-cap glass bottles are supplied with suitable closures. For culture tubes and flasks, traditionally cotton plugs, sometimes wrapped in cheese-cloth, have been used. However, if the use of such stoppers is found time consuming and inconvenient, a wide choice of alternative closures exists. A number of plastic (polypropylene) and metallic (aluminium and stainless steel) cap closures are available. Transparent, autoclavable, polypropylene caps with a membrane built into the top, produced by KimKaps (Kimble, Division of Ownes, IL), are claimed to be very effective in preventing moisture loss from tubes. Local availability and cost influence the selection of a culture tube closure. However, it is important to ensure that the closure does not inhibit the growth of the cultured plant materials.

With a better understanding of the role a culture vessel plays in the growth and developmental behaviour of plant tissues enclosed in them has resulted in the development of culture vessels made of different synthetic materials. It is possible to buy vessels made of polypropylene which transmits about 65% light and those made of polycarbonate which transmits almost 100% light. Gas permeable fluorocarbonate vessels have been used in experiments with plant materials sensitive to gaseous build up within the culture vials (Kozai, 1991a). Osmotek Ltd., Israel, has introduced repeatedly autoclavable, polypropylene 'liferafts', provided with interfacial membrane and floats to culture plant materials in liquid medium without submerging them. The membrane is treated with

a surfactant to make it hydrophilic. The surfactant is removed during cleaning, and must be reapplied prior to the next use. These rafts are available in different sizes to fit culture tubes, magenta boxes and round jars. Osmotek Ltd. is also producing vented polypropylene lids which ensures better gas exchange in plant tissue cultures, thereby reducing the hyperhydration problem. The vent is covered with a membrane with 0.3 μm pores.

2.2.4. Growth room

The room for incubating cultures is maintained at a controlled temperature. Usually air-conditioners and heaters, attached to a temperature controller, are used to maintain the temperature around 25 ± 2°C. For higher or lower temperature treatments, special incubators with built-in fluorescent lights can be used. These may be installed even outside the culture room, in the corridor or in any other laboratory. However, when kept in the corridor, precautions must be taken to avoid the risk of people tampering with the adjustment knobs. In commercial companies which have more than one growth room, it may be possible to maintain different growth conditions in different rooms. Since cleanliness is paramount in this area, enough care should be taken to prevent any direct contact with the outside. The paint on the walls and the flooring should be able to withstand repeated cleaning. Desirably, the junction of the walls should be rounded rather than angular to prevent cob webs.

Cultures are generally grown in diffuse light (less than 1 klx). Some provision should also be made for maintaining cultures under higher light intensities (5–10 klx), and total darkness. Diurnal control of illumination of the lamps (fluorescent tubes) can be achieved by using automatic time-clocks.

If the relative humidity in the culture room falls below 50%, provision to increase humidity should be made to prevent the medium from drying rapidly. With very high humidity, cotton plugs become damp and the chances of contamination of cultures increase.

The culture room should be provided with specially designed shelves to store cultures (see Figs. 2.1 and 2.2). While some laboratories have shelves on the wall along the sides of the room, others have them fitted onto angular iron frames (culture racks) placed conveniently in the room. The culture racks may be provided with wheels for more efficient utilization of space. The shelves can be made of glass or rigid wire mesh. Each shelf is provided with a separate set of fluorescent tubes. Insulation between the lamps and the shelf above ensures a more even temperature around the cultures. To prevent a build-up of hot air in the shelves due to

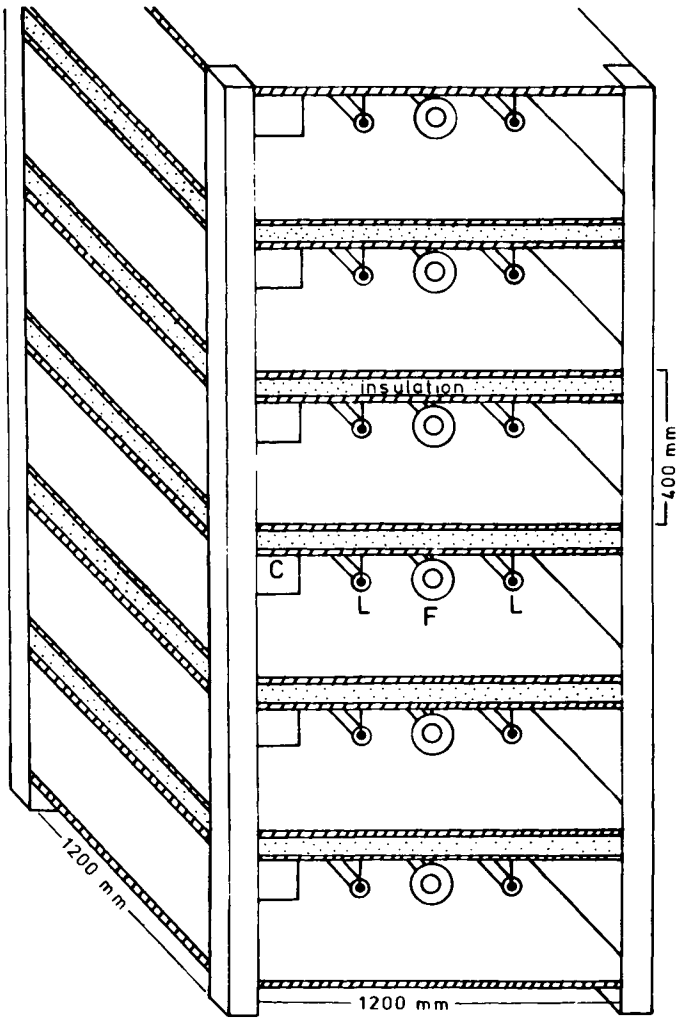


Fig. 2.1. Diagram of a shelving unit especially designed for storing cultures. C, control panel; F, fan; L, light source.

the lamps, ventilation of the individual shelves can be provided by fitting a small fan at one end of the shelf and blowing air through a plastic pipe running the length of shelf. Holes are drilled on the sides of the pipe at appropriate distances to allow even air flow along the length of the pipe. Another point to consider is the heat generated by the ballast of the fluorescent tubes. This could be obviated by mounting all the ballast on a panel outside the room and having flexible wiring. Alternatively, electronic ballast may be used, which are expensive initially but will save on the wiring. They are also energy efficient and do not heat up much.

Fig. 2.2. Illuminated shelves of culture racks with culture jars held in plastic trays (courtesy of Dr Vibha Dhawan, TERI, New Delhi).

While flasks, jars and petri-dishes can be placed directly on the shelf or trays of suitable sizes, culture tubes require some sort of support. Metallic wire racks, each with a holding capacity of 20 or 24 tubes, are suitable for this purpose. In commercial companies, the handling of culture jars can also be made convenient by using autoclavable plastic/ metallic trays (Fig. 2.2). On one face of the culture tube racks and trays, there should be a label giving details of the experimental or production details (e.g. name of the plant, explant, medium, date of culture, name of operator).

The culture room should also have a shaking machine, either of the horizontal type or the rotatory type if cell suspensions are grown. Shakers with temperature and light controls are also available.

It is desirable to have emergency power points attached to a generator, to maintain both light and temperature in the culture room, and also to eliminate the risk of suspension cultures dying due to stoppage of the shakers in the event of a major power breakdown at the mains. Such a catastrophe may ruin important experiments. Some temperature-sensitive strains of tissues may even die.

2.2.5. Greenhouse

In order to grow the mother plants and to acclimatize in vitro produced plants, the tissue culture laboratory should invariably have a green house/glass house/plastic house attached to it. The sophistication of this facility will depend on the funds available. However, minimum facilities for maintaining high humidity by fogging, misting or a fan and pad system, reduced light, cooling system for summers and heating system for winters must be provided. It would be desirable to have a potting room adjacent to this facility.

2.3. TECHNIQUES

This section deals with techniques other than media preparation which is discussed in Chapter 3. Techniques specific to various other areas of cell, tissue and organ culture have been described in the respective chapters.

2.3.1. Glassware and plasticware washing

Detergents especially designed for washing laboratory glassware and plasticware are available. After soaking in detergent solution for a suitable period (preferably overnight) the apparatus is thoroughly rinsed first in tap water and then in distilled water. If the glassware used has dried agar sticking to the sides of the tubes or jars, it would be better to melt it by autoclaving at low temperature. To recycle glassware that had contaminated tissues or media, it is extremely important to autoclave them without opening the closure so that all the microbial contaminants are destroyed. Even the disposable culture vials should be autoclaved prior to discarding them, in order to minimize the spread of bacteria and fungi in the laboratory. The washed apparatus is placed in wire baskets or trays to allow maximum drainage and dried in an oven or hot-air cabinet at about 75°C and stored in a dust-proof cupboard. Half of one or more shelves in the oven or hot-air cabinet may be lined with filter paper on which instruments and more fragile and small objects (e.g. filter holders, sieves, etc.) can be laid out. Glassware washing can also be done using domestic or industrial dishwashers.

2.3.2. Sterilization

Plant tissue culture media, which is rich in sucrose and other organic nutrients, support the growth of many micro-organisms (like bacteria

and fungi). On reaching the medium these microbes generally grow much faster than the cultured tissue and finally kill it. The contaminants may also give out metabolic wastes which are toxic to plant tissues. It is, therefore, absolutely essential to maintain a completely aseptic environment inside the culture vessels. For this, two obvious general precautions are: (1) not to share the plant tissue culture working area with microbiologists and pathologists, and (2) to remove contaminated cultures from the culture area as soon as detected.

There are several possible sources of contamination of the medium: (a) the culture vessel, (b) the medium itself, (c) the explant, (d) the environment of the transfer area, (e) instruments used to handle plant material during inoculation and subculture, (f) the environment of the culture room, and (g) the operator. In the following few pages some measures taken to guard the cultures against contamination from any of these sources are discussed. The reader should refer to the excellent reviews by Cassells (1991), Leifert and Waites (1990), and Leifert et al. (1994) for a detailed exposition on contamination in cultures.

(i) *Medium.* The microbial contaminants are normally present in the medium right from the start. To destroy them, the mouth of the culture vial containing the medium is properly closed with a suitable bacteria-proof closure and the vial is autoclaved (steam heating under pressure) at 1.06 kg cm^{-2} (121°C) for 15–40 min from the time the medium reaches the required temperature. If an autoclave is not available, a domestic pressure cooker may be used. Sterilization depends on the temperature and not directly on the pressure. The exposure time varies with the volume of the liquid to be sterilized (see Table 2.1). Monnier (1976) reported that heating at 120°C decreased the nutritive value of the culture medium for young *Capsella* embryos. Best results were obtained when the medium was autoclaved at 100°C for 20 min. Care must be taken while cooling the solution. A rapid loss of pressure, exceeding the rate of reduction in temperature will make the liquid boil vigorously. The pressure gauge of the autoclave should be at zero (temperature not higher than 50°C) before the autoclave is opened.

It has been observed that 2–5% of media are contaminated during manual pouring after autoclaving (Leifert et al., 1994). Moreover, certain *Bacillus* species have been shown to survive even after autoclaving of the medium at 110–120°C for 20 min. It is, therefore, advisable to store the medium for about 7 days before use.

Some of the plant growth regulators (e.g. GA_3, zeatin, ABA) urea, certain vitamins, pantothenic acid, antibiotics, colchicine, plant extracts and enzymes used in tissue culture are thermolabile. These compounds

TABLE 2.1

Minimum time necessary for steam sterilization of media as suggested by Biondi and Thorpe (1981)

Volume/container (ml)	Minimum sterilization time at 121°C (min)
20–50	15
75	20
250–500	25
1000	30
1500	35
2000	40

should not be autoclaved. When using such a compound the whole medium minus the heat-labile compound is autoclaved in a flask and kept in the sterilized hood to cool down. The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium when the latter has cooled to around 40°C in the case of a semi-solid medium (just before the setting of agar) or to room temperature when using a liquid medium. For filter sterilization of the solutions, bacteria-proof filter membranes of pore size 0.45 μm or less are used. The membranes are fitted into filter holders of appropriate size and autoclaved after wrapping in aluminium foil, or enclosed in screwcap glass jars of a convenient size. Sterilization temperature for filters is critical; it should not exceed 121°C. A graduated syringe (need not be sterilized) carrying the liquid is fixed to one end of the sterilized filter assembly (see Fig. 2.3) and the solution is gradually pushed through the membrane present in the middle of the assembly. The sterilized solution dripping out from the other end of the assembly is added to the medium or collected in a sterilized jar and added to the medium using a sterilized, graduated pipette. Large filter assemblies are also available for filter sterilization. The solution to be filter-sterilized should first be clarified by passing through a No. 3 porosity sintered glass filter. This facilitates filter-sterilization by reducing the plugging of membrane filter pores.

(ii) *Glassware and plasticware.* Glass culture vials are mostly sterilized together with the medium. For pre-sterilized nutrient medium the glassware (culture vessels and other labware) may be sterilized by autoclaving or dry-heating in an oven at 160–180°C for 3 h (De Fossard, 1976). Disadvantages of dry-heat sterilization are poor circulation and slow penetration. Therefore, proper loading of the oven is essential. The glassware is allowed to cool before removal from the oven. If removed before suffi-

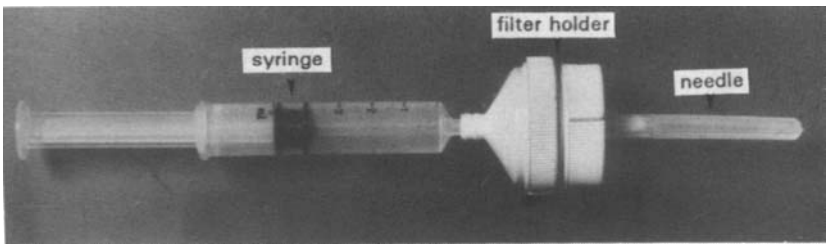


Fig. 2.3. 'Swinnex' Millipore filter assembly for sterilizing small volumes of liquids. The needle is not always required.

cient cooling has taken place, cool air from the exterior may be sucked into the oven, exposing the load to bacterial contamination and the risk of cracking.

Certain types of plastic labware can also be heat sterilized. Polypropylene, polymethylpentene, polyallomer, Tfzel ETFE and Teflon FEP may be repeatedly autoclaved at 121°C (Biondi and Thorpe, 1981). Of these, only Teflon FEP may be dry-heat sterilized. Polycarbonate shows some loss of mechanical strength with repeated autoclaving, and sterilization cycles for it should be limited to 20 min. A large variety of presterilized culture vessels are also available which could be directly used to pour autoclaved media.

(iii) *Instruments*. The instruments used for aseptic manipulations, such as forceps, scalpels, needles, and spatula, are normally sterilized by dipping in 95% ethanol followed by flaming and cooling. This is done at the start of the transfer work and several times during the operation. De Fossard (1976) has suggested the use of 70% alcohol because 95% and 100% alcohol can harbour bacterial spores without killing them. However, for flame sterilization of instruments 95% alcohol has been found entirely satisfactory. The alcohol should be regularly changed as *Bacillus circulans* strains persist in alcohol for more than a week (Leifert and Waites, 1990).

Effective sterilization of instruments can be achieved by flaming in a Bunsen burner. However, the heat liberated by a Bunsen burner is enormous, and the air currents generated could increase incidence of contamination during sub-culture. In recent times the glass bead sterilizer (steripot) and infra-red sterilizer have become available for sterilizing the instruments. In the glass bead sterilizer (Fig. 2.4) a high watt element heats up the glass beads contained in a brass crucible at the centre of the box. The temperature of the beads is raised to 250°C in 15–20 min. Sterilization of instruments is effected by pushing them into the beads for 5–7 s. A regulator maintains the temperature at 250°C by a 15 s cut off and

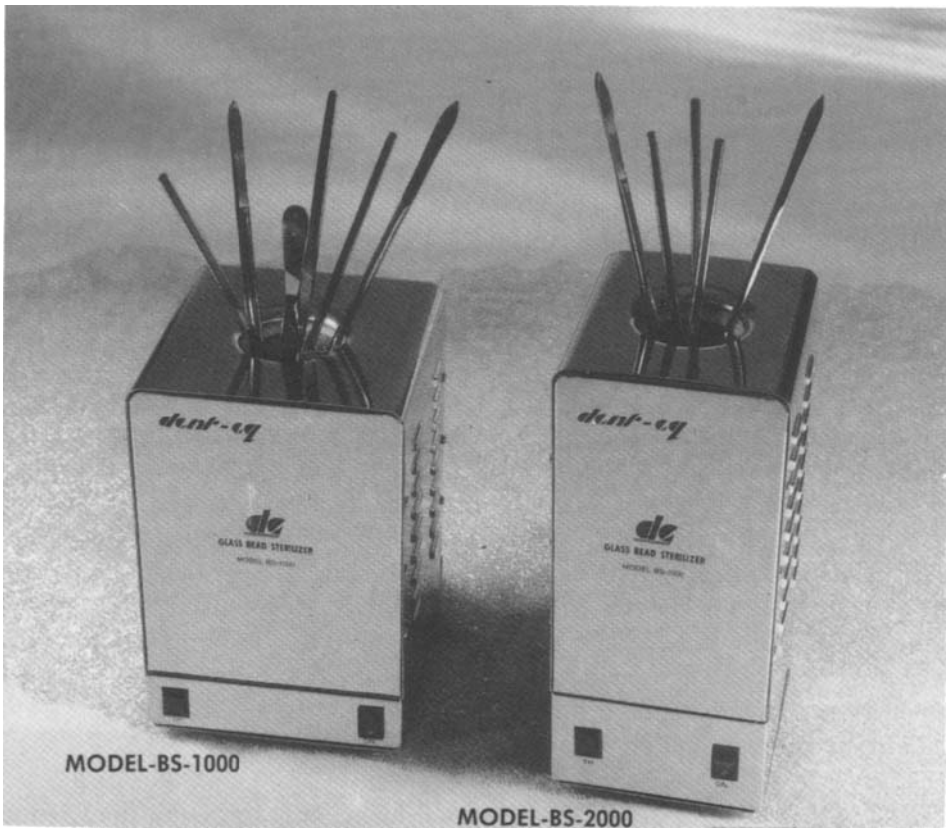


Fig. 2.4. Glass bead sterilizers (courtesy of Mrs Nanda Prasad, Dent-eq, Bangalore).

an indicator light or a dial thermometer indicates the temperature. Glass beads need to be replaced when they turn black. The infrared sterilizer has a cavity where temperature rises to almost 700°C. Here sterilization is effected by a 2–5 s exposure at this temperature. Being well insulated, these sterilizers do not spill out large quantities of heat. These instruments are also safe compared to a Bunsen burner which could cause heat burns and may also be a fire hazard.

(iv) *Plant material*. Surfaces of plant parts carry a wide range of microbial contaminants. To avoid this source of infection the tissue must be thoroughly surface sterilized before planting it on the nutrient medium; tissues with systemic fungal or bacterial infection are usually discarded in tissue culture studies.

To disinfect plant tissues various sterilizing agents have been used (see Table 2.2). Hypochlorite solutions (sodium or calcium) have proved to

TABLE 2.2

Effectiveness of some surface sterilizing agents[a]

Sterilizing agent	Concentration used (%)	Duration (min)	Effectiveness
Calcium hypochlorite	9–10	5–30	Very good
Sodium hypochlorite	2[b]	5–30	Very good
Hydrogen peroxide	10–12	5–15	Good
Bromine water	1–2	2–10	Very good
Silver nitrate	1	5–30	Good
Mercuric chloride	0.1–1	2–10	Satisfactory
Antibiotics	4–50 mg l^{-1}	30–60	Fairly good

[a]After Yeoman and Macleod (1977).
[b]20% (v/v) of a commercial solution.

be effective in most cases. For example, 0.3–0.6% sodium hypochlorite treatment for 15–30 min will decontaminate most tissues. It is important to realize that a surface sterilant is also toxic to the plant tissue. Therefore, the concentration of the sterilizing agent and the duration of treatment should be chosen to minimize tissue death.

Ethyl and isopropyl alcohol have also been used to surface sterilize some plant tissues (methanol should never be used). After rinsing in ethanol for a few seconds the material is either left exposed in the sterile hood until the alcohol evaporates (Kao and Michayluk, 1980) or, if fairly hardy, flamed (Bhojwani, 1980a).

In general, if the explant is fairly hard and large enough to be easily handled, the inoculum can be directly treated with the disinfectant. For example, in the culture of mature seeds, mature endosperm of euphorbiaceous plants or nodal explants, whole seeds, decoated seeds or stem pieces, respectively, are surface sterilized. However, when immature ovules, embryos or endosperm are to be cultured, the customary method is to surface sterilize the ovary or the ovule, as the case may be, and dissect out the explant under aseptic conditions so that the soft tissues of the inoculum are protected from the toxic effects of the sterilizing agent. Similarly, for raising cultures of delicate shoot apices and pollen grains, shoot buds or flower buds, respectively, are surface sterilized and the explant excised aseptically. Such explants are usually free of microbial contaminants. A 30 s rinse of the plant material in 70% ethanol before treatment with the sterilant or the addition of a few drops of surfactant, such as Triton-X and Tween-80, to the sterilization solution enhances its efficiency. A careful dissection of shoot apices may give high frequency of healthy cultures even without a surface sterilization treatment (Quak,

1977). However, dissection of wet material should be avoided. After surface sterilization treatment (not applicable when using alcohol), the plant material must be rinsed three or four times in sterile, distilled water to remove all traces of the sterilizing agent.

Treating wheat seeds in a 1% (v/v) solution of cetavlon (Cetrimide, ICA) for 2 min before hypochlorite treatment was found very effective in reducing bacterial contamination of cultures (Bhojwani and Hayward, 1977). In cases where the explant carries a heavy load of micro-organisms on its surface it may pay to wash it in running tap water for an hour or more. Often aseptic seedlings are raised through seed culture and their various parts (roots, stem pieces, leaves, etc.) are utilized for initiating cultures.

Antibiotics and antifungal compounds have been used by several workers to control explant contamination. Arbitrary use of antibiotics might not yield any useful results as the majority of the bacteria infecting plant materials are gram-negative, which are less sensitive to the commonly used antibiotics (Leifert et al., 1994). The mode of action and effectivity of the antimicrobial agents should be fully understood before use (Table 2.3). Micro-organisms can be accurately identified by fatty acid profile, pattern of carbon compound utilization, and nucleic acid studies (Buckley et al., 1995). However, if these procedures are found expensive the classical method of using liquid medium or an enriched agar medium may be employed. Reed et al. (1995) reported that streptomycin at 1000 g l^{-1} for a period of 10 days was effective against endophytic bacteria and less phytotoxic to *mentha* spp. than gentamicin, neomycin and rifampicin. However, antibiotics have been shown to restrict rooting, general growth and multiplication in plant cultures (Leifert et al., 1994). Antifungals, such as binomyl has been shown to reduce fungal infection when used with mercuric chloride (Mederos and Lopez, 1991).

Interestingly, Attree and Scheffield (1986) found that it was physically possible to separate micro-organisms from plant cells and protoplasts by using a sucrose gradient centrifugation. This could be combined with dilute hypochlorite and/or antibiotic treatment of cells before or after centrifugation (Bradley, 1988; Finner et al., 1991).

(v) *Transfer area*. Finally, it is very essential that all precautions are taken to prevent the entry into the culture vial of any contaminant when its mouth is opened either for subculture or for planting fresh tissues (inoculation). To achieve this, all transfer operations are carried out under strictly aseptic conditions.

In most laboratories laminar air-flow cabinets (see Fig. 2.5), which are available in various shapes and sizes are used for aseptic manipulation.

TABLE 2.3

Mode of action of some antimicrobial agents[a]

Antimicrobial compound	Mode of action	Comments
Aminoglycosides		Bactericidal
Streptomycin		
Kanamycin	Inhibit protein synthesis	
Neomycin	by interaction with 30S	
Gentamicin	50S ribosomes	
Tobramycin		
Amikacin		
Spectinomycin		(an aminocyclitol)
Quinolones		Bactericidal
Nalidixic acid		
Ofloxacin	Interfere with DNA	
Norfloxacin	replication by inhibition	
Enoxacin	of DNA gyrase	
Ciprofloxacin		
β-Lactams		Bactericidal
Penicillin		
Ampicillin		
Carbenicillin		
Cephradine		
Cephamandole	Inhibit bacterial cell	
Cefuroxime	wall synthesis	
Ceftazidime		
Sublactam		(sulphone)
Imipenem		(carbapenem)
Aztreonam		(monobactam)
Tetracyclines	Inhibit protein synthesis by acting on 30S ribosome	Bacteriostatic
Trimethoprim and suplhanamides	Inhibit synthesis of tetrahydrofolate (at different sites)	Bacteriostatic
Chloramphenicol	Inhibit protein synthesis by acting on 50S ribosome	Bacteriostatic
Macrolides and lincosamides		Bacteriostatic
Erythromycin	Inhibit protein synthesis	
Lincomycin	by acting on 50S ribosome	
Glycopeptides		
Vancomycin	Interferes with bacterial cell wall synthesis	Bactericidal for Gram +ves only

TABLE 2.3 (*continued*)

Antimicrobial compound	Mode of action	Comments
Polymixins		Bactericidal
Polymixin B Polymixin E	Attach to cell membrane and modify ion flux, resulting in cell lysis	for Gram –ves esp. *Pseudomonas* (*Proteus* resistant)
Rifampicin	Interferes with mRNA formation by binding to RNA polymerase	Resistance emerges readily

[a]After Falkiner (1990). According to the author, the agents which act specifically on bacterial cell walls would be more suitable to control infection in plant tissue cultures.

Work can be started within 10–15 min of switching on the air flow, and one can work uninterrupted for long hours.

Essentially, a laminar air-flow cabinet has a small motor to blow air which first passes through a coarse filter, where it loses large particles, and subsequently through a fine filter. The latter, known as the 'high efficiency particulate air (HEPA)' filter, removes particles larger than 0.3 μm, and the ultraclean air (free of fungal and bacterial contaminants) flows through the working area. The velocity of the air coming out of the fine filter is about 27 ± 3 m min^{-1} which is adequate for preventing the contamination of the working area by the worker sitting in front of it. All contaminants such as hairs, salts, flakes, etc., are blown away by the ultraclean air flow, and a completely aseptic environment is maintained in the working area as long as the cabinet is kept on. The flow of air does not in any way hamper the use of a spirit lamp or a Bunsen burner.

In temperate countries, air-flow cabinets are used in ordinary laboratories. However, in tropical and sub-tropical countries, where atmospheric dust is very high, it would be better to house the cabinet in a culture room fitted with double doors in order to prolong the effective life of the filters. Under no circumstances should the hood be kept opposite a door or a window which is frequently used.

Maximum chances of cultures getting infected occur when the culture vials are opened during initiation of fresh cultures or subculture of established cultures. Therefore, most of the commercial tissue culture companies have a separate 'transfer room' in the clean area and maximum cleanliness and least air turbulence is maintained in this room. The movement of people in this area should be restricted. In this room the walls should be made smooth so that dust does not settle. The walls can

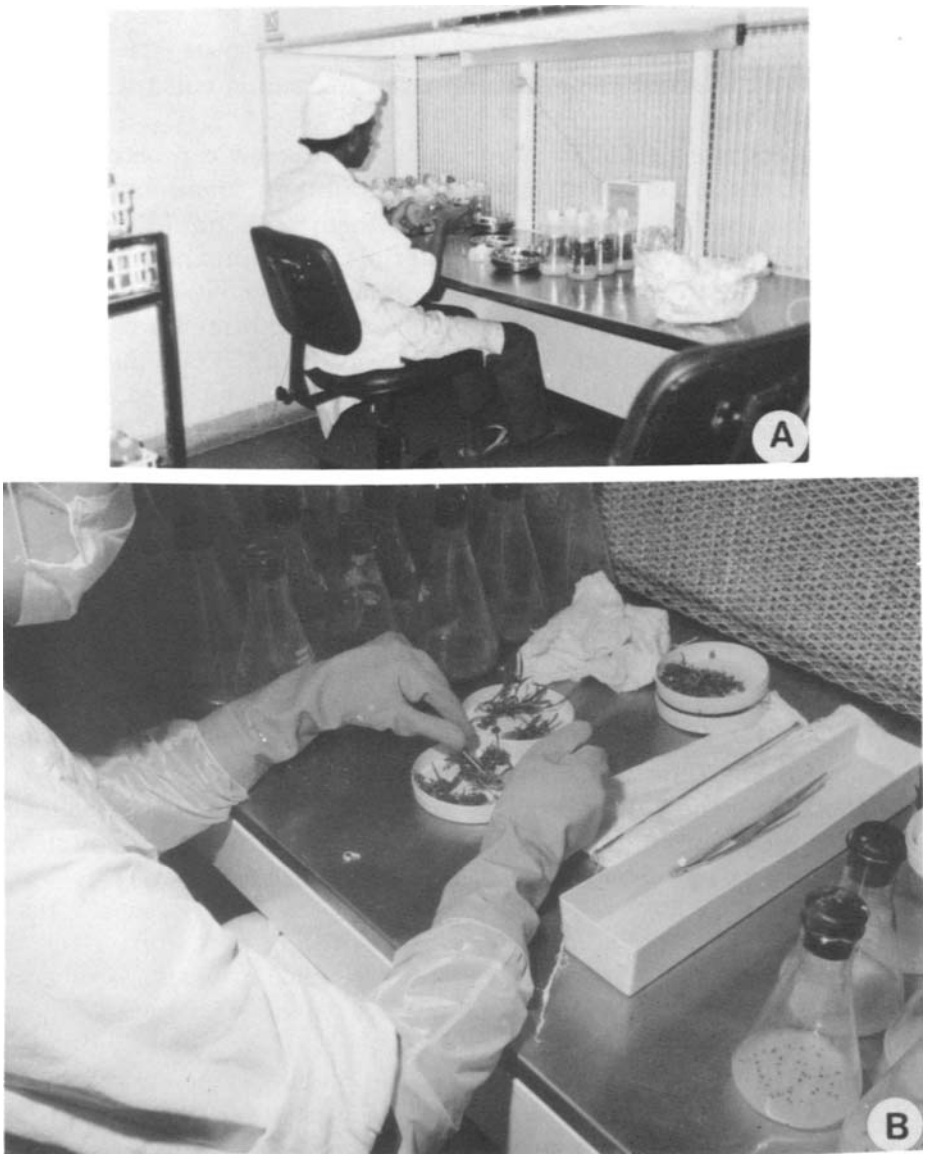


Fig. 2.5. Laminar air-flow cabinets in use. (A) Courtesy of Dr Vibha Dhawan, TERI, New Delhi; (B) courtesy of South Pacific Orchids Ltd., New Zealand.

be painted with high gloss and water tolerant paint to withstand repeated cleaning. A one-piece floor covering of linoleum extending a few centimetres up the walls is easier to clean. The transfer hoods, light and air-conditioners in the transfer room should be connected to an emergency source of power.

APPENDIX 2.I

The sequence of steps commonly involved in aseptic culture of plant tissues[1] is as follows:

(a) Pieces of plant material are collected in a screw-cap bottle and a dilute solution of the disinfectant, containing a small amount of a suitable surfactant, is poured onto them. The liquid should be enough to fully immerse the material. After putting on the closure, the bottle is taken to the aseptic transfer hood. During the sterilization period the bottle is shaken two to three times.

(b) After sterilization treatment, the cap of the bottle is removed and the liquid poured out. An adequate quantity of sterilized, distilled water is poured onto the material and the cap replaced. After shaking a few times, the water is discarded. Such washings with sterile distilled water are repeated three to four times.

(c) The material is then transferred to a pre-sterilized petri-dish.

(d) While the plant material is being treated for disinfection the instruments required are sterilized by dipping them in 95% ethanol and flaming, and allowed to cool. It may be necessary to sterilize the instruments each time after handling tissue.

(e) Suitable explants are prepared from the surface sterilized material using sterilized instruments (scalpels, needles, cork-borer, forceps, dissecting microscope, etc.).

(f) Closure of the culture vial is removed, the inoculum transferred onto the medium, the neck of the vial flamed (in the case of glass vials only), and the closure replaced in quick succession.

[1] From step (b) onwards all the operations are performed under aseptic conditions. Precise methods for raising aseptic cultures of various plant tissues and organs are described at appropriate places in subsequent chapters.

APPENDIX 2.II

A list of apparatus required for tissue culture work.

1. flasks (100 ml, 250 ml, 500 ml, 1 l, 5 l);
2. volumetric flasks (500 ml, 1 l, 2 l, 3 l);
3. measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml, 1 l);
4. graduated pipettes (1 ml, 2 ml, 5 ml, 10 ml);
5. Pasteur pipettes and teats for them;
6. culture vials (culture tubes, screw-cap bottles of various sizes, petri-dishes, nipple flasks, etc.) with suitable closure;

} glassware or plasticware for media preparation

7. plastic or steel buckets, to soak labware for washing;
8. hot-air cabinet, to dry washed labware;
9. oven, to dry washed labware, and dry-heat sterilization of glassware;
10. wire-mesh baskets, to autoclave media in small vials and for drying labware;
11. water distillation unit, demineralization unit, Milli Q unit or reverse osmosis unit for water purification;
12. plastic carboys (10 l and 20 l), to store high quality water;
13. balances, one to weigh small quantities and the other to weigh comparatively larger quantities;
14. hot plate-cum-magnetic stirrer, to dissolve chemicals;
15. exhaust pump, to facilitate filter sterilization;
16. plastic bottles of different sizes, to store and deep-freeze solutions;
17. refrigerator, to store chemicals, stock solutions of media, plant materials, etc.;
18. deep freeze, to store stock solutions of media for longer periods, certain enzymes, coconut milk, etc.;
19. steamer or microwave oven to dissolve agar and melt media;
20. pH meter, to adjust pH of media and solutions;
21. autoclave or domestic pressure cooker, for steam sterilization of media and apparatus;
22. heat-regulated hot plate or gas stove for steam sterilization in domestic pressure cooker;
23. filter membranes and their holders, to filter sterilize solutions;
24. hypodermic syringes, for filter sterilization of solution;
25. medium dispenser, to pour medium;
26. trolley with suitable trays, to transport cultures, media and apparatus;

27. laminar air-flow cabinet, for aseptic manipulations;
28. spirit lamp, burner, glass bead sterilizer or infra-red sterilizer to sterilize instruments;
29. atomizer, to spray spirit in the inoculation chamber;
30. screw-cap bottles, to sterilize plant material;
31. instrument stand, to keep sterilized instruments during aseptic manipulations;
32. large forceps with blunt ends, for inoculation and subcultures;
33. forceps with fine tips, to peel leaves;
34. fine needles, for dissections;
35. stereoscopic microscope with cool light, for dissection of small explants;
36. table-top centrifuge, to clean protoplast and isolated microspore preparations, etc.;
37. incubator shaker, for liquid cultures.

Chapter 3

Tissue Culture Media

3.1. INTRODUCTION

Nutritional requirements for optimal growth of a tissue in vitro may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962). As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. When starting with a new system, it is essential to work out a medium that will fulfil the specific requirements of that tissue. During the past 25 years, the need to culture diverse tissues and organs has led to the development of several recipes (Table 3.1).

Some of the earliest plant tissue culture media, e.g. root culture medium of White (1943) and callus culture medium of Gautheret (1939), were developed from nutrient solutions previously used for whole plant culture. White evolved the medium from Uspenski and Uspenskaia's medium (1925) for algae, and Gautheret's medium is based on Knop's (1865) salt solution. All subsequent media formulations are based on White's and Gautheret's media.

While some calli (carrot tissue, blackberry tissue, most tumour tissues) may grow on simple media containing only inorganic salts and a utilizable sugar, for most others it is essential to supplement the medium with vitamins, amino acids and growth substances in different qualitative and quantitative combinations. Often, complex nutritive mixtures have been added to plant tissue culture media. A medium containing only 'chemically-defined' compounds is referred to as a 'synthetic medium'[1].

In tissue culture literature the concentrations of inorganic and organic constituents of the medium are generally expressed in mass values (mg l^{-1} and ppm are synonymous but only mg l^{-1} is now acceptable). This has been followed in Table 3.1. However, the International Association

[1]Even in a synthetic medium one knows only what has been added. Breakdown of certain compounds (sucrose, vitamins) during autoclaving and interaction between various ingredients may occur during preparation, thus changing the final composition of the medium.

TABLE 3.1

Composition of some plant tissue culture media[a]

Constituents	Media (amount in mg l^{-1})						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Inorganic							
NH_4NO_3	–	–	1650	1200	–	720	825
KNO_3	80	–	1900	1900	2527.5	950	950
$CaCl_2 \cdot 2H_2O$	–	75	440	440	150	–	220
$CaCl_2$	–	–	–	–	–	166	–
$MgSO_4 \cdot 7H_2O$	750	250	370	370	246.5	185	1233
KH_2PO_4	–	–	170	340	–	68	680
$(NH_4)_2SO_4$	–	–	–	–	134	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	300	–	–	–	–	–	–
$NaNO_3$	–	600	–	–	–	–	–
Na_2SO_4	200	–	–	–	–	–	–
$NaH_2PO_2 \cdot H_2O$	19	125	–	–	150	–	–
KCl	65	750	–	–	–	–	–
KI	0.75	0.01	0.83	–	0.75	–	0.83
H_3BO_3	1.5	1	6.2	0.63	3	10	6.2
$MnSO_4 \cdot 4H_2O$	5	0.1	22.3	2.23	–	25	22.3
$MnSO_4 \cdot H_2O$	–	–	–	–	10	–	–
$ZnSO_4 \cdot 7H_2O$	3	1	8.6	–	2	10	–
$ZnSO_4 \cdot 4H_2O$	–	–	–	–	–	–	8.6
$ZnNa_2 \cdot EDTA$	–	–	–	15	–	–	–
$Na_2MoO_4 \cdot 2H_2O$	–	–	0.25	0.025	0.25	0.25	0.25
MoO_3	0.001	–	–	–	–	–	–
$CuSO_4 \cdot 5H_2O$	0.01	0.03	0.025	0.0025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	–	–	0.025	0.0025	0.025	–	–
$CoSO_4 \cdot 7H_2O$	–	–	–	–	–	–	0.03
$AlCl_3$	–	0.03	–	–	–	–	–
$NiCl_2 \cdot 6H_2O$	–	0.03	–	–	–	–	–
$FeCl_3 \cdot 6H_2O$	–	1	–	–	–	–	–
$Fe_2(SO_4)_3$	2.5	–	–	–	–	–	–
$FeSO_4 \cdot 7H_2O$	–	–	27.8	27.8	–	27.8	27.8
$Na_2EDTA \cdot 2H_2O$	–	–	37.3	37.3	–	37.3	37.3
Sequestrene 330Fe	–	–	–	–	28	–	–
Organic							
Inositol	–	–	100	–	100	100	100
Nicotinic acid	0.05	–	0.5	0.5	1	5	–
Pyridoxine·HCl	0.01	–	0.5	0.5	1	0.5	–
Thiamine·HCl	0.01	–	0.1	0.5	10	0.5	1
Glycine	3	–	2	2	–	2	–
Folic acid	–	–	–	–	–	0.5	–
Biotin	–	–	–	–	–	0.05	–

TABLE 3.1 (*continued*)

Constituents	Media (amount in mg l^{-1})						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Sucrose	2%	–	3%	4%	2%	2%	1%
D-Mannitol	–	–	–	–	–	–	12.7%

[a]Growth regulators and complex nutrient mixtures described by various authors are not included here. The compositions of several media recommended for specific tissue and organ are given in relevant chapters.
[b]Concentrations of mannitol and sucrose are expressed in percentage.
[c]White (1963).
[d]Heller (1953).
[e]Murashige and Skoog (1962).
[f]Eriksson (1965).
[g]Gamborg et al. (1968).
[h]Nitsch (1969).
[i]Nagata and Takebe (1971).

for Plant Physiology has recommended the use of mole values. Mole is an abbreviation for gram molecular weight which is the formula weight of a substance in grams. The formula weight of a substance is equal to the sum of the weights of the atoms in the formula of a substance. One litre of solution containing 1 mole of a substance is said to be 1 Molar (1 M) or a 1 mol l^{-1} solution of the substance (1 mol l^{-1} = 1000 or 10^3 mmol l^{-1} = 1 000 000 or 10^6 μmol l^{-1}). According to the recommendations of the International Association for Plant Physiology, mmol l^{-1} should be used for expressing the concentration of macronutrients and organic nutrients and μmol l^{-1} for micronutrients, hormones, vitamins and other organic constituents in the plant tissue culture medium. One of the reasons for using mole values is that the number of molecules per mole is constant for all compounds.

When preparing medium according to a published recipe the original mole values can be used irrespective of the number of water molecules in the sample of the salt. This cannot be done when the concentrations are expressed in mass values.

3.2. MEDIA CONSTITUENTS

3.2.1. Inorganic nutrients

Mineral elements are very important in the life of a plant. For example, magnesium is a part of chlorophyll molecules, calcium is a constitu-

ent of the cell wall, and nitrogen is an important part of amino acids, vitamins, proteins and nucleic acids. Similarly, iron, zinc and molybdenum are parts of certain enzymes. Besides C, H, and O, 12 elements are known to be essential for plant growth: nitrogen, phosphorus, sulphur, calcium, potassium, magnesium, iron, manganese, copper, zinc, boron and molybdenum. Of these, the first six elements are required in comparatively large quantities and are, therefore, termed macro- or major elements. The other six elements are necessary in only small amounts and are called micro- or minor elements. According to the recommendations of the International Association for Plant Physiology the elements required by plants in concentrations greater than 0.5 mmol l^{-1} are referred to as macroelements and those in concentrations less than 0.5 mmol l^{-1} are microelements (De Fossard, 1976). Essentially, the 15 elements found important for whole plant growth have also proved necessary for tissue cultures. A survey of Tables 3.1 and 3.2 shows that the chief difference in the composition of various commonly used tissue culture media lies in the quantity of various salts and ions, respectively. Qualitatively, the inorganic nutrients required for various plant tissues appear to be fairly constant.

When mineral salts are dissolved in water they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt. For example, in Murashige and Skoog's (1962) medium (MS) NO_3^- ions are contributed by NH_4NO_3 as well as KNO_3, and K^+ ions are contributed by KNO_3 and KH_2PO_4. Therefore, a useful comparison between the two media can be made by looking into total concentrations of different types of ions in them. 'Balance sheets' of ions for the seven media given in Table 3.1 are presented in Table 3.2.

White's medium, one of the earliest plant tissue culture media, includes all the necessary nutrients and is widely used for root culture. Experience of various investigators has, however, revealed that quantitatively the inorganic nutrients in this medium are inadequate for good callus growth (Murashige and Skoog, 1962). This deficiency was overcome by enriching the medium with complex mixtures like yeast extract, casein hydrolysate, coconut milk, amino acids, etc. (Reinert and White, 1956; Risser and White, 1964). With the objective of evolving suitable synthetic media, later investigators have effectively replaced the nutritive mixtures by increasing the concentrations of the various inorganic nutrients, particularly potassium and nitrogen. Most plant tissue culture media that are now being widely used (Tables 3.1 and 3.2) are richer in mineral salts (ions) compared to White's medium. Aluminium and nickel used by Heller (1953) could not be demonstrated as essential and, there-

TABLE 3.2

Balance sheet of ions for the media included in Table 3.1

Ions	Units	Media[a]						
		White's	Heller's	MS	ER	B_5	Nitsch's	NT
NO_3	mM	3.33	7.05	39.41	33.79	25.00	18.40	19.69
NH_4	mM	–	–	20.62	15.00	2.00	9.00	10.30
Total N	mM	3.33	7.05	60.03	48.79	27.03	27.40	29.99
P	mM	0.138	0.90	1.25	2.50	1.08	0.50	5.00
K	mM	1.66	10.05	20.05	21.29	25.00	9.90	14.39
Ca	mM	1.27	0.51	2.99	2.99	1.02	1.49	1.50
Mg	mM	3.04	1.01	1.50	1.50	1.00	0.75	5.00
Cl	μM	0.87	11.08	5.98	5.98	2.04	2.99	3.00
Fe	μM	12.50	3.70	100.00	100.00	50.10	100.00	100.00
S	μM	4502.00	1013.50	1730.00	1610.00	2079.90	996.80	5236.50
Na	μM	2958.00	7966.00	202.00	237.20	1089.00	202.00	202.00
B	μM	24.20	16.00	100.00	10.00	48.50	161.80	100.00
Mn	μM	22.40	0.40	100.00	10.00	59.20	112.00	100.00
Zn	μM	10.40	3.40	30.00	37.30	7.00	34.70	36.83
Cu	μM	0.04	0.10	0.10	0.01	0.10	0.10	0.10
Mo	μM	0.007	–	1.00	0.1	1.00	1.00	1.00
Co	μM	–	–	0.10	0.01	0.10	–	0.10
I	μM	4.50	0.06	5.00	–	4.50	–	5.00
Al	μM	–	0.20	–	–	–	–	–
Ni	μM	–	0.10	–	–	–	–	–

[a]For references, see Table 3.1.

fore, were dropped by subsequent workers. The indispensability of sodium, chloride and iodide has also not been established.

A detailed study of inorganic nutrients of plant tissue cultures was made by Heller (1953). He gave special emphasis to iron and nitrogen. In the original White's medium (1943), iron was added in the form of $Fe_2(SO_4)_3$, but Street and co-workers replaced it with $FeCl_2$ for root cultures because the former contained Mn and some other metallic ions as impurities (Street and Henshaw, 1966). However, $FeCl_2$ also did not prove to be an entirely satisfactory source of iron. In this form iron is available to the tissues at and around a pH of 5.2. It is known that in root cultures, within a week of inoculation, the pH of the medium drifts from the initial value of 4.9–5.0 to 5.8–6.0, and roots start showing iron-deficiency symptoms. To overcome this problem, in most media, iron is now used as Fe·EDTA. In this form iron remains available up to a pH of 7.6–8.0. Incidentally, unlike roots, callus cultures can utilize $FeCl_2$ up to a pH of 6.0 by secreting natural chelates which bind with iron (Heller,

1953). Fe·EDTA may be prepared by using $FeSO_4 \cdot 7H_2O$ and Na_2·EDTA as described in Table 3.6, or it may be possible to buy NaFe·EDTA.

(i) *Macroelements*. Nitrogen is one of the main elements contributing to the growth of plants in vitro and in vivo. It is a constituent of the amino acids, proteins, certain hormones and chlorophyll. The source of nitrogen in vitro could be either organic or inorganic. An indirect effect of nitrogen on tissue growth is through its influence on the pH of the medium (Dougall, 1980; Congard et al., 1986). The form of nitrogen, as NH_4^+ or NO_3^-, has a dramatic influence on the morphogenic response of plant tissues in vitro (see Section 6.3.4). Development of anthocyanin in vitro has been attributed to deficiency of NO_3^- ions (Heller, 1965).

Phosphorus is vital for cell division as well as in storage and transfer of energy in plants. Its role in photosynthesis is also important. Kozai et al. (1991) reported that in autotrophic cultures of strawberry, uptake of PO_4^{3-} is much greater than that of other minerals. Too little phosphorus causes plants to be abnormal and sickly.

Potassium is necessary for normal cell division, for synthesis of proteins, chlorophyll, and for nitrate reduction. The level of K^+ in vitro is rarely a problem but certain species are sensitive to high levels. Anderson (1975) showed that *Rhododendron* shoots grew better, without browning, when K^+ level was reduced.

Sulphur is present in some proteins. It is quite often present as an impurity in agar (Pochet et al., 1991).

Calcium as calcium pectate is an integral part of the walls of plant cells and helps maintain integrity of the membrane. High levels of calcium have been shown to promote callose deposition thereby inhibiting cell extension (Eklund and Eliasson, 1990). Atkinson (1991) found that stomata were more open in plants grown in the presence of high Ca^{2+}. Cytoplasmic Ca^{2+} is also involved in the regulation of hormone responses and mediates in responses to environmental factors such as temperature and light (Williams, 1995). Calcium could be having a pre-emptive role in morphogenesis (Hush et al., 1991). Calcium is not very mobile in plants. As a result, it is the new growth that suffers when there is a calcium deficiency either absolutely or because of poor mobility. The leaf tips and growing points tend to die back under such conditions.

Magnesium is a component of chlorophyll and a co-factor for many enzyme reactions. Magnesium uptake is not usually limited, except at low pH.

(ii) *Microelements*. The majority of the micro-elements are required in trace quantities and quite often may get carried into the medium as im-

purities in other ingredients. They may also get carried-over with the explant or tissues and support growth for several weeks without showing any deficiency symptoms. This and the interaction amongst the microelements makes the study of individual elements slightly complicated. The microelements are essential as catalysts for many biochemical reactions. Microelement deficiency symptoms include reduced lignification (Cu, Fe), rosetting (Zn, Mn), leaf chlorosis (Fe, Zn, Mn) and shoot tip necrosis (B). Certain elements, such as Co and Ni, can inhibit ethylene synthesis.

The availability of ions becomes critical sometimes because of the solubility problems. Dalton et al. (1983) suggested that an imbalance between Fe and EDTA can cause precipitation and make 45% of Fe, 20% of Zn and 13% of original PO_4^{3-} in MS medium unavailable within 2 days of media preparation. Interpretation of Fe status is complicated by the interactions between Fe and Mn or Zn. Excess Mn can lead to Fe deficiency while excess Fe or EDTA can reduce Zn uptake (Williams, 1995).

3.2.2. Organic nutrients

Most cultured plant cells are capable of synthesizing all essential vitamins but, apparently, in sub-optimal quantities (Czosnowski, 1952; Paris, 1955, 1958). To achieve the best growth of the tissue it is often essential to supplement the medium with one or more vitamins and amino acids. Various standard media show wide differences in their composition with respect to vitamins and amino acids (see Table 3.1).

(i) *Vitamins*. Animals require minor quantities of vitamins as necessary ancillary food factors which they get from extraneous sources. Plants, on the other hand, can produce their requirements of vitamins. However, plant cell cultures need to be supplemented with certain vitamins. The most widely used vitamins are thiamine (vitamin B_1), niacin (vitamin B_3), pyridoxine (vitamin B_6), and myo-inositol (a member of the vitamin B complex). Certain other vitamins which find specific uses in cell cultures are pantothenic acid, vitamin C, vitamin D and vitamin E.

The widely used Murashige and Skoog's (1962) medium lists four vitamins as necessary for tobacco callus growth. However, in a subsequent study, Linsmaier and Skoog (1965) removed niacin and pyridoxine but retained myo-inositol and increased the quantity of thiamine to 4 mg l^{-1}. Several later modifications of MS medium use only myo-inositol and thiamine.

Myo-inositol or meso-inositol is a natural constituent of plants and as phosphatidyl-inositol could be a crucial factor in the functioning of mem-

branes (Jung et al., 1972; Harran and Dickinson, 1978). In plants inositol as inositol phosphate may be acting as a second messenger to the primary action of auxins. It probably has a role as a carrier and in storage of IAA as IAA-myo-inositol ester. In plant tissue cultures myo-inositol could be a crucial precursor in the biosynthetic pathways leading to the formation of pectin and hemicelluloses needed in the cell wall synthesis (Loewus et al., 1962; Verma and Dougall, 1979) and may have a role in the uptake and utilization of ions (Wood and Braun, 1961).

Thiamine is involved in the direct biosynthesis of certain amino acids and is an essential co-factor in carbohydrate metabolism. Certain plant cultures appear to be self-sufficient for thiamine but most cultures do benefit by minute quantities of it, with the requirement increasing with consecutive passages. Thiamine could be having a synergistic interaction with cytokinins (Digby and Skoog, 1966).

Vitamin E is used as an anti-oxidant while vitamin C is useful to prevent blackening during explant isolation. Vitamin D has a growth regulatory effect on plant tissue cultures. Riboflavin has been found to inhibit callus formation and improve growth and quality of shoots (Drew and Smith, 1986).

(ii) *Amino acids*. There is little substantive evidence for the necessity or role of amino acids in plant tissue cultures. Even the often used glycine has little benefit in the sustained growth of tobacco callus (Linsmaier and Skoog, 1965) and may even be inhibitory at higher levels. Amino acids may be directly utilized by the plant cells or may serve as a nitrogen source. However, an organic source of nitrogen is preferred only when an inorganic source is lacking or exhausted (Williams, 1995). Cysteine has been included in media as an antioxidant to control the oxidation of phenolics and prevent blackening of tissue. The in vitro produced shoots of dwarf apple rootstocks formed more roots in the presence of arginine (Orlikowska, 1992).

(iii) *Undefined supplements*. Numerous complex nutritive mixtures of undefined composition, like casein hydrolysate (CH), coconut milk (CM), corn milk, malt extract (ME), tomato juice (TJ), and yeast extract (YE), have also been used to promote the growth of certain calli and organs. However, the use of these natural extracts should be avoided as far as possible. Different samples of these substances, especially the fruit extracts, may affect the reproducibility of results because the quality and quantity of the growth-promoting constituents in these extracts often vary with the age of the tissue and the variety of the donor organism. Moreover, it should be possible to effectively replace these substances by

a single amino acid. For example, for maize endosperm callus Straus (1960) could substitute yeast extract and tomato juice by L-asparagine alone. Similarly, Risser and White (1964) demonstrated that L-glutamine could replace a mixture of 18 amino acids earlier used by Reinert and White (1956) for tissue cultures of *Picea glauca*.

(iv) *Carbon source.* Haberlandt (1902) attempted to culture green mesophyll cells probably with the idea that green cells would have simpler nutritive requirements but this did not prove to be true. We now know that, as a rule, tissues which are initially green gradually lose their green pigments in cultures and depend on an external source of carbon. Even those tissues which acquire pigments through sudden changes or under special conditions during culture period are not autotrophs for carbon. Fully organized, green shoots in cultures also show better growth and proliferation with the addition of a suitable carbon source in the medium. Thus, it is essential to add a utilizable source of carbon to the culture medium.

The most commonly used carbon source is sucrose, at a concentration of 2–5%. Glucose and fructose are also known to support good growth of some tissues. Ball (1953, 1955) observed that autoclaved sucrose was better than filter-sterilized sucrose for the growth of *Sequoia* callus. Autoclaving seems to bring about hydrolysis of sucrose into more efficiently utilizable sugars, such as fructose and glucose. Bretzloff (1954) found that in a fungal medium sucrose breakdown during autoclaving was dependent on pH, with no hydrolysis occurring on setting the pH to 6.0.

In general, excised dicotyledonous roots grow best with sucrose whereas those of monocots do best with dextrose (glucose). Tissue cultures of *Malus pumila* (var. McIntosh) grow as well with sorbitol as with sucrose or glucose (Chong and Taper, 1972). Some other forms of carbon that plant tissues are known to utilize include maltose, galactose, mannose, and lactose (Gautheret, 1959). Tissue cultures of *Sequoia* (Ball, 1955) and maize endosperm (Straus and LaRue, 1954) can even metabolize starch as the sole carbon source.

Kozai (1991b) suggested that sucrose could either be reduced or completely eliminated from medium if autotrophic conditions of high CO_2 and light intensity could be maintained. However, despite such autotrophic conditions sucrose might become a limiting factor in the growth of certain cultures (Debergh et al., 1992a).

Sucrose in the medium is necessary for various metabolic activities. It is required for differentiation of xylem and phloem elements in cultured cells (Aloni, 1980). Sugars also represent the major osmotic component of the medium. Brown et al. (1979) replaced a third of sucrose in tobacco

callus medium with mannitol without effecting callus growth. Mannitol is an osmotic agent and is not taken into the plant cells or metabolized because of its molecular size.

3.2.3. Growth hormones

In addition to the nutrients, it is generally necessary to add one or more growth substances, such as auxins, cytokinins, and gibberellins, to support good growth of tissues and organs. However, the requirement for these substances varies considerably with the tissue, and it is believed that it depends on their endogenous levels.

The growth regulators are required in very minute quantities (μmol l^{-1} values). There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species.

(i) *Auxins*. In nature, the hormones of this group are involved with elongation of stem and internodes, tropism, apical dominance, abscission, rooting, etc. In tissue cultures auxins have been used for cell division and root differentiation. The auxins commonly used in tissue culture are: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), naphthoxyacetic acid (NOA), *para*-chlorophenoxyacetic acid (*p*-CPA), dichlorophenoxyacetic acid (2,4-D), and trichlorophenoxyacetic acid (2,4,5-T). Of these, IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation. 2,4-D and 2,4,5-T are very effective for the induction and growth of callus. 2,4-D is also an important factor for the induction of somatic embryogenesis. Auxins are usually dissolved in either ethanol or dilute NaOH.

(ii) *Cytokinins*. These hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance. More commonly used cytokinins are: benzylamino purine (BAP), isopentenyl-adenine (2-ip), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin. Compared to the other cytokinins, thidiazuron is generally used at very low concentrations (0.1–5 μg l^{-1}). Cytokinins are generally dissolved in dilute HCl or NaOH. For thidiazuron, DMSO may be used as the solvent.

(iii) *Gibberellins*. There are over 20 known gibberellins. Of these, generally, GA_3 is used. Compared to auxins and cytokinins, gibberellins are used very rarely. They are reported to stimulate normal development of plantlets from in vitro formed adventive embryos. GA_3 is readily soluble in cold water up to 1000 mg l^{-1}.

(iv) *Ethylene*. All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced abiologically when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation (Matthys et al., 1995).

Pure ethylene or chemical compounds which release ethylene during their decomposition, such as 2-chloroethylphosphonic acid (marketed under the trade names Ethrel, Ethaphon, Floridimex, Camposan), can be applied to study the effect of this gaseous growth regulator on plant tissue cultures. Ethylene exerts various morphogenic influences on cultured tissues but its effects are not clear cut (Matthys et al., 1995). It may be promotory or inhibitory for the same process in different systems. For example, it promoted somatic embryogenesis in maize (Vain et al., 1989a,b) but the same process was inhibited in *Hevea brasiliensis* (Auboiron et al., 1990).

(v) *Others*. Abscisic acid is most often required for normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Ammirato, 1988). It is also known to promote morphogenesis in *Begonia* cultures.

More recently, there has been some interest in the application of growth retardants, such as paclobutrazol, during the acclimatization stage of micropropagation to reduce hyperhydricity and regulate leaf growth and function in relation to control of water stress (Smith and Krikorian, 1990a; Ziv, 1992). Ancymidol has been used to inhibit leaf formation and promote shoot formation in gladiolus (Ziv, 1989; Ziv and Ariel, 1991).

3.2.4. Gelling agents

In static cultures if liquid medium is used the tissue would get submerged and die due to lack of oxygen. A gelling agent is generally used to circumvent this problem. The most desirable property of a gelling agent is that it should withstand sterilization by autoclaving, and the medium should be liquid when hot but form a semisolid gel when cool. Plant tissues have been shown to do better when cultured on a semi-solid support

TABLE 3.3

The concentration of minerals ($\mu g\ g^{-1}$) in a range of gelling agents[a]

Minerals	Gelling agents					
	Merck agar[1,b]	Bacto-agar[2]	Phyta-agar[3]	TC-agar[4]	BiTek agar[5]	Gelrite gellan[6]
Na	1200	7194	1244	596	10949	6800
S	5900	–	–	–	7120	220
K	2000	317	86	24	885	28000
Ca	110	1997	2097	2542	90	4900
P	1300	42	331	51	1005	2100
Mg	62	1002	635	478	110	1530
Fe	31	8.3	226	25	26	280
Al	7.7	6.2	75	16	–	185
B	23	109	57	80	34	1.4
Mn	0.6	0.3	46	2.2	0.5	5.3
Zn	1.5	6.6	4.5	5.7	2.2	19
Cu	0.3	0.8	0.8	0.2	0.1	2.9

[a]Source of the gelling agent: 1, Merck, Germany; 2, Difco Laboratories, USA; 3, GIBCO, USA; 4, K.C. Biologicals, USA; 5, ?; 6, Kelco, USA.
[b]1,6, after Scherer et al. (1988); 2–4, after Singha et al. (1985); 5, after Williams (1993).

than otherwise. Most of the gelling agents (agar, agarose, gelrite) used in plant tissue culture media are biological products. Being natural products and subjected to varying degrees of processing and purification the composition of these gelling agents varies with the brand and the batch, particularly their mineral composition (Table 3.3).

(i) *Agar*. This most commonly used gelling agent is obtained from red algae, especially *Gelidium amansii* It is a complex mixture of related polysaccharides built up from the sugar, galactose. These include the neutral polymer fraction, agarose, which gives strength to the gel and the highly charged anionic polysaccharides agaropectins which give agar its viscosity (Araki and Arai, 1967). The agar quality and purity vary from batch to batch as it depends a lot on the culture conditions of the algae and the varying degrees of processing and purification. The proportion of agarose to total polysaccharides, which can vary from 50 to 90%, influences the gel strength (Adrian and Assoumani, 1983). Firmness of the gel produced by a given concentration of agar varies according to the brand and the pH during autoclaving. Agar is partly hydrolyzed if it is autoclaved in an acidic medium. Agar is used at varying concentrations from 0.8 to 1%.

(ii) *Agarose*. Agarose consists of β-D(1–3) galactopyranose and 3,6-anhydro-α-L(1–4) galatopyranose linked into polymer chains of 20–160 monosaccharide units. Agarose is obtained by purifying agar to remove agaropectins with its sulphate side groups. As the process is tedious, the cost of agarose is much higher than agar. It is only used where high gel strength is required, such as in single cell or protoplast cultures. Agarose is adequate at 0.4%.

(iii) *Gelrite*. Gelrite (Kelco Division, Merck & Co.) or Phytagel (Sigma Chemical Co.), a gellan gum, is a linear polysaccharide produced by the bacterium *Pseudomonas elodea*. It comprises of linked K-glucuronate, rhamnose and cellobiose molecules (Kang et al., 1982). The commercial product contains significant quantities of K, Na, Ca and Mg (Scherer et al., 1988) but it is said to be free of organic impurities found in agar. Gelrite requires a minimum level of cations in the solution for gelling. Unlike agar, which requires heating, gelrite can be readily prepared in cold solution. To prevent clumping it should be added to rapidly stirring culture medium at room temperature.

Gelrite is a good alternative to agar not only because of its low cost per litre of medium (0.1–0.2% is sufficient) but also for the many advantages it offers. Gelrite sets as a clear gel which assists easy observation of cultures and their possible contamination. Unlike agar, the gel strength of gelrite is unaffected over a wide range of pH (Bonga and Von Aderkas, 1992). Various plant species have shown as good results on gelrite as on agar, and sometimes gelrite proved to be better. However, certain plants show hyperhydricity on gelrite, apparently due to more freely available water (Debergh, 1983). This problem could be rectified by mixing small quantities of agar with gelrite (Pasqualetto et al., 1986). Kyte (1987) has recommended the use of a mixture of gelrite and agar in a ratio of 3:1.

3.2.5. pH

The pH of the medium is usually adjusted between 5.0 and 6.0 before sterilization. However, Straus and LaRue (1954) observed that the growth of maize endosperm callus on a fresh weight basis was best at pH 7.0 and on a dry weight basis pH 6.1 proved optimal. In general, a pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar.

The pH of the medium changes at various stages of preparation and culture. pH of the medium set after the addition of the gelling agent shows a remarkable drop on autoclaving. The pH of the medium further

changes once plant tissue is placed on it. The plant tissue and the medium interact to adjust the pH to an equilibrium irrespective of the initial pH adjusted (Skirvin et al., 1986; Williams et al., 1990). The ratio of NH_4^+ and NO_3^- ions in the medium also influences the pH. When NH_4^+ is predominantly taken up the medium gets acidified due to liberation of H^+ ions, while uptake of NO_3^- ions increases pH due to liberation of OH^- ions (Dougall, 1980; Congard et al., 1986). Such pH changes then influence the availability of various mineral ions in the medium and their uptake by the plant tissue.

3.3. MEDIA SELECTION

There is no one ideal approach to formulate a suitable medium for a new system. A convenient approach could be to select three media from the available recipes, that represent high, medium and low salt media and combine them factorially with different levels of plant growth regulators suitable for the desired response. For shoot proliferation or adventitious shoot bud differentiation a commonly used auxin (NAA) and cytokinin (BAP) may be used, each at five concentrations (0, 0.5, 2.5, 5, 10 μmol l^{-1}). All possible combinations of the five concentrations of the two substances would lead to an experiment with 25 treatments (Table 3.4) with each basal medium. Select the best of the 75 treatments and test some of the available auxins and cytokinins at that concentration. While varying cytokinins, keep the auxin constant and vice versa. Test a range of sucrose concentrations (2–6%) to decide its optimal level. However, there are limitless opportunities to further improve the selected medium by manipulating its nutrient salts and plant growth regulators.

De Fossard et al. (1974) have described a 'broad spectrum experiment' to select a suitable medium for an untested system. In this approach all the components of the medium are divided into four broad categories: (a) minerals, (b) auxins, (c) cytokinins, and (d) organic nutrients (sucrose, amino acids, inositol, etc.). For each group of substances three concentrations are chosen: low (L), medium (M), and high (H) (see Table 3.5). Trying various combinations of the four categories of substances at three different concentrations leads to an experiment with 81 treatments. The best of the 81 treatments is denoted by a four-letter code. For example, the treatment with medium salts, low auxin, medium cytokinin, and high organic nutrients would be represented as MLMH. Having reached this stage it would be desirable to test different auxins and cytokinins to find the best types.

TABLE 3.4

NAA (μM)	BAP (μM)				
	0	0.5	2.5	5	10
0	1	2	3	4	5
0.5	6	7	8	9	10
2.5	11	12	13	14	15
5	16	17	18	19	20
10	21	22	23	24	25

3.4. MEDIA PREPARATION

The most simple method of preparing media today is to use commercially available dry powdered media, containing inorganic salts, vitamins, and amino acids. The powder is dissolved in purified water (10% less than the final volume of the medium), and after adding sugar, agar, and other desired supplements, the final volume is made up with purified water. The pH is adjusted, and the medium autoclaved. Several standard plant tissue culture media and a range of complete mixes (together with agar) for micropropagation of specific plant species are now available as dry powder. The powdered media may be useful for routine purposes, such as micropropagation of plant species for which composition of the medium required is well established. In such cases the use of a powdered medium should save time. However, in experimental work where it is necessary to make major qualitative and quantitative changes in the organic and/or inorganic constituents of the medium, or where powdered media are either not available or considered expensive, there are two possible ways of preparing medium. One method is to weigh and dissolve the required quantities of the ingredients separately and mix them before the preparation of the medium. A more convenient and popular method, however, is to prepare a series of concentrated stock solutions. For example, to prepare Murashige and Skoog's basal medium, four different stock solutions may be prepared (Table 3.6): (a) major salts (20× concentrated); (b) minor salts (200× concentrated); (c) iron (200× concentrated), and (d) organic nutrients except sucrose (200× concentrated). For the preparation of stock solutions (a)–(d) each component should be separately dissolved to the last particle and then mixed with the others. Separate stock solutions are prepared for each growth regulator by dissolving it in a minimal quantity of the appropriate solvent (if insoluble in water; see Section 3.2.3) and making up the final volume with purified

TABLE 3.5

Constituents and concentrations of minerals, auxin, cytokinin, and organic nutrients of the broad spectrum experiment of De Fossard et al. (1974)

Constituents	Concentration range (mM)		
	Low	Medium	High
Minerals			
NH_4NO_3	5	10	20
KNO_3	–	10	20
KH_2PO_4	0.1	–	–
NaH_2PO_4	–	1	2
KCl	1.9	–	–
$CaCl_2$	1	2	3
$MgSO_4$	0.5	1.5	3
H_3BO_3	0.01	0.05	0.15
$MnSO_4$	0.01	0.05	0.1
$ZnSO_4$	0.001	0.02	0.04
$CuSO_4$	0.00001	0.0001	0.0015
Na_2MoO_4	0.00001	0.0001	0.001
$CoCl_2$	0.0001	0.0005	0.001
KI	0.0005	0.0025	0.005
$FeSO_4$	0.01	0.05	0.1
Na_2·EDTA	0.01	0.05	0.1
Auxin	0.0001	0.001	0.01
Cytokinin	0.0001	0.001	0.01
Organic nutrients			
Inositol	0.1	0.3	0.6
Nicotinic acid	0.004	0.02	0.04
Pyridoxine·HCl	0.0006	0.003	0.006
Thiamine·HCl	0.0001	0.002	0.04
Biotin	0.00004	0.0002	0.001
Folic acid	0.0005	0.001	0.002
D-Ca-pantothenate	0.0002	0.001	0.005
Riboflavin	0.0001	0.001	0.01
Ascorbic acid	0.0001	0.001	0.01
Choline chloride	0.0001	0.001	0.01
L-Cysteine·HCl	0.01	0.06	0.12
Glycine	0.0005	0.005	0.05
Sucrose	6	60	120

water. Depending on the levels of growth regulators used, their stock solutions may be prepared at the strength of 1 mmol l^{-1} or 10 mmol l^{-1}. All the stock solutions are stored in proper plastic or glass bottles under refrigeration. The iron stock must be stored in an amber-coloured bottle.

TABLE 3.6

Stock solutions for Murashige and Skoog's medium (MS)[a]

Constituents	Amount (mg l^{-1})
Stock solution I	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2 \cdot 2H_2O$	8800
$MgSO_4 \cdot 7H_2O$	7400
KH_2PO_4	3400
Stock solution II	
KI	166
H_3BO_3	1240
$MnSO_4 \cdot 4H_2O$	4460
$ZnSO_4 \cdot 7H_2O$	1720
$Na_2MoO_4 \cdot 2H_2O$	50
$CuSO_4 \cdot 5H_2O$	5
$CoCl_2 \cdot 6H_2O$	5
Stock solution III[b]	
$FeSO_4 \cdot 7H_2O$	5560
$Na_2EDTA \cdot 2H_2O$	7460
Stock solution IV	
Inositol	20000
Nicotinic acid	100
Pyridoxine·HCl	100
Thiamine·HCl	20
Glycine	400

[a]To prepare 1 l of medium, take 50 ml of stock I, 5 ml of stock II, 5 ml of stock III, and 5 ml of stock IV.

[b]Dissolve $FeSO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$ separately in 450 ml distilled water by heating and constant stirring. Mix the two solutions, adjust the pH to 5.5, and add distilled water to make up the final volume to 1 l.

For storing coconut milk (liquid endosperm) the water collected from fruits is boiled to deproteinize it, filtered, and stored in plastic bottles in a deep freeze at –20°C. As a rule, before using the stocks the bottles must be shaken gently and if any of the solutions show a suspension of a precipitate or a biological contaminant they should be immediately discarded.

For preparing stock solutions and media, glass, distilled or purified water and chemicals of high purity (AnalaR grade) should be used.

The sequence of steps involved in preparing a medium is as follows:

(a) Required quantities of agar and sucrose are weighed and dissolved in water, about 3/4 the final volume of the medium, by heating them in a water bath or an autoclave at low pressure. This step is not necessary for a liquid medium because sucrose would dissolve even in lukewarm water.

(b) Appropriate quantities of the various stock solutions, including growth regulators and other special supplements are added. Some workers feel that it is better to add vitamins and auxins after autoclaving. If there is a special reason to do so the substance may be sterilized by filtering their solutions (adjusted to the desired pH) through microfilters[2] of pore size 0.22–0.45 μm (see Section 2.3.2).

(c) The final volume of the medium is made up with purified water.

(d) After mixing well, the pH of the medium is adjusted using 0.1 N NaOH and 0.1 N HCl.

(e) The medium is poured into the desired culture vessels. About 15 ml of the medium is dispensed in a 25 × 150 mm culture tube, and about 50 ml in a 150-ml flask. If during steps (b)–(e) the medium starts to gel, the flask containing the medium should be heated in a water bath or microwave oven and poured only when it is in a uniformly liquid state.

(f) Mouth of the culture vessels are closed with non-absorbent cotton wrapped in cheese-cloth (such closures exclude microbial contaminants but allow free gas exchange), or any other suitable closure.

(g) The culture vessels containing medium are transferred to appropriate baskets, covered with aluminium foil to check wetting of plugs during autoclaving, and sterilized by autoclaving at 120°C (1.06 kg cm^{-2}) for 15 min. If pre-sterilized, unautoclavable, plastic culture vials (petri-plates or jars) are being used the medium may be autoclaved in 250 or 500 ml flasks with suitable closures (large flasks are inconvenient for pouring) or narrow-mouthed bottles. The medium is allowed to cool to around 60°C before pouring into the vials under aseptic conditions.

(h) The medium is allowed to cool at room temperature and is stored at 4°C. When preparing a solid medium in culture tubes it is desirable to make slants by keeping the tubes tilted during cooling. Such slants provide a larger surface area for tissue growth. It is also easier to photograph cultures grown on such slants.

[2]Filter assemblies of various sizes and filter membranes of various porosity are manufactured by Millipore Intertech Inc., P.O. Box 255, Bedford, MA 01730, USA.

MEDIA NO.	SUMMARY COMPOSITION	VOLUME	PURPOSE	DATE:
				PREPARED BY:

CONSTITUENTS		TYPE	STOCK CONC.	AMOUNT ADDED
Minerals	Macro		20 x	
	Micro		200 x	
	Iron		200 x	
Organics			200 x	
Sugar				
Auxin			1 mmol.l^{-1}	
			10 mmol.l^{-1}	
Cytokinin			1 mmol.l^{-1}	
			10 mmol.l^{-1}	
Other				
Agar				
Container		pH Required pH Original pH Adjusted		

Fig. 3.1. A reference sheet used for media preparation.

3.5. CONCLUDING REMARKS

Recent studies have shown that a correct nutrient balance in the culture medium may be a prerequisite for any response of explants to plant growth regulators, and optimization of inorganic nutrients may reduce or eliminate the use of plant growth regulators (Preece, 1995). The number of adventitious buds differentiated from leaf explants of *Juinperus oxycedrus* was doubled by using SH (Schenk and Hildebrandt, 1972) medium instead of MS medium (Gomez and Segura, 1994). Whereas on MS medium optimum response was obtained in the presence of BAP and NAA, on SH medium it did not require NAA. Likewise, on full strength MS

medium the number of olive petiole explants that formed adventitious shoots was twice as many as on half strength MS medium and the amount of thidiazuron required in full MS medium was one quarter of that required with 1/2 MS (Mencuccini and Rugini, 1993). The best in vitro rooting of *Leucopogon obtectus* microshoots occurred on a agar-water medium devoid of an auxin but on media with increasing concentration of MS salts a requirement for auxin also increased (Bunn et al., 1989). Thus, it may be possible to reduce the use of plant growth regulators which sometime cause undesirable effects, such as callusing, vitrification and poor quality roots, by optimization of nutrient salts in the medium.

Street (1977a) remarked 'More false trials have been laid by mistakes in media preparation than by any other fault of technique'. To minimize human error all the steps listed above should be followed very carefully. The ingredients of the medium should be listed on paper and, after adding a component, it should be cancelled on the sheet. A reference sheet routinely used in some laboratories is depicted in Fig. 3.1. All the tubes, jars, flasks and petri-plates containing the medium should be clearly marked in such a manner that they can be identified even after autoclaving and long storage under light.

APPENDIX 3.I

Molecular weights of the compounds commonly used in tissue culture media

Compound	Chemical formula	Molecular weight
Macronutrients		
Ammonium nitrate	NH_4NO_3	80.04
Ammonium sulphate	$(NH_4)_2SO_4$	132.15
Calcium chloride	$CaCl_2 \cdot 2 \cdot H_2O$	147.02
Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$	236.16
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	246.47
Potassium chloride	KCl	74.55
Potassium nitrate	KNO_3	101.11
Potassium dihydrogen *ortho*-phosphate	KH_2PO_4	136.09
Sodium dihydrogen *ortho*-phosphate	$NaH_2PO_4 \cdot 2H_2O$	156.01
Micronutrients		
Boric acid	H_3BO_3	61.83
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	237.93
Cupric sulphate	$CuSO_4 \cdot 5H_2O$	249.68
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	223.01
Potassium iodide	KI	166.01
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	241.95
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	287.54
Sodium EDTA	$Na_2.EDTA \cdot 2H_2O$ $(C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O)$	372.25
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	278.03
Ferric-sodium EDTA	FeNa·EDTA $(C_{10}H_{12}FeN_2NaO_8)$	367.07
Sugars and sugar alcohols		
Fructose	$C_6H_{12}O_6$	180.15
Glucose	$C_6H_{12}O_6$	180.15
Mannitol	$C_6H_{14}O_6$	182.17
Sorbitol	$C_6H_{14}O_6$	182.17
Sucrose	$C_{12}H_{22}O_{11}$	342.31
Vitamins and amino acids		
Ascorbic acid (vitamin C)	$C_6H_8O_6$	176.12
Biotin (vitamin H)	$C_{10}H_{16}N_2O_3S$	244.31
Calcium pantothenate (Ca salt of vitamin B_5)	$(C_9H_{16}NO_5)_2Ca$	476.53
Cyanocobalamine (vitamin B_{12})	$C_{63}H_{90}CoN_{14}O_{14}P$	1357.64
L-Cysteine·HCl	$C_3H_7NO_2S \cdot HCl$	157.63
Folic acid (vitamin B_c, vitamin M)	$C_{19}H_{19}N_7O_6$	441.40
Inositol	$C_6H_{12}O_6$	180.16
Nicotinic acid or niacin (vitamin B_3)	$C_6H_5NO_2$	123.11

APPENDIX 3.I (*continued*)

Compound	Chemical formula	Molecular weight
Pyridoxine HCl (vitamin B_6)	$C_8H_{11}NO_3 \cdot HCl$	205.64
Thiamine HCl (vitamin B_1)	$C_{12}H_{17}ClN_4OS \cdot HCl$	337.29
Glycine	$C_2H_5NO_2$	75.07
L-Glutamine	$C_5H_{10}N_2O_3$	146.15
Glutathione	$C_{10}H_{17}N_3O_6S$	307.33
Hormones		
Auxins		
p-Chlorophenoxyacetic acid (p-CPA)	$C_8H_7O_3Cl$	186.59
3,6-Dichloro-*o*-anisic acid (Dicamba)	$C_8H_6Cl_2O_3$	221.04
2,4-Dichlorophenoxyacetic acid (2,4-D)	$C_8H_6O_3Cl_2$	221.04
Indole-3-acetic acid (IAA)	$C_{10}H_9NO_2$	175.18
3-Indolebutyric acid (IBA)	$C_{12}H_{13}NO_2$	203.23
2-Methyl-4-chlorophenoxyacetic acid (MCPA)	$C_9H_9ClO_3$	200.62
α-Naphthaleneacetic acid (NAA)	$C_{12}H_{10}O_2$	186.20
β-Naphthoxyacetic acid (NOA)	$C_{12}H_{10}O_3$	202.20
4-Amino-3,5,6-Trichloropicolinic acid (Picloram)	$C_6H_3Cl_3N_2O_2$	241.46
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	$C_8H_4Cl_3O_3$	255.49
Cytokinins		
Adenine (Ad)	$C_5H_5N_5.3H_2O$	189.13
Adenine sulphate ($AdSO_4$)	$(C_5H_5N_5)_2.H_2SO_4 \cdot 2H_2O$	404.37
6-Benzyladenine or 6-benzylamino purine (BA or BAP)	$C_{12}H_{11}N_5$	225.20
6-γ,γ-Dimethylallylamino purine or *N*-isopentenylamino purine (2-ip)	$C_{10}H_{13}N_5$	203.3
6-Furfurylamino purine (kinetin)	$C_{10}H_9N_5O$	215.21
6-(Benzylamino)-9-(2-tetrahydropyranyl)-9H-purine (SD8339)	$C_{17}H_{19}N_5O$	309.40
n-Phenyl-*N*-1,2,3- thiadiazol-5-urea (thidiazuron)	$C_9H_8N_4OS$	220.2
6-(4-Hydroxy-3methylbut-2-enylamino)-purine (zeatin)	$C_{10}H_{13}N_5O$	219.20
Gibberellins		
Gibberellic acid (GA_3)	$C_{19}H_{22}O_6$	346.37
Other compounds		
Abscisic acid	$C_{15}H_{20}O_4$	264.31
2′-Isopropyl-4′-(trimethylammonium chloride)-5′methylphenyl piperidine carboxylate (Amo 1618)		

APPENDIX 3.I (*continued*)

Compound	Chemical formula	Molecular weight
α-Cyclopropyl-α-4-methoxyphenyl (ancymidol)	$C_{15}H_{16}N_2O_2$	256.3
β-Chloroethyltrimethyl ammonium chloride (CCC)	$C_5H_{13}Cl_2N$	158.07
Colchicine	$C_{22}H_{25}NO_6$	399.43
N-Dimethylaminosuccinamic acid (paclobutrazol)	$C_{15}H_{20}ClN_3O$	293.80
Phloroglucinol	$C_6H_6O_3$	126.11
1,4-Diaminobutane; tetramethylene-diamine (putrescine dihydrochloride)	$C_4H_{12}N_2 \cdot 2HCl$	161.1
N-(3-Aminopropyl)-1,4-butanediamine (spermidine)	$C_7H_{19}N_3$	145.2
N,*N*′-(Bis 3-aminopropyl)-1,4-butane-diamine (spermine)	$C_{10}H_{26}N_4$	202.3
2,3,5-Tri-iodobenzoic acid (TIBA)	$C_7H_3I_3O_2$	499.81

APPENDIX 3.II

Atomic weights

Name	Symbol	Atomic weight
Aluminium	Al	26.98
Boron	B	10.82
Calcium	Ca	40.08
Carbon	C	12.011
Chlorine	Cl	35.457
Cobalt	Co	58.94
Copper	Cu	63.54
Hydrogen	H	1.008
Iodine	I	126.91
Iron	Fe	55.85
Magnesium	Mg	24.32
Manganese	Mn	54.94
Molybdenum	Mo	95.95
Nickel	Ni	58.71
Nitrogen	N	14.008
Oxygen	O	16.00
Phosphorus	P	30.975
Potassium	K	39.10
Sodium	Na	22.991
Sulphur	S	32.066
Zinc	Z	65.38

Chapter 4

Cell Culture

4.1. INTRODUCTION

At the turn of the 19th century Haberlandt (1902) made pioneering attempts to isolate and culture single cells from the leaves of flowering plants. He had envisaged that such a system would provide an excellent opportunity for investigating the properties and potential of plant cells and also to understand the interrelationships and complementary influences of cells in multicellular organisms (see Krikorian and Berquam, 1969). Although Haberlandt failed to achieve the division of free cells for various reasons (see Chapter 1), his detailed paper of 1902 stimulated several workers to pursue this line of investigation. To date the progress in this field has been so spectacular that it is possible not only to culture free cells but also to induce divisions in a cell cultured in complete isolation and to raise whole plant from it.

Plant physiologists and plant biochemists have recognized the merits of a single cell system over intact organs and whole plants for studying cell metabolism and the effects of various substances on cellular responses. The free cell system permits quick administration and withdrawal of diverse chemicals and radioactive substances (Gnanam and Kulandaivelu, 1969; Edwards and Black, 1971; Harada et al., 1972). The cloning of single cells permits crop improvement through the extension of the techniques of microbial genetics to higher plants. Large scale cultivation of plant cells in vitro provides a viable alternative for the production of vast arrays of commercially important phytochemicals (Chapter 17).

4.2. ISOLATION OF SINGLE CELLS

4.2.1. From intact plant organs

(i) *Mechanical method.* Leaf tissue is the most suitable material for the isolation of single cells. Ball and Joshi (1965), Joshi and Noggle (1967), and Joshi and Ball (1968) isolated cells from mature leaves of *Arachis hypogaea* by first tearing across the leaf to expose the mesophyll cells, followed by scraping of the cells with a fine scalpel. The isolated cells

were directly placed into liquid medium (for composition see Table 4.2). Many of the free cells were viable and underwent sustained divisions in culture. This is the first report demonstrating that free cells capable of dividing in artificial medium can be isolated from intact plant organs. However, these workers were unable to isolate viable cells from the leaves of most other plants they tested.

Gentle grinding of the leaves followed by cleaning the cells by filtration and centrifugation is now widely used for the isolation of mesophyll cells. Gnanam and Kulandaivelu (1969) isolated mesophyll cells active in photosynthesis and respiration from mature leaves of several species. The procedure involved mild grinding of 10 g leaves in 40 ml of the grinding medium (20 μmol sucrose, 10 μmol $MgCl_2$, 20 μmol Tris–HCl buffer, pH 7.8) with a mortar and pestle. After filtering the homogenate through two layers of fine muslin cloth the released cells were washed by low centrifugation in the grinding medium. Besides dicots, many monocots, including grasses (unidentified), yielded intact mesophyll cells by this procedure. Edwards and Black (1971) used a similar method to isolate metabolically active mesophyll cells and bundle sheath cells from crabgrass (*Digitaria sanguinalis*), and mesophyll cells of spinach.

Rossini (1969, 1972) described a method for the large-scale mechanical isolation of free parenchymatous cells from the leaves of *Calystegia sepium*. This method was later used successfully with *Asparagus officinalis* and *Ipomoea hederifolia* by Harada et al. (1972). The details of the procedure followed by these workers are given in Appendix 4.I.

Mechanical isolation of cells has at least two distinct advantages over the enzymatic method: (a) it eliminates the exposure of cells to the harmful effect(s) of enzymes, and (b) in this method the cells need not be plasmolyzed which is often desirable in physiological and biochemical studies. Schwenk (1980) isolated viable cells from soybean cotyledons in sterile distilled water. In several cases the mechanically isolated cells have been reported to divide and form callus (Ball and Joshi; 1965; Kohlenbach, 1966; Rossini, 1972; Schwenk, 1980).

Although Gnanam and Kulandaivelu (1969) were able to isolate cells from the leaves of several species, the mechanical method of cell isolation is not universally applicable. Mesophyll cells could be successfully isolated by this method only in such cases where the parenchymatous tissue was loosely arranged, having few points of cell to cell contact (Rossini, 1972).

(ii) *Enzymatic method.* Enzymatic isolation of single cells has been practised for quite some time by plant physiologists and biochemists (Chayen, 1952; Zaitlin, 1959; Sato, 1968). A procedure for the large-scale

isolation of metabolically active mesophyll cells of tobacco by pectinase treatment was first reported by Takebe et al. (1968) and later extended by Otsuki and Takebe (1969) to 18 other herbaceous species. For the details of their method, see Appendix 4.II.

Takebe et al. (1968) demonstrated that the presence of potassium dextrane sulphate in the maceration mixture improved the yield of free cells. The enzyme macerozyme used to isolate cells not only degrades the middle lamella but also weakens the cell wall. It is, therefore, essential that in the enzymatic method of cell isolation the cells are provided with osmotic protection. Tobacco protoplasts collapsed within the cell wall when mannitol was used at a concentration below 0.3 M (Takebe et al., 1968).

A special feature of enzymatic isolation of cells is that in some cases it has been possible to obtain pure preparations of spongy parenchyma and palisade parenchyma (Takebe et al., 1968). However, some plant species, especially *Hordeum vulgare*, *Triticum vulgare* and *Zea mays*, have proved difficult materials for cell isolation through the enzymatic methods (Zaitlin, 1959; Otsuki and Takebe, 1969). According to Evans and Cocking (1975) the mesophyll cells in these cereals appear elongated with a number of constrictions. Within the leaf these cells may form an interlocking structure preventing their isolation.

Rubos (1985) described a method to isolate viable single cells from zygotic embryos of cabbage, carrot and lettuce. The excised embryos were treated with 1% macerozyme, at 32°C, for 2 h, on a shaker (50 rev min^{-1}). The treated embryos were forced through a 5 ml hypodermic syringe several times to release single cells from the confines of the surrounding cuticular layer.

4.2.2. From cultured tissues

Traditionally, the single cell systems used in basic and applied research are isolated from cultured tissues because this approach is convenient and widely applicable. Cultures are initiated by simply placing freshly cut sections from surface-sterilized plant organs on a nutrient medium containing suitable hormones; generally auxins and cytokinins are used. On such a medium the explant exhibits callusing which usually starts at the cut ends and gradually extends over the entire surface of the tissue. The callus is separated from the parent explant and transferred to a fresh medium of the same composition to build up a reasonable amount of tissue. Repeated subculture on the agar medium may also improve the friability of the tissue which is highly desirable for raising a fine cell suspension in liquid medium (Wilson and Street, 1975; Noguchi et al., 1977). Wilson and Street (1975) observed that when freshly isolated callus of

Hevea brasiliensis was transferred to a liquid medium and agitated, it broke up into small pieces but all efforts to raise a fine suspension of cells from it failed. They transferred the callus pieces grown in liquid medium back to the agar medium. After 2 months a very friable callus was formed which on transfer to the liquid medium gave a fine suspension.

To obtain free cells, pieces of undifferentiated and friable calli are transferred to liquid medium in flasks or some other suitable vial and the medium is continuously agitated by a suitable device. Such cultures are called 'suspension cultures'. Agitation of the medium serves at least two functions. First, it exerts a mild pressure on cell aggregates, breaking them into smaller clumps and single cells and, secondly, agitation maintains uniform distribution of cells and cell clumps in the medium. Movement of the medium also provides gaseous exchange between the culture medium and culture air.

4.3. SUSPENSION CULTURES

4.3.1. General techniques

Basically there are two types of suspension cultures: batch cultures and continuous cultures.

(i) *Batch cultures.* These are used for initiating single cell cultures. Cell suspensions are grown in 100–250 ml flasks each containing 20–75 ml of culture medium. The cultures are continuously propagated by routinely taking a small aliquot of the suspension and transferring it to a fresh medium (ca. 5× dilution).

During the incubation period the biomass of the suspension cultures increases due to cell division and cell enlargement. This continues for a limited period after which the growth stops due to the exhaustion of some factors or the accumulation of certain toxic metabolites in the culture medium. If at this stage a small aliquot of the cell suspension, with uniformly dispersed cells and cell aggregates, is transferred to a fresh medium of the same composition (subculture), the cell growth is revived. The biomass growth in batch cultures follows a fixed pattern as shown in Fig. 4.1. Initially, the culture passes through a lag phase, followed by a brief exponential growth phase during which active cell divisions occur. After three to four cell generations the growth declines and finally the culture enters the stationary phase. The duration of the lag phase would largely depend on the growth phase of the stock culture at the time of subculture and the size of the inoculum (Stuart and Street, 1969). Cultures can be maintained continuously in exponential phase by frequent

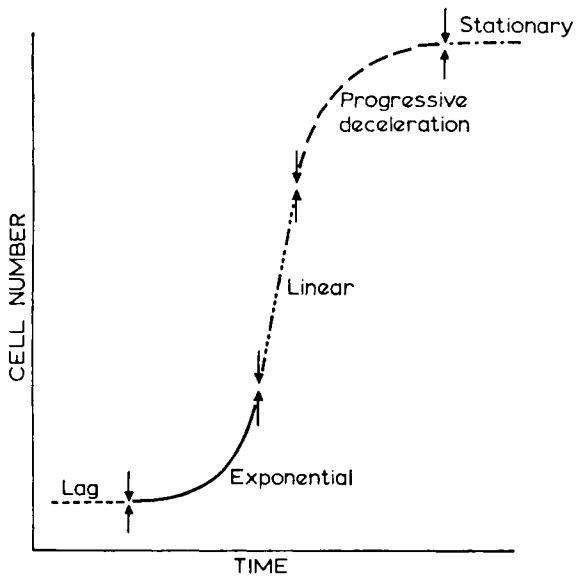


Fig. 4.1. Model curve relating cell number per unit volume of culture to time in a batch-grown cell suspension culture. Growth phases are labelled (after Wilson et al., 1971).

(every 2–3 days) subculture of the suspensions. Prolonged maintenance of cultures in the stationary phase may result in extensive death and lysis of cells. It is, therefore, critical that suspensions are subcultured soon after they have reached their maximum dry-weight yield (Street, 1977b). Addition of conditioned medium (in which cell cultures have been grown before) reduces the lag phase dramatically (Bergmann, 1977). Cell doubling time in suspension cultures varies with the tissue: *Nicotiana tabacum*, 48 h; *Acer pseudoplatanus*, 40 h; *Rosa* sp. 36 h; *Haplopappus gracilis*, 22 h (Butcher and Ingram, 1976).

For subculture of the suspension cultures a pipette or syringe with an orifice fine enough to allow single cells and small aggregates (2–4 cells) to pass through, but excluding larger cell clumps, is used. At the time of subculture the flask is allowed to stand for a few seconds to allow the large colonies to settle down, and suspension is taken from the upper part of the culture. Regular practice of this procedure should allow the build-up of a fine suspension.

The texture of a callus is genetically controlled and often it may be difficult to obtain a good dispersion of cells under any conditions. Generally, however, it has been possible to improve the tissue dissociation by manipulating the media composition and subculture routine. Addition of 2,4-D (Negrutiu and Jacobs, 1977), small amounts of hydrolytic enzymes

such as cellulase and pectinase (Street, 1977b), or substances like yeast extract (Noguchi et al., 1977), had a promotory effect on cell dispersion. Negrutiu and Jacobs (1977) reported that maximum dissociation of cells was achieved by permanently maintaining the cultures in the late log phase by adding fresh medium every other day in a proportion that the biomass/medium volume was kept at 2. To obtain a fine suspension culture it is of prime importance that, as far as possible, a friable callus is used initially. As mentioned earlier, friability of the callus tissue often increases if it is maintained on a semi-solid medium for 2–3 passages. However, it must be borne in mind that even the finest cell suspensions carry cell aggregates and no suspension culture is comprised exclusively of free cells.

Owing to certain inherent drawbacks of the system, batch cultures are not ideal for studies of cell growth and metabolism (Fowler, 1977; Street, 1977b; Wilson, 1980). Batch cultures are characterized by a constant change in the pattern of cell growth and metabolism, and the composition of the nutrient medium. In these cultures the exponential growth with a constancy of cell doubling time may be achieved for a short time but there is no period of steady-state growth in which the relative cell concentrations of metabolites and enzymes are constant (Wilson, 1980). To a certain extent these problems are overcome by continuous cultures.

(ii) *Continuous cultures.* A number of culture vessels have been designed to grow large-scale cultures under steady state for long periods by adding fresh medium and draining out the used medium (Street, 1977b). Continuous cultures may be of the close type or the open type. In the former, the addition of fresh medium is balanced by outflow of the old medium. The cells from the outflowing medium are separated mechanically and added back to the culture. Thus, in 'close continuous cultures' cell biomass continues to increase as the growth proceeds. In contrast, in the 'open continuous cultures' the inflow of the medium is accompanied by a balancing harvest of an equal volume of the culture (medium and cells). The rate of inflow of medium and culture harvest are so adjusted that the cultures are maintained at a constant, submaximal growth rate indefinitely (Fowler, 1977; Street, 1977b). Two major types of open continuous culture have been used: chemostat and turbidostat. In chemostat cultures, a steady state of cell growth is maintained by a constant inflow of the fresh medium in which the concentration of a chosen nutrient (nitrogen, phosphorus or glucose) has been adjusted so as to be growth-limiting. In such a medium all constituents other than the growth-limiting nutrient are present at concentrations higher than that required to maintain the desired rate of cell growth. The level of the growth-

TABLE 4.1

Culture medium for suspension cultures of tobacco[a]

Constituents	Amounts (mg l^{-1})
Inorganic salts	As in MS medium
Thiamine·HCl	10
Pyridoxine·HCl	10
Nicotinic acid	5
myo-Inositol	100
Casein hydrolysate	1000
2,4-D	2
Kinetin	0.1
Sucrose	30000
pH	5.7

[a]After Reynolds and Murashige (1979).

limiting factor is so adjusted that any increase or decrease in it is reflected by corresponding increase or decrease in the growth rate of cells. In turbidostat cultures, the input of fresh medium is intermittent, controlled by an increase in the turbidity of the culture from cell growth. A preselected biomass density is maintained by the wash-out of cells.

Chemostat cultures offer the possibility of maintaining a steady-state of cell growth and metabolism, and to determine the effect of individual growth-limiting nutrients on cell growth. However, despite the clear-cut advantages, continuous cultures are not used widely for large scale plant cell culture, probably because these cultures require a lot of attention, and the equipment is not generally available (Wilson, 1980).

4.3.2. Culture medium for suspensions

The medium used for raising fast growing friable callus should generally prove suitable for initiating suspension cultures of that species provided, of course, agar is omitted from it. Manipulation of the auxin/cytokinin ratio to achieve better cell dispersion is desirable. For tobacco, increasing the concentration of 2,4-D from 0.3 mg l^{-1} to 2 mg l^{-1} and supplementing the callus medium with additional vitamins and casein hydrolysate (see Table 4.1) have been recommended (Reynolds and Murashige, 1979).

In actively growing suspension cultures the inorganic phosphate is rapidly utilized and, consequently, it soon becomes a limiting factor (Eriksson, 1965; Noguchi et al., 1977). Noguchi et al. (1977) have demonstrated that in tobacco suspension cultures maintained in a medium with

standard MS salts the phosphate concentration declines to almost zero within 3 days of the initiation of culture. When the phosphate concentration in the medium was raised three times the original level, it was completely utilized by the cells within 5 days. B_5 and ER media given in Table 3.1 were developed for suspension cultures of higher plants. These and other synthetic media are normally suitable only if the initial population density is around 5×10^4 cells ml^{-1} or higher. With a lower cell density the medium needs to be enriched with various other components (see Section 4.4.2).

4.3.3. Agitation of the medium

To achieve movement of the culture medium in suspension cultures various types of set-up have been used. Muir (1953), who for the first time demonstrated that cells of tobacco and *Tagetes erecta* can be cultured in suspension cultures, used an orbital platform shaker. This is still the most popular method of growing batch suspension cultures. The platform of the shaker is fitted with clips of appropriate size for holding the flasks. Often the clips are interchangeable to permit the use of flasks of different sizes. An orbital shaker with a variable speed of 30–150 rev. min^{-1} is satisfactory (speed above 150 rev. min^{-1} is unsuitable; Street, 1977b) with stroke in the range of 2–3 cm orbital motion.

4.3.4. Synchronization

A synchronous culture is one in which the majority of cells proceed through each cell cycle phase (G_1, S, G_2 and M) simultaneously. The degree of synchrony is expressed as percentage synchrony.

For studying cell cycle and cell metabolism in suspension cultures it is advantageous to use synchronous or partially synchronous cultures, which exhibit amplification of each event of the cell cycle as compared to non-synchronous cultures (King, 1980). Since cell suspension cultures are normally asynchronous, considerable efforts have been made to achieve a reasonable synchrony. Synchronization of asynchronous cultures is characterized by sequential alternation in frequency distribution of different cell cycle events. It has been emphasized by King and Street (1977) and King (1980) that the degree of synchrony should not be determined solely by mitotic index; it should be based on a number of parameters determined independently, e.g. (a) the percentage of cells at a specific point in the cycle at one moment in time, (b) the percentage of cells passing a specific point in the cycle during a brief specific period, and (c) the percentage of total time required for all the cells to pass a specific point in

the cycle. The methods used to achieve synchronization of cell suspensions fall under two categories: starvation and inhibition.

(i) *Starvation.* In this method cells are first arrested in the G_1 or G_2 phase of the cell cycle by starving them of a nutrient or hormonal factor required for cell division. After a period of starvation when the limiting factor is supplied into the medium the stationary cells enter division synchronously.

Up to 80–90% of the cells in the explants of tuber tissue of *Helianthus tuberosus* excised in low intensity green light and cultured in the dark on a nutrient medium containing 2,4-D divided synchronously (Mitchell, 1967; Yeoman and Evans, 1967; Davidson and Yeoman, 1974; Fraser and Loening, 1974; Aitchison et al., 1977). Suspension cultures of *Acer pseudoplatanus* from the stationary phase entered synchronous division when they were inoculated into fresh medium at low density (Street, 1968). The cells in the stationary phase are locked up in the G_1 phase of the cell cycle (Bayliss and Gould, 1974; King and Street, 1977), which is probably due to the depletion of nitrate ions in the medium (King, 1977). In large-scale cell cultures of *A. pseudoplatanus* a high level of cell synchrony was maintained over five cell cycles, as revealed by the step-wise increase in cell number at each successive cytokinesis. Komamine et al. (1978) achieved synchrony in *Vinca rosea* cultures by starving them of phosphate for 4 days and then transferring to phosphate-containing medium.

Growth hormone starvation of cells has been used to synchronize cell cultures of *Nicotiana tabacum* var. Wisconsin 38 (cytokinin; Jouanneau, 1971; Peaud-Lenoel and Jouanneau, 1971) and *Daucus carota* (auxin; Nishi et al., 1977).

(ii) *Inhibition.* Inhibitors of DNA synthesis, such as 5-aminouracil (Eriksson, 1966; Mattingley, 1966; Kovacs and Van't Hof, 1970; Butenko et al., 1974), FUdR (Blaschke et al., 1978; Cress et al., 1978), hydroxyurea and thymidine (Eriksson, 1966), have been used to synchronize cell cultures. When cells are treated with these chemicals the cell cycle proceeds only up to the G_1 phase and the cells are collected at the G_1/S boundary. Removal of the inhibitor is followed by synchronous division of cells. In this method cell synchrony is limited to only one cell cycle (King, 1980). Periodic flushing of chemostat cultures of *Glycine max* with nitrogen (Clowes, 1976) or ethylene (Constabel et al., 1977) are also reported to induce cell synchrony. However, in all these cases the only evidence of synchrony produced was fluctuation in the mitotic index which is not entirely satisfactory (see King, 1980).

4.3.5. Assessment of growth in suspension cultures

Growth in plant cell suspension cultures is commonly measured by cell counting, determination of total cell volume (packed cell volume, PCV), and fresh and dry-weight increase of cells and cell colonies.

(i) *Cell counting*. Since suspension cultures invariably carry cell colonies of various sizes it is difficult to make a reliable counting of cell numbers by taking samples directly from the flask. The accuracy of cell counting may be improved if the cells and cell aggregates are first dispersed by treating them with chromic acid (5–8%) or pectinase (0.25%). The method followed by Street and co-workers (Street, 1977b) to count sycamore cells is as follows: add 1 volume of culture to 2 volumes of 8% chromic trioxide and heat to 70°C for 2–15 min (the duration is determined by the growth of the culture). Cool the mixture and shake vigorously for 10 min before counting the cells in a haemocytometer.

(ii) *Packed cell volume* (PCV). To determine PCV transfer a known volume of uniformly dispersed suspension to a 15-ml graduated centrifuge tube and spin at 200 $\times g$ for 5 min. PCV is expressed as ml pellet ml^{-1} culture.

(iii) *Cell fresh weight* (FW). This can be determined by collecting cells on a pre-weighed (in wet condition) circular filter of nylon fabric supported in a Hartley funnel, washing the cells with water to remove the medium, draining under vacuum, and reweighing.

(iv) *Cell dry weight* (DW). Follow as above using a pre-weighed dry nylon filter and after collecting the cells on the filter dry them for 12 h at 60°C and reweigh. Cell weight is expressed as per culture or per 10^6 cells.

(v) *Non-invasive methods*. All four methods described above require withdrawal of culture samples. Recently two non-invasive methods to characterize growth in batch cultures have been described.

Schripsema et al. (1990) observed a direct correlation between loss of weight of the contents of a culture vessel and the curve representing dissimilation of sugar into CO_2. The dissimilation curve clearly shows the different growth phases, viz. lag phase, exponential phase and stationary phase. Therefore, by periodic weighing of a culture flask, provided with a stable closure (e.g. Silicosen T-type plugs) for accurate correlation of water loss, a dissimilation curve can be prepared and the growth characterized.

In the method suggested by Blom et al. (1992), the culture flask, fitted with a ruler, is tilted at an angle of 30° or 60° (same each time) for 5 min and the height of the sediment recorded. The change in the height of the sediment with the age of the culture would represent the change in fresh weight of the cells as there is a direct correlation between the two parameters.

4.3.6. Assessment of viability of cultured cells

(i) *Phase contrast microscopy*. Microscopic assessment of cell viability is based on cytoplasmic streaming and the presence of a healthy nucleus (Negrutiu and Jacobs, 1977). While the phase contrast microscopy gives a better picture of these feature, it is often not difficult to observe them under bright field microscopy.

(ii) *Reduction of tetrazolium salts*. In this test the respiratory efficiency of cells is measured by reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the red dye formazan. Formazan can be extracted and measured spectrophotometrically. Although this method allows quantification of observations, it alone may not always give a reliable picture of the cell viability (Withers, 1980).

(iii) *Fluorescein diacetate* (FDA) method. This technique offers a quick visual assessment of percentage viability of cells. Stock solution of FDA at a concentration of 0.5% is prepared in acetone and stored at 0°C. To test viability it is added to the cell or protoplast suspension (for protoplasts, an appropriate osmotic stabilizer is added to the FDA solution) at a final concentration of 0.01%. After about 5 min incubation the cells are examined, using a mercury vapour lamp with a suitable excitation filter and suppression filter. FDA is non-fluorescing and non-polar, and freely permeates across the plasma membrane. Inside the living cell it is cleaved by esterase activity, releasing the fluorescent polar portion fluorescein. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cells, but in dead and broken cells it is lost. When illuminated with UV light it gives green fluorescence (Widholm, 1972; Larkin, 1976).

(iv) *Evan's blue staining*. The stain can be used complementary to FDA. When the cells are treated with a dilute (0.025%) solution of Evan's blue the damaged cells take up the stain but the intact and viable cells exclude it and, thus, remain unstained.

4.4. CULTURE OF SINGLE CELLS

In his pioneering attempt to culture mechanically isolated mesophyll cells, Haberlandt (1902) was successful in maintaining the cells alive for about 10 days. During this period cell swelling and wall thickening occurred but the cells failed to divide. Schmucker (1929) reported that mechanically isolated cells from the leaves of *Macleaya cordata* divided repeatedly in filter-sterilized sap of the same leaves. Kohlenbach (1959, 1965) confirmed the ability of these cells to undergo sustained divisions. Since then steady progress has been made in this field of research.

Ball and Joshi (1965) used time-lapse photomicrography and studied the development of an individual mesophyll cell of peanut in liquid medium. They noted that after 3–5 days in culture the leaf cell increased in size such that it no longer resembled a palisade cell. Accumulation of plastids around the nucleus (systrophy) preceded actual cell division. According to these authors only palisade cells divided, whereas the spongy parenchyma cells died. Later, Jullien (1970) demonstrated that spongy cells of peanut are also capable of dividing provided the isolation of the cells has been carried out properly. Similarly, Rossini (1972), who made cinematographic studies of the cultures of mesophyll cells of *Calystegia*, observed that both palisade and spongy parenchyma cells undergo division (see Fig. 4.2). Under optimum conditions 60% of these cells divided. However, the division of spongy cells occurs slightly later than that of palisade cells.

4.4.1. Techniques of single cell culture

The most popular technique of single cell culture is Bergmann's cell plating technique (Bergmann, 1960; see Fig. 4.3). Free cells are suspended in a liquid medium at a density twice the finally desired plating cell density. Melted agar (0.6–1%)-containing medium of otherwise the same composition as the liquid medium is cooled and maintained at 35°C in a water bath. Equal volumes of the two media are mixed and rapidly spread out in petri dishes in such a manner that the cells are evenly distributed and fixed in a thin layer (ca. 1 mm thick) of the medium after it has cooled and solidified. The dishes are sealed with parafilm. Suspension cultures which carry cell aggregates in addition to free cells should be filtered through a sieve which would allow only single cells and small cell aggregates to pass through. The large cell aggregates are discarded and only the fine suspension is plated. The plates may be observed under an inverted microscope and single cells marked on the outside of the plate by a fine marker to ensure the isolation of pure single cell clones.

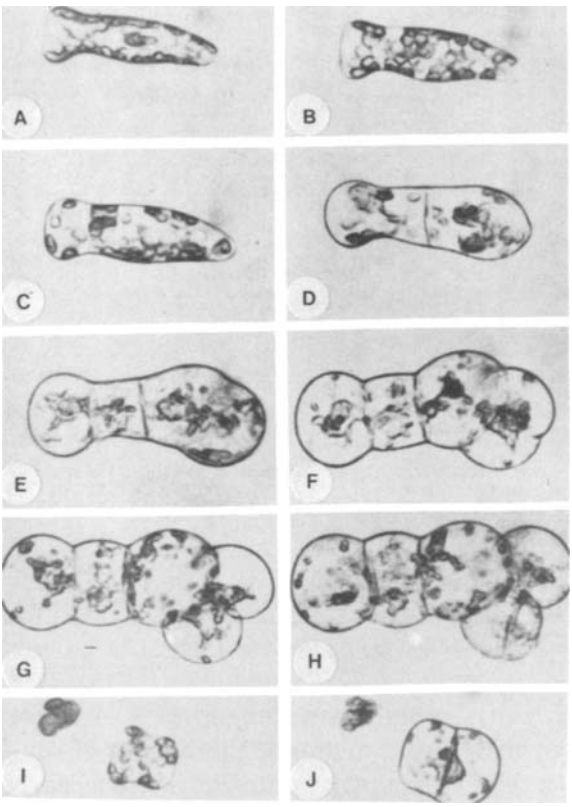


Fig. 4.2. Time-lapse pictures of divisions in an isolated palisade (A–H) and a spongy cell (I,J) of *Calystegia sepium*. A,I. Cells on the day of inoculation. B–H. 1,2,3,4,5,6 and 7 days after inoculation. J. On the fifth day of inoculation (after Rossini, 1972).

The culture plates are incubated in the dark at 25°C. It has been a common experience that frequent inspection of plates under the microscope light during the incubation period adversely affects the development of the colonies (Street, 1977b). In such cases it would be advisable to keep the observations to a bare minimum.

Free single cells can also be plated in a thin layer of liquid medium as commonly practised for protoplast culture (see Chapter 12). Cells isolated directly from plant organs have been frequently cultured in a liquid medium (Ball and Joshi, 1965; Kohlenbach, 1965; Rossini, 1969). A disadvantage in using a liquid medium is that the follow-up of an individual cell and its derivatives is extremely difficult because the cells are not in a fixed position.

If the plating cell density is determined at the time of culturing and a known volume of suspension is transferred to each plate it should be

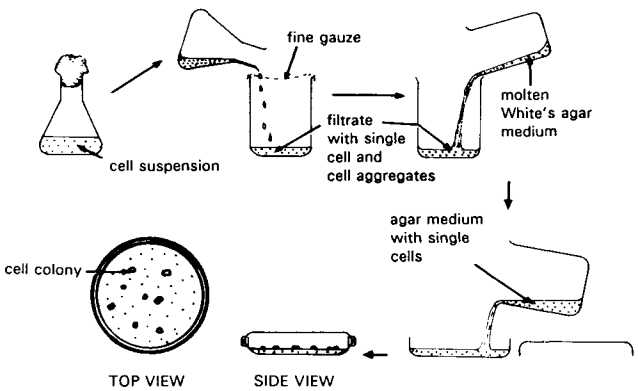


Fig. 4.3. Diagrammatic summary of steps involved in Bergmann's technique of cell plating (after Konar, 1966).

possible to make a quantitative assessment of plating efficiency using the formula:

$$\text{Plating efficiency} = \frac{\text{No. colonies / plate at the end of the experiment}}{\text{No. of cell units initially / plate}} \times 100$$

When cells are plated at an initial population density of 1×10^4 or 1×10^5 cells ml^{-1}, either in agar or in liquid medium, the mixing of colonies derived from neighbouring cells frequently occurs at a fairly early stage, much before they can be successfully diluted. This complicates the isolation of pure cell clones. The problem can be minimized if the effective plating cell density can be reduced or individual cells can be cultured in complete isolation. However, as in suspension cultures, under normal conditions there is a plating density optimum for each species, and cells fail to divide below a critical cell density. To grow single cells at low densities or individually, special requirements need to be fulfilled. Various methods have been described to grow individual cells.

(i) *The filter paper raft-nurse technique*. This method was developed by Muir et al. (1954) to culture single cells from suspension cultures and friable calli of tobacco and marigold. It involves cultivating individual cells on top of an actively growing callus separated by a piece of filter paper (see Fig. 4.4). In practice, individual cells are isolated from suspension cultures or a friable callus with the aid of a micropipette or microspatula. Several days before cell isolation, sterile 8 × 8 mm squares of filter paper are placed aseptically on top of the established callus of the same or different species. The filter paper is wetted by liquid and nutrients from the

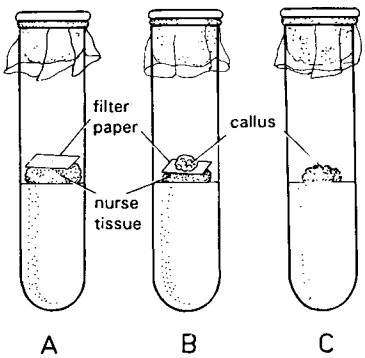


Fig. 4.4. Nurse-tissue technique to raise single cell clones. (A) A single cell from callus placed on filter paper lying on the top of a large callus (nurse tissue). (B) The cell has divided and formed a small tissue. (C) The tissue of single-cell origin has grown into a big callus after transfer from the filter paper to the medium directly (after Muir et al., 1958).

nurse tissue piece. The isolated single cell is placed on the wet filter paper raft. Cell transfer should be rapid in order to avoid excessive drying of the cell and the raft. After a macroscopic colony develops from the cell it is transferred to agar medium for further growth and maintenance of the cell clone.

An isolated cell which generally fails to divide when plated directly on the medium used for callus cultures is able to divide under the nursing effect of the callus. Apparently, the callus supplies the cell with not only the nutrients from the culture medium but something more that is critical for cell division. The cell division factor(s) can diffuse through the filter paper. The effect of callus tissue in stimulating the division of isolated cells can also be demonstrated by putting two callus pieces on an agar plate and seeding single cells around them. In such cultures cells close to the calli divide first. The beneficial effect of conditioned medium in single cell culture at low density is yet another evidence of the release of metabolites by growing tissues which are essential for cell division (see Section 4.4.2).

The feeder layer technique for low density cell plating used by some workers (Raveh et al., 1973; Vardi, 1978; Cella and Galun, 1980) is also based on the same principle as the raft-nurse technique (see Chapter 12).

(ii) *The microchamber technique.* This method was developed by Jones et al. (1960). They replaced the nurse tissue by a conditioned medium and grew single cells in a microchamber (see Fig. 4.5). In this method a drop of the medium carrying a single cell is isolated from suspension cultures, placed on a sterile microscope slide and ringed with sterile mineral

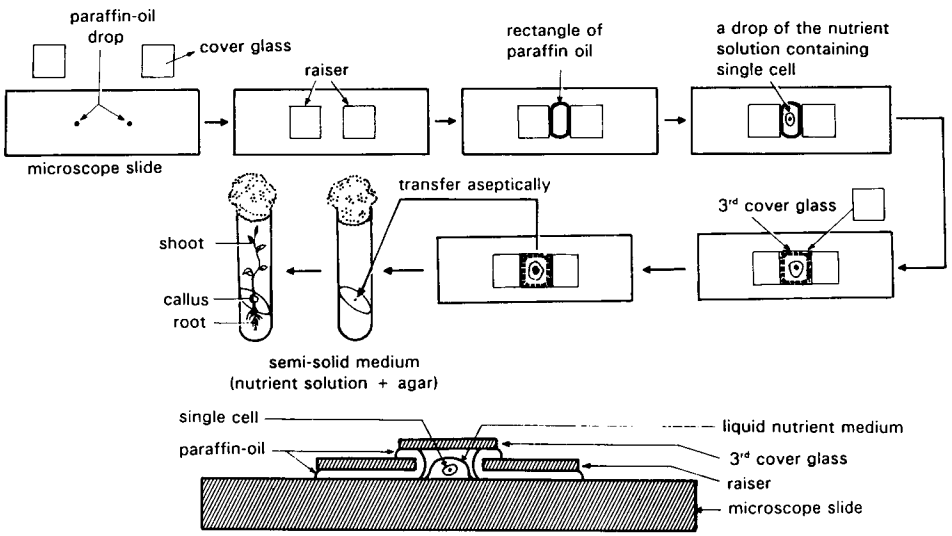


Fig. 4.5. Diagrammatic summary of the steps involved in the microchamber technique of cell cloning (after Jones et al., 1960).

oil. A drop of oil is placed on either side of the culture drop and a coverglass placed on each drop. A third coverglass is then placed on the culture drop bridging the two coverglasses and forming a microchamber to enclose the single cell aseptically within the mineral oil. The oil prevents water loss from the chamber but permits gaseous exchange. The whole microchamber slide is placed in a petri-dish and incubated. When the cell colony becomes sufficiently large the coverglass is removed and the tissue is transferred to fresh liquid or semi-solid medium.

The microchamber technique permits regular observation of the growing and dividing cell. Vasil and Hildebrandt (1965) used the microchamber method and demonstrated that a complete flowering plant can be raised starting from an isolated single cell (see Fig. 4.6). Unlike Jones et al. (1960), they used a fresh medium containing mineral salts, sucrose, vitamins, Ca-pantothenate and coconut milk to culture a single cell.

(iii) *Microdrop method.* This method has been especially useful for culturing individual protoplasts but there is no reason why it cannot be equally effective for single cell culture (for details of this technique see Chapter 12).

4.4.2. Factors affecting single cell culture

The composition of the medium and the initial plating cell density are

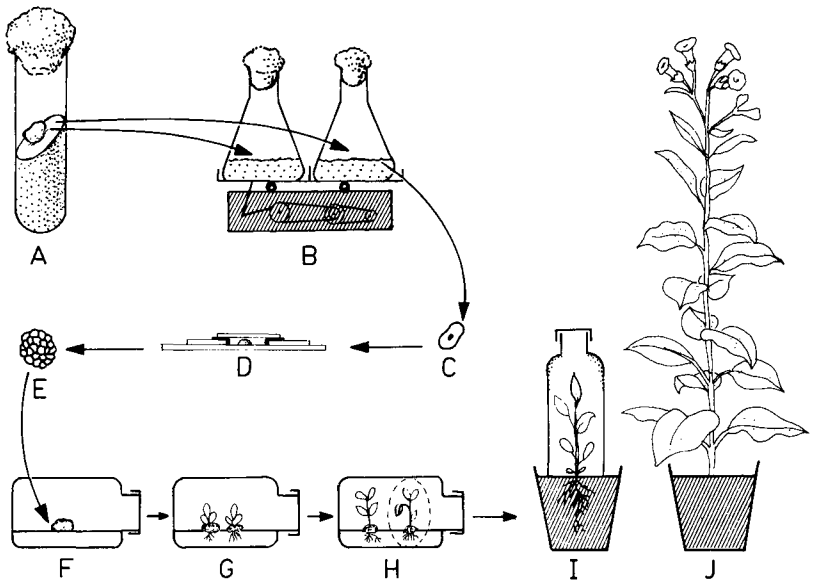


Fig. 4.6. Development of a tobacco plant from a single cell. A callus is raised from a small piece of tissue excised from the pith (A). By transferring it to a liquid medium and shaking the culture flasks (B) the callus is dissociated into single cells. A cell (C) is mechanically removed from the flask and placed in a drop of culture medium in micro-chamber (D). A small tissue (E) derived from the cell through repeated divisions is then transferred to a semi-solid medium where it grows into a large callus (F), and eventually differentiates plants (G,H). When transferred to soil (I,J) these plants grow to maturity, flower and set seeds (from the work of Vasil and Hildebrandt, 1965).

critical for single cell culture. These two factors are interdependent. When cells are plated at a high density (5×10^4 or 1×10^5 cells ml^{-1}) a purely synthetic medium with a composition similar to that used for suspension cultures or callus cultures is generally satisfactory. The composition of three media employed for the culture of isolated mesophyll cells is given in Table 4.2.

The culture requirements of cells become increasingly complex as the plating cell density is decreased. This population effect on cell division can be effectively replaced by the addition to the minimal medium of undefined factors such as coconut milk, casein hydrolysate and yeast extract. Efforts have been made to develop synthetic culture medium for cells plated at low density. *Convolvulus* cells plated at low density required a cytokinin and amino acids that were not necessary for the callus culture of that species (Earle and Torrey, 1965a). Similarly, for sycamore cells plated at low density it was necessary to add a cytokinin, gibberellic acid and amino acids to the medium that was otherwise satisfactory for callus cultures of that plant (Stuart and Street, 1971). Kao and Michay

TABLE 4.2

Composition of media recommended for the culture of isolated mesophyll cells[a]

Constituents	Amounts (mg l^{-1})		
	Rossini (1972)	Joshi and Ball (1968)	Kohlenbach (1984)
KNO_3	950	–	950
KCl	–	750	–
NH_4NO_3	725	–	720
$NaNO_3$	–	600	–
$MgSO_4 \cdot 7H_2O$	187	250	185
$CaCl_2$	169	–	–
$CaCl_2 \cdot 6H_2O$	–	112	166
KH_2PO_4	69	–	68
$NaH_2PO_4 \cdot 2H_2O$	–	141	–
NH_4Cl	–	5.35	–
$MnSO_4 \cdot 4H_2O$	12.5	–	25
$MnCl_2 \cdot 4H_2O$	–	0.036	–
H_3BO_3	5	0.056	10
$ZnSO_4 \cdot 4H_2O$	5	–	10
$ZnCl_2$	–	0.15	–
$NaMoO_4 \cdot 2H_2O$	0.125	0.025	0.25
$CuSO_4 \cdot 5H_2O$	0.0125	–	0.025
$CuCl_2 \cdot 2H_2O$	–	0.054	–
$CoCl_2$	–	0.02	–
$FeSO_4 \cdot 7H_2O$	13.9	–	27.85
$FeCl_3 \cdot 6H_2O$	–	0.5	–
Na·EDTA	18.6	–	37.25
Disodium salt of ethylene dinitrilotetraacetic acid	–	0.8	–
Adenine	–	–	20.25
Glutamine	–	–	14.7
Glycine	2	–	2
Nicotinic acid	5	–	5
Pyridoxine·HCl	0.5	–	0.5
Thiamine·HCl	0.5	–	0.5
Biotin	0.05	–	0.05
Folic acid	0.5	–	0.5
Casein hydrolysate (acid hydrolysate, acid and vitamin free)	–	400	–
myo-Inositol	100	–	100
BAP	0.1	–	–
Kinetin	–	0.1	1
2,4-D	1	1	1
Sucrose	10000	20000	10000

TABLE 4.2 (*continued*)

Constituents	Amounts (mg l^{-1})		
	Rossini (1972)	Joshi and Ball (1968)	Kohlenbach (1984)
pH	5.0	?	5.5

[a]Rossini (1972), for *Calystegia sepium*; Joshi and Ball (1968), for *Arachis hypogaea*; Kohlenbach (1984), for *Macleaya cordata*, *Zinnia elegans*, etc.

luk (1975) developed a rich but synthetic medium containing mineral salts, sucrose, glucose, a mixture of 14 vitamins, glutamine, alanine, glutamic acid and cysteine, a mixture of six nucleic acid bases, and a mixture of four organic acids of the TCA cycle, which supported division in cell cultures of *Vicia hajastana* at a density as low as 25–50 cells ml^{-1}. With the addition of casamino acids (250 mg l^{-1}) and coconut milk (20 ml l^{-1}) in place of the amino acids and nucleic acid bases in the above medium the effective plating cell density could be further reduced to 1–2 cells ml^{-1}. On a similar medium (for composition see Table 12.3) it was possible to culture individual protoplasts in separate dishes (each dish contained 4 ml of the medium). However, this medium proved ineffective for low density (80 or 800 protoplasts ml^{-1}) protoplast culture of *Solanum tuberosum* and *S. cardiophyllum*. Hunt and Helgeson (1989) succeeded in cultivating isolated single cells of these two species on a modified KM8p medium, in which sodium pyruvate, citric acid, malic acid and fumaric acid were omitted, the phosphate level was raised from 1.2 to 1.5 mM and 0.2% bovine serum albumin was added.

The cell density effect on cell division has been explained on the basis that cells synthesize certain compounds necessary for their division. The endogenous concentration of these compounds should reach a threshold value before a cell can embark on division. The cells continue to lose their metabolites into the medium until an equilibrium is reached between the cell and the medium. As a result, at high cell density the equilibrium is attained much earlier than at low density, and hence the lag phase is longer under the latter condition. Below a critical cell density the equilibrium is never reached and cells fail to divide. However, a conditioned medium which is rich in the essential metabolites is able to support divisions at a fairly low cell density. The inability to culture individual cells in a purely synthetic medium is due to our lack of adequate knowledge about the exact nature of the factor(s) responsible for cell division. A detailed analysis of conditioned medium should give some clues in this direction. Preliminary studies suggest that the conditioning factor/s are

TABLE 4.3

Comparison of the characteristics of microbial and plant cells

Characteristics	Typical microbial cell	Typical plant cell
Size (μm long)	2–10	50–100
Doubling time	1 h	2–6 days
Growth pattern	Single cell, pellets, mycelia	Clumps
Fermentation time	2–10 days	2–3 weeks
Oxygen requirement	1–3 mmol g^{-1} h^{-1}	10–100 mmol g^{-1} h^{-1}
Shear sensitivity	Insensitive	Sensitive
Water content (%)	Approx. 80	>90
Regulatory mechanism	Complex	Highly complex
Genetic makeup	Stable	May be highly variable
Product accumulation	Often extracellular	Mostly intracellular

After Panda et al. (1989) and Scragg (1991).

molecules which are stable at 25°C, non-volatile, acid and alkali tolerant and very polar with high molecular weight (700–1200 kDa), such as oligosaccharides and their derivatives (Bellincampi and Morpurgo, 1987; Birnberg et al., 1988; Schroder et al., 1989).

4.5. PLANT CELL REACTORS

Mass culture of plant cells in vitro has been proposed as a viable alternative for the production of vast arrays of high value, low volume phytochemicals (see Chapter 17). Therefore, during the past two decades considerable work has been done to design bioreactors for plant cell culture (Panda et al., 1989; Bisaria and Panda, 1991, Taticek et al., 1991; Scragg, 1991, 1994). A bioreactor is a glass or steel vessel in which organisms are cultured. Ideally, bioreactors are fitted with probes to monitor the pH, temperature and dissolved oxygen in the culture and have provisions to sample the cultures, add fresh medium, adjust pH, air supply, mixing of cultures and controlling the temperature, without endangering the aseptic nature of the culture. It, thus, allows closer control and monitoring of culture conditions than is possible using shake cultures.

Although the basic requirements for suspension cultures of plant cells are similar to those of submerged microbial cultures, the fermentors used for microbial cell cultures are not suitable for plant cell cultures because of striking differences in the nature and growth pattern of the two types of cells (Table 4.3).

Efficient mixing of plant cells cultured on large scale is extremely important to provide uniform physiological conditions inside the culture vessel. Mixing promotes better growth by enhancing the transfer of nutrients from liquid and gaseous phases to the cells and by break-off and dispersion of air bubbles for effective oxygenation. Although, plant cells have higher tensile strength in comparison to microbial cells, their large size, rigid cellulosic wall and extensive vacuole make them sensitive to the shear stress restricting the use of high agitation for efficient mixing. Plant cells are, therefore, often grown in modified stirred-tank bioreactors at very low agitation speeds. Air-lift reactors may provide even better and uniform environmental conditions at low shear.

All plant cells are aerobic and require continuous supply of oxygen. However, plant cells require less oxygen (1–3 mmol O_2 g^{-1} h^{-1}) than micro-organisms (10–100 mmol O_2 g^{-1} h^{-1}) because of their slow metabolism. In some cases, high oxygen concentration is even toxic to the metabolic activities of cells. Air is normally sparged or blown in at the base of the bioreactor.

Plant cells in suspension culture tend to form aggregates of 2–200 cells. During the late exponential phase of growth, cells become more sticky because of increased excretion of polysaccharides into the culture vessel. This leads to the adhesion of plant cells to the reactor wall, probes and stirring device and the formation of larger aggregates. Mixing is affected, as the aggregates tend to sediment or stick to the reactor surface, forming extensive wall growth. Large aggregates also create rheological problems by creating dead zones in the culture vessel and can block the opening and pipe lines of the reactor. Cell aggregation adversely affects the operation of the probes used to monitor culture conditions during growth and product formation. Diffusion-limited biochemical reactions may occur in large aggregates when nutrients can no longer penetrate to the aggregate's central core. In spite of these effects, certain degrees of cell aggregation (cell–cell contact) and cell differentiation seem to be essential for secondary metabolite production. Hence, controlled aggregation of plant cells is of interest from the process engineering point of view.

4.5.1. Selection of a bioreactor

The suitability of a particular bioreactor for plant cell cultivation could be evaluated by considering the following factors:

1. capacity of oxygen supply and intensity of air bubble dispersion in broth;

2. intensity of hydrodynamic stresses generated inside the reactor and their effect on the plant cell system;
3. adequacy of mixing of culture broth at high cell concentration;
4. ability to control temperature, pH, and nutrient concentration inside the reactor;
5. ability to control aggregate size (which may be helpful for increasing product formation);
6. ease of scale-up;
7. simplicity of aseptic operation for long durations.

4.5.2. Bioreactor designs

The large scale cultivation of plant cell suspension started in 1959 with NASA sponsored research on the possibility of using the cultures to supply food during space flight (Tulecke and Nickell, 1959, 1960). The vessels first used were large carboys and bottles which were either rolled or bubbled to give good mixing. These make-shift bioreactors were soon replaced by stainless steel bioreactors that are fitted with a motor and agitator. An air-lift bioreactor was introduced for plant cell culture in the

TABLE 4.4

The range of cell lines grown in bioreactors of different designs and capacities since 1959

Bioreactor system	Capacity (l)	Cell lines cultured	Period
Sparged carboy	3–10	*Ginkgo, Lolium, Mentha, Zea mays, Hyoscyamus niger*	1959–1975
Bubble-column	1.8–1500	*Glycine max Nicotiana tabacum*	1971–1975
Stirred-tank	2–15500	*N. tabacum, G. max, Petroselinum, Morinda citrifolia, Spinancia oleracea, Phaseolus vulgaris, Cudrania tricuspidata, Catharanthus roseus, Helianthus annuus, Coleus blumei*	1971–present
Air-lift	7–100	*M. citrifolia C. roseus, Theobroma, C. tricuspidata, Berberis wilsoneae, H. annuus, Cinchona ledgeriana*	1977–present
Rotating-drum		*C. roseus*	1983

After Scragg (1991).

TABLE 4.5

Comparison of reactor performance for plant cells

Reactor type	Oxygen transfer	Hydro-dynamic stress	Mixing	Scale-up	Limitations
Stirred-tank (ST)	High	Highly destructive	Completely uniform	Difficult	Cell death; contamination due to moving parts
ST-low agitation and modified impeller	Medium	Low	Reasonably uniform	Difficult	Insufficient mixing at very high cell densities
Bubble-column	Medium	Low	Non-uniform	Easy	Dead zones; settling of cells due to poor mixing
Air-lift	High	Low	Uniform	Easy	Dead zones at high cell densities
Rotating-drum	High	Low	Uniform	Difficult	Non-uniform mixing at very large scale

After Panda et al. (1989).

mid-1970s. Some of the bioreactors used for plant cell culture and their merits and demerits are listed in Tables 4.4 and 4.5.

Mostly the large scale plant cell cultures have been run as batch systems (Scragg, 1991). Up to 10-l bioreactors may be sterilized by autoclaving. In the case of larger reactors, the vessels are steam sterilized (2–3 exposures of 2 h each). Autoclaved or filter-sterilized medium is added to the vessel. Cultures are initiated with an inoculum ratio of about 1:10. Generally, the pH of the plant cell culture is not controlled. It is initially adjusted between 5 and 6. It often drops soon after inoculation to 4.5, rising slowly to 5–6 or above as growth proceeds. Temperature of the culture is generally maintained between 25° and 35°C. Growth optimum for *Catharanthus roseus* is 35°C (Scragg, 1991).

The major types of bioreactors currently in use for suspension culture of plant cells are stirred-tank, bubble column, air-lift, and rotating-drum reactors (Fig. 4.7).

(i) *Stirred-tank reactor.* The stirred-tank reactor (Fig. 4.7A), in which air is dispersed by mechanical agitation, represents the classical bioreac-

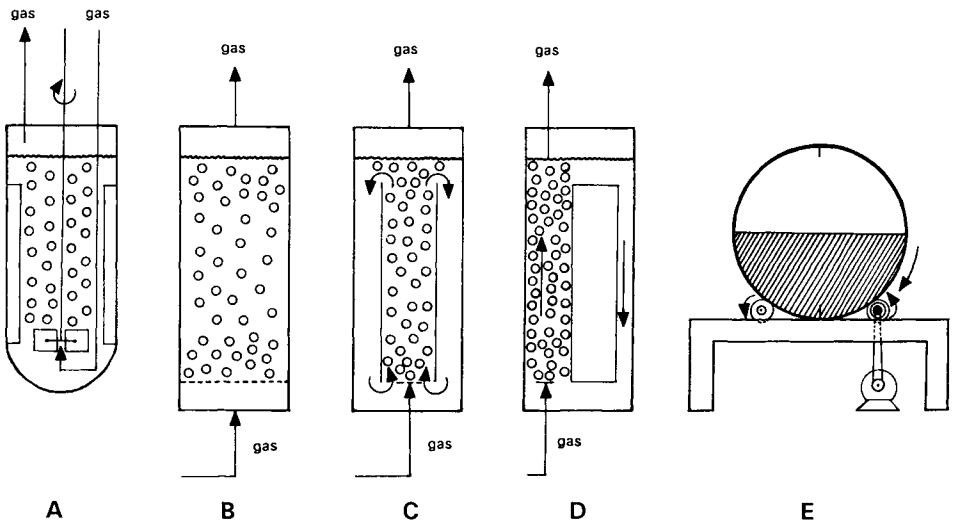


Fig. 4.7. Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.

tor for aerobic fermentations. Its behaviour has been well studied in a number of biological systems. Temperature, pH, amount of dissolved oxygen, and nutrient concentration can be controlled better within this reactor than any other reactor.

A major drawback of the stirred-tank reactor is the shearing stress generated by its stirring device to which plant cells may be sensitive. Despite this, most existing laboratory and commercial bioreactors are of the stirred-tank design which have been suitably modified for plant cell culture, such as: (1) the impeller speed is reduced to 50–150 rev. min^{-1} and in some cases the turbine impeller is replaced by a marine screen or paddle; (2) removal of baffles, pH probe and other probes not required; and (3) the sample ports are enlarged to about 1 cm to reduce blockage caused by cell aggregates. The two largest reactors (75 000 and 5000 l) used to date for plant cell culture are stirred-tank type (Scragg, 1994). For the first industrial plant cell culture process, in which shikonin was produced from *Lithospermum erythrorhizon*, stirred-tank reactors of 200 l and 500 l capacities were used (see Fig. 17.2; Tabata and Fujita, 1985). Cells of *Catharanthus roseus* have been grown in stirred-tank reactors by Wagner and Vogelmann (1977), Kurz et al. (1981), Pareilleux and Vinas (1983), Ducos and Pareilleux (1986) and Drapeau et al. (1986). Except for Wagner and Vogelmann, all others reported favourable results in terms of growth and product formation. It is interesting to note that some recent studies have revealed that many plant cell cultures are remarkably

tolerant to shear levels (1000 rps, shear rate 167 s^{-1}) once thought to be lethal (Scragg, 1994).

Some other disadvantages of stirred-tank reactors are their high energy requirements and complexity of construction and the fact that they are difficult to scale up.

(ii) *Bubble-column reactor.* The bubble-column reactor (Fig. 4.7B) is one of the simplest types of gas-liquid bioreactors used for aerobic fermentation. It consists of a cylindrical vessel aerated at the bottom. In such a system the gas is dispersed pneumatically through a deep pool of liquid by means of nozzles or perforated plates. A 1.5 kl bubble-column reactor has been used for the cultivation of *N. tabacum*, but insufficient mixing at such a scale reduced the specific growth rate of the cells.

Some of the merits of bubble-column reactor are: (i) it facilitates sterile operation because of the absence of moving parts and the fact that non-sealing parts are required, (ii) it provides high mass and heat transfer areas without the input of mechanical energy and may, thus, be suitable for shear-sensitive systems such as plant and animal cell culture, and (iii) the scale-up is relatively easy, and the reactor requires minimum maintenance.

The disadvantages of the bubble-column reactor are the undefined fluid flow pattern inside the reactor and its non-uniform mixing. Data on gas holdup and mass transfer characteristics for non-Newtonian fermentation are scanty.

(iii) *Air-lift reactor.* In air-lift reactor, as its name implies, compressed air is used for aeration and mixing of the contents of the reactor vessel. Its operation is based on the draught tube principle. Air sparged into the base of the reactor lowers the density of the medium which rises up the draft tube pulling fresh medium in at the base and, therefore, a flow is achieved. Schematic diagrams of the draught tube (inner loop and outer loop) air-lift vessels are given in Fig. 4.7C,D. A more uniform flow pattern is achieved in the air-lift reactor compared with the bubble column reactor, where a random flow pattern exists.

Air-lift reactors up to 100 l capacity have been used extensively by Fowler and his co-workers for cultivation of *C. roseus* cells. The cells of *Berberis wilsoneae*, *Cinchona ledgeriana*, *Cudrania tricuspidata*, *Dioscorea deltoidea*, *Digitalis lanata*, *Morinda citrifolia*, *Tripterygium wildordii* and, recently, *L. erythrorhizon* have also been cultured in air-lift reactors. In all cases, the reactor configuration gave favourable results in terms of biomass and product formation. Vienne and Marison (1986) have

reported successful continuous culture of *C. roseus* cells in an air-lift reactor.

The air-lift reactor is one of the most suitable bioreactor types for cultivation of plant cells on a large scale. It provides reasonable mixing and oxygen transfer at low shear, and less contamination occurs because there are no moving parts and no intrusion of impeller shaft. The operating cost, compared to the stirred-tank reactor, is low because of its simple design and it does not require power input for the stirrer. Despite these advantages air-lift reactors have not been used as extensively as stirred-tank reactors.

The disadvantages of air-lift reactors are the development of dead zones inside the reactor and insufficient mixing at high cell densities. Moreover, little information is available on the engineering analysis of the reactor behaviour in complex systems such as the plant cell cultures.

(iv) *Rotating-drum reactor.* The rotating-drum reactor consists of a horizontally rotating-drum on rollers connected to a motor (Fig. 4.7E). The rotating motion of the drum facilitates good mixing and aeration without imposing a high shear stress on the cultured cells. Baffles in the inner wall of the drum help to increase oxygen supply. This type of reactor has the capacity to promote high oxygen transfer to cells at high density. It has been used to grow cultures of *C. roseus* and *L. erythrorhizon* up to 1000 l in volume.

In a comparative study of the performance of rotating-drum and stirred-tank reactors for the cultivation of *Vinca rosea* the former was found to be superior on the basis of increased oxygen transfer at high cell densities (Tanaka et al., 1983). The rotating-drum reactor facilitates better growth and imparts less hydrodynamic stress. In the stirred-tank reactor growth rate was low at low agitation speed because of insufficient oxygen supply, while at high agitation speed the cells died. Hence, for cultivation of cells at high densities, the rotating-drum reactor was preferred. The rotating-drum reactor has also been shown to be superior to air-lift and modified stirred-tank reactors for the cultivation of *L. erythrorhizon* (Tanaka, 1987).

The major disadvantage of this reactor type is the restriction in scale-up.

(v) *Immobilized plant cell reactors.* Immobilization of plant cells into a suitable carrier and cultivation of the immobilized cells in different types of reactors has been developed as an alternative to free cell culture systems for the production of industrial phytochemicals (see Chapter 17). Entrapment in natural (alginate, agar, agarose, carrageenan) or syn-

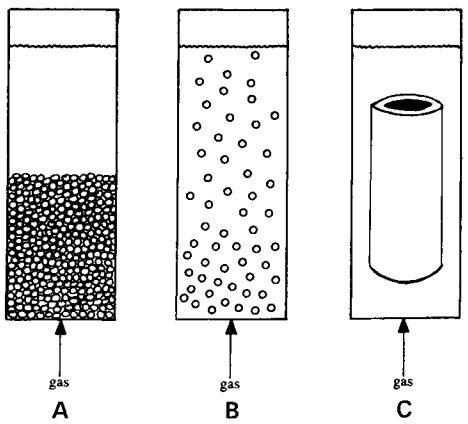


Fig. 4.8. Configuration of some reactors used to culture immobilized plant cells. (A) Packed-bed reactor; (B) fluidized-bed reactor; (C) polyurethane draft tube reactor.

thetic (polyacrylamide) polymers, adhesion to reticulate polyurethane foam, and confinement behind semi-permeable membranes have been employed to immobilize plant cells.

Alginate has been the most popular polymer used to immobilize plant cells. Cell suspension in 4% sodium alginate solution is allowed to fall as 2 mm drops in a beaker containing 0.2 M solution of $CaCl_2$. Ca-alginate is formed by ion exchange reaction and the drops harden as beads within 20–30 min. Alginate entrapped cells can be cultured in packed-bed (Fig. 4.8A), fluidized-bed (Fig. 4.8B) or air-lift bioreactors (Fig. 4.7C).

Polyurethane foam has been used to immobilize a range of cell lines. The cells are immobilized in these matrices by flowing cells and medium through the foam or by adding sterile foam to growing cultures. The foam can be cut into various shapes. Polyurethane entrapped cells have been cultured in both packed and fluidized beds as cubes, shaped into draft tube (Fig. 4.8C) or threaded as strips on stainless steel rods.

Membrane reactors (e.g. hollow-fibre units and flat membrane reactors), in which cells are separated from the growth medium by membrane, are particularly suitable for fragile cells which can be entrapped more readily on membrane and allow better control over cell density. The environment in a membrane reactor is more homogeneous; pressure drop and fluid dynamics are more easily controlled and are relatively independent of the scale of operation. In a hollow-fibre reactor (Fig. 4.9) cells are introduced into the shell side of the hollow-fibre cartridge and the medium is circulated through the fibre lumen and aerated using a separate reservoir. Since the cells do not stick to the fibre membrane the reactors may retain their mechanical integrity for a longer period of time and

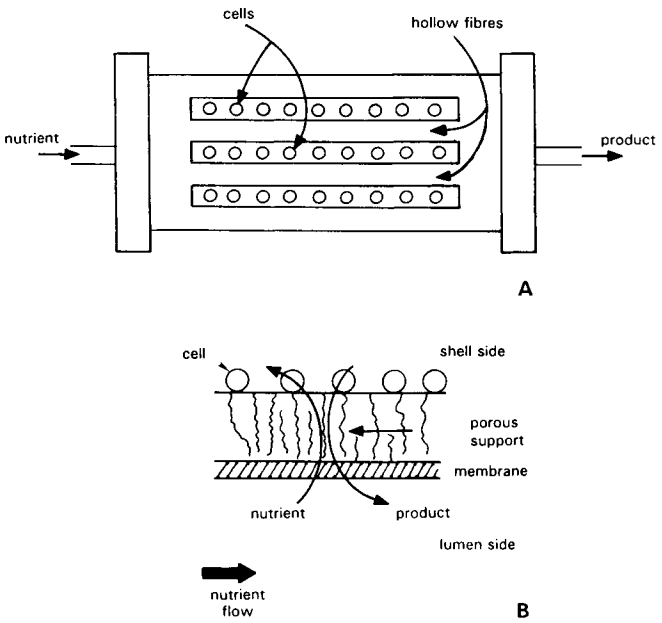


Fig. 4.9. (A) Hollow-fibre reactor for immobilized cell culture; (B) the portion marked in (A) enlarged to show the details of the reactor and the flow of nutrients and products across the membrane and the porous support of the hollow-fibre cartridge (adapted from Prenosil and Pederson, 1983).

may be reusable. When cells are no longer productive, when an experiment is over, or when a new cell-product combination is desired, it is potentially possible to flush out the old cells and refill the device with the new cells.

In flat-plate membrane reactor systems (Fig. 4.10), with one side flow and two side flow, the cells are loaded manually into the membrane cell layer and direct sampling can be achieved through a removal cap plate. Substrate enters the cell layer by diffusion or pressure driven flow and is converted into product which diffuses into the cell-free compartment. Multimembrane reactors have also been proposed for immobilized plant cell cultures. The main advantage of this reactor is that the desired metabolites are produced and selectively separated from the reactant simultaneously.

4.6. APPLICATIONS OF CELL CULTURE

4.6.1. Mutant selection

The occurrence of a high degree of spontaneous variability in cell cul-

tures and its exploitation in mutant selection in relation to crop improvement is discussed in Chapter 9. The frequency of certain phenotypes could be increased several fold by treating the cells with mutagens (Sung, 1976; Muller and Grafe, 1978; Miller and Hughes, 1980).

One of the major drawbacks of mutation breeding in higher plants is the formation of chimeras following the mutagenic treatment of multicellular organisms. In this regard cell culture methods of mutant selection are more efficient. Millions of potential plants can be handled in a minimal space; 100 ml of rapidly growing suspension cultures of tobacco contain over 1×10^7 cells.

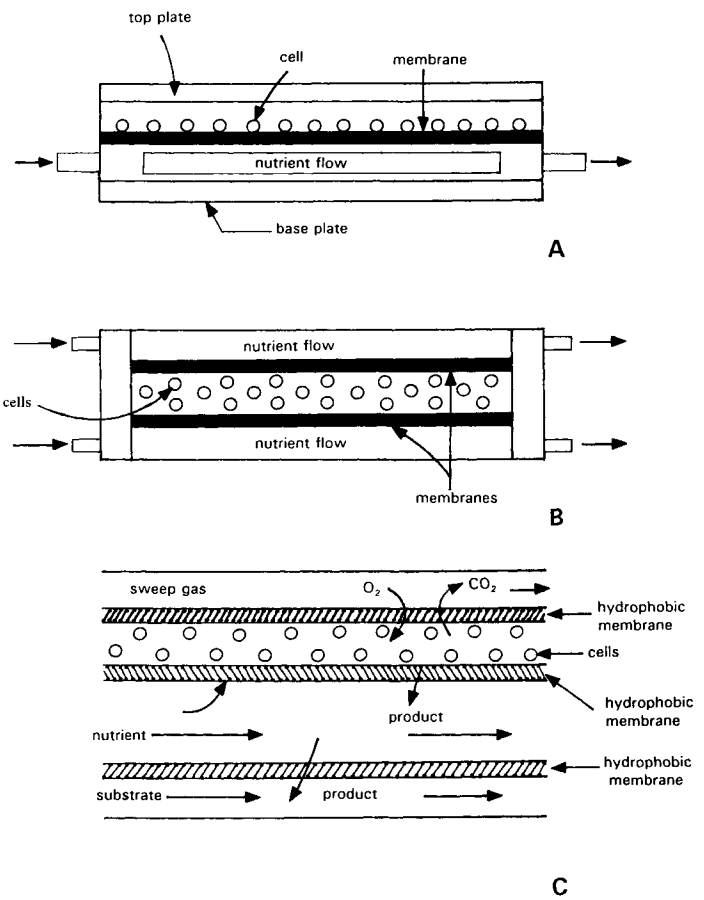


Fig. 4.10. Membrane reactor systems for immobilized plant cell culture. (A) Flat plate membrane reactor with one side flow of nutrients; (B) flat plate membrane reactor with two side flow of nutrients; (C) multimembrane reactor system. (A,B) are adapted from Shuler et al. (1984) and (C) from Shuler et al. (1986).

4.6.2. Industrial uses

Since the early 1950s many researchers have investigated the production of useful compounds by plant tissue cultures, and remarkable technological advances have been made to culture plant cells in large bioreactors for the commercial production of plant metabolites (see Chapter 17)

4.6.3. Induction of polyploidy

Doubling of chromosome number is frequently required to overcome the problem of sterility associated with hybrids of unrelated plants. In the genus *Saccharum* a large number of genetically sterile hybrids that exist could well be utilized in the breeding programmes if their fertility can be restored through doubling of their chromosome number (Heinz and Mee, 1970). Attempts using seeds and vegetative cuttings failed to accomplish this objective. Heinz and Mee (1970) demonstrated that a large number of polyploid plants of sugarcane could be produced through the use of cell cultures. They regenerated over 1000 plants from cell suspension cultures of a complex *Saccharum* species hybrid treated with 50 mg l^{-1} colchicine for 4 days. Cytological investigations revealed that about 48% of these plants were with uniformly doubled chromosome number. In this regard cell cultures should prove useful with other crop plants also.

Duplication of chromosomes in cell cultures also occurs spontaneously (Murashige and Nakano, 1966). This is one of the methods recommended for raising homozygous diploids from pollen-derived haploids (see Chapter 7).

4.7. CONCLUDING REMARKS

The methods of cell and callus culture are reasonably well developed. It is now possible to establish such cultures from most plant tissues. Several methods to culture plant cells in large bioreactors and at low plating densities or in complete isolation, under defined conditions, have been described (see also Chapter 12).

Potential applications of cell culture in mutant selection and the production of natural plant products are discussed in Chapters 9 and 17, respectively.

APPENDIX 4.I

Protocol for mechanical isolation of mesophyll cells from the leaves of *Calystegia sepium* (after Rossini, 1972):

(a) Surface sterilize the leaves by rapid immersion in 95% ethanol followed by rinsing for 15 min in filter-sterilized 7% solution of calcium hypochlorite. Wash in sterile distilled water.

(b) Cut the leaves into small pieces (less than 1 cm^2).

(c) Homogenize 1.5 g of leaf material with 10 ml of culture medium (for composition see Table 4.2) in a Potter-Elvehjem glass homogenizer tube.

(d) Filter the homogenate through two sterile metal filters, the upper and lower filters with mesh diameters of 61 and 38 μm, respectively.

(e) Fine debris can be removed by slow-speed centrifugation of the filtrate which would sediment the free cells. Remove the supernatant and suspend the cells in a volume of the medium sufficient to achieve the required cell density.

(f) Plate the cells in a thin layer of agar medium or liquid medium.

APPENDIX 4.II

Protocol for enzymatic isolation of mesophyll cells from tobacco leaves (after Takebe et al., 1968, as modified by Evans and Cocking, 1975):

(a) Take fully expanded leaves from 60–80-day-old plants and surface sterilize them by immersion in 70% ethanol for 30 s followed by rinsing for 30 min in 3% sodium hypochlorite solution containing 0.05% Teepol or cetavlon.

(b) Wash the leaves with sterile distilled water and peel off the lower epidermis with the aid of sterile fine jeweller's forceps.

(c) Excise peeled areas as 4 cm^2 pieces with a sterile scalpel blade.

(d) Transfer 2g of peeled leaf pieces to 100 ml Erlenmeyer flasks containing 20 ml filter sterilized enzyme solution containing 0.5% macerozyme, 0.8% mannitol, and 1% potassium dextran sulphate (MW source dextrin 560, sulphur content 17.3%; Meito Sangyo Co. Ltd., Japan).

(e) Infiltrate the enzyme into the leaf tissue by briefly evacuating the flasks with a vacuum pump.

(f) Incubate the flasks at 25°C for 2 h on a reciprocating shaker with a stroke of 4–5 cm at the rate of 120 cycles min^{-1}.

(g) Change enzyme solution after the first 30 min. The enzyme solution after the second 30 min should contain largely spongy parenchyma cells, and those after the third and the fourth 30 min periods should contain predominantly palisade cells.

(h) Wash the cells twice with culture medium and culture.

Chapter 5

Cellular Totipotency

5.1. INTRODUCTION

Unlike animals, where differentiation is generally irreversible, in plants even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus. Sieve tube elements and xylem elements whose nuclei have started to disintegrate, or fibres with cell walls thicker than 2 μm (mature tracheids have 7 μm thick walls) would, obviously, not divide any more. According to Gautheret (1966) the degree of regression a cell can undergo would depend on the cytological and physiological state it has reached in situ (see Table 5.1).

When non-dividing, quiescent cells from differentiated tissues are grown on a nutrient medium that supports their proliferation, the cells first undergo certain changes to achieve the meristematic state. These include replacement of non-functional cellular components damaged by lysosomal activity during the processes of cytoquiescence (Bornman, 1974). The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed 'dedifferentiation'. A multicellular explant generally comprises cells of diverse

TABLE 5.1

Degree to which different cell types may dedifferentiate[a]

Vegetative point	Cambium	Companion and secretory cells	Parenchyma	Thick-walled cells (collenchyma, lignified cells)	Fibre	Degenerating cells (vascular elements, sieve tubes)
•←———	•←———	•←				
•←———	•←———	•←———	•←			
	•←———			———	•	
	•←———				———	•
	•←———					——— •

[a]After Gautheret (1966).

types. As a result, the callus derived from it would be heterogeneous with respect to the ability of its component cells to form a whole plant or plant organs ('redifferentiation'). The inherent potentiality of a plant cell to give rise to a whole plant, a capacity which is often retained even after a cell has undergone final differentiation in the plant body, is described as 'cellular totipotency'. For a differentiated cell to express its totipotency it first undergoes dedifferentiation followed by redifferentiation. Mostly dedifferentiation involves embryonization of cells leading to callus formation. However, embryonic explants often exhibit differentiation of roots, shoots or embryos without an intervening callus phase.

Tissue culture techniques offer not only an excellent opportunity to study the factors that elicit the totipotentiality of cells but also allows investigation of factors controlling cytological and histological differentiation.

5.2. CYTODIFFERENTIATION

In the area of cytodifferentiation in vitro as well as in vivo the main emphasis has been on vascular differentiation, particularly the tracheary elements (TEs). Phloem has received less attention because of technological problems. Whereas TEs can be easily stained and scored in macerated preparations of the tissue, this is not possible with the small and delicate sieve tubes.

In an intact plant, tissue differentiation goes on in a fixed manner which is characteristic of the species and the organ. Torrey and coworkers have done considerable work on the control of the pattern and extent of vascular differentiation in excised organized roots (Torrey, 1966).

Kohlenbach and Schmidt (1975) observed that mechanically isolated mesophyll cells of *Zinnia elegans* differentiated into TEs without cell division when cultured on a suitable medium. The *Zinnia* system was further refined by Fukuda and Komamine (1980a,b) and Church and Galston (see Church, 1993), who achieved relatively synchronous differentiation of a high percentage (50–65%) of the cells within 72 h. Since then *Zinnia* cells have been extensively used as a model system to understand the process of TE differentiation. The special merits of this system are: (1) freshly isolated cells of *Zinnia* provide a very homogeneous single cell system composed of uniformly non-polyploid cells held in G_1 phase of the cell cycle (Fukuda and Komamine, 1981), (2) controlled differentiation can be achieved by exogenous supply of phytohormones and other chemicals into the medium, avoiding the problem of hormonal and nutritional

gradient in multicellular system, (3) since cell division is not a prerequisite for differentiation, the inductive factors influence TE differentiation directly rather than cell division, and (4) the differentiation occurs synchronously and at high frequency.

Fukuda and Komamine (1983) have shown that under inductive conditions for TE differentiation from mesophyll cells of *Zinnia*, synthesis of two proteins is shut off and two new polypeptides appear (within 48–60 h of culture) before any detectable morphological change in the mesophyll cells. These novel proteins can be regarded as biochemical markers for TE differentiation. More recently, Demura and Fukuda (1994) have isolated 3 cDNA clones for the genes (TED2, TED3, TED4) expressed preferentially in mesophyll cells of *Zinnia* during their redifferentiation into TE. For detailed reviews of the subject, see Roberts (1976), Fukuda and Komamine (1985), Fukuda (1989), Fukuda and Kobayashi (1989), Sugiyama and Komamine (1990) and Church (1993).

5.2.1. Factors affecting vascular tissue differentiation

Two substances that have a profound effect on vascular tissue differentiation are auxin and sucrose. They affect vascular differentiation qualitatively as well as quantitatively. Some evidence also points towards the involvement of cytokinins and gibberellins in the process of xylogenesis.

(i) *Growth regulators.* Camus (1949), a French botanist, grafted small vegetative buds on the upper surface of cultured root tissues of *Cichorium*, and after a few days observed the differentiation of vascular strands in the parenchymatous tissue below the bud. These strands connected the vascular tissue of the bud to the other vascular tissues in the explant. The differentiation of vascular tissue occurred even if the physical contact between the callus and the bud was broken by placing cellophane paper at the site of the graft. This suggested that the stimulus provided by the bud for vascular tissue differentiation was of the nature of diffusable chemical(s). This work was later confirmed by Wetmore and Sorokin (1955) using the undifferentiated callus (completely lacking in vascular elements) of *Syringa vulgaris* as the experimental system. Callus pieces (400 mg fresh weight) were planted on a medium which did not favour the differentiation of vascular tissue. The trimming of the callus and its orientation was such that its upper surface was fairly flat and smooth. A V-shaped incision was made on the upper surface of the callus and a vegetative bud, with its basal end cut wedge-shaped, was inserted

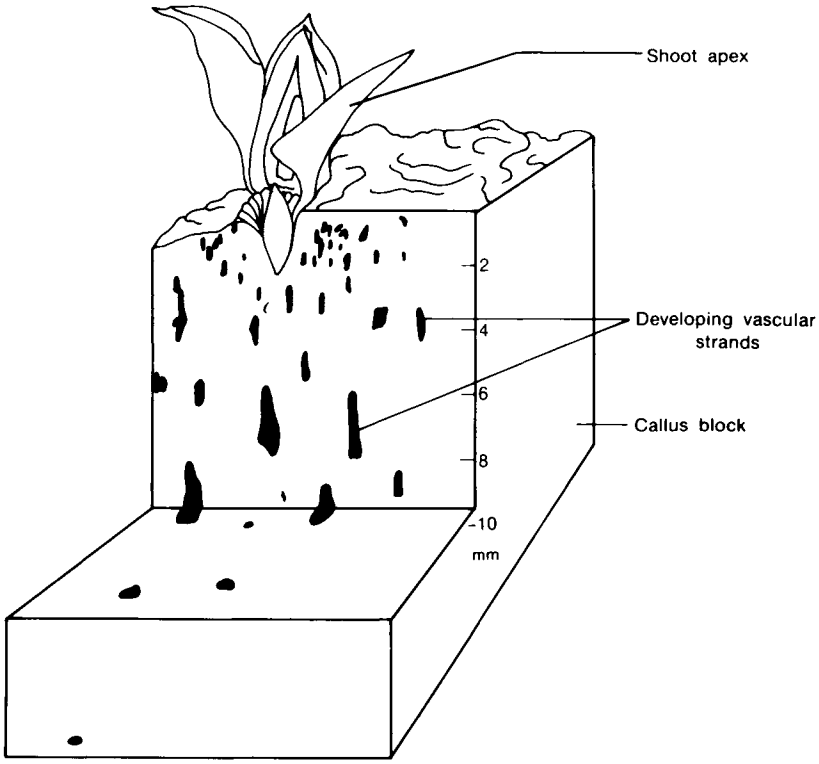


Fig. 5.1. Induction of vascularization in callus tissue of *Syringa* by grafting a stem apex bearing two or three leaf primordia. Drawing made 54 days after grafting (after Wetmore and Sorokin, 1955).

in the cavity (see Fig. 5.1). To prevent desiccation at the point of graft the cavity was filled with 1% non-nutrient agar before inserting the bud. Within 20–30 days the bud induced divisions in the cells underneath it, resulting in the appearance of short vertical columns of cells which later developed into a ring of vascularized nodules. Agar containing sucrose and auxin could effectively replace the bud for vascular differentiation (see Fig. 5.2) (Jeffs and Wetmore, 1967).

Fosket and Torrey (1969) reported a stimulatory effect of cytokinin on xylogenesis in cotyledonary callus cultures of soybean. The observations of Mizuno et al. (1971, 1973) and Mizuno and Komamine (1978) with cultured root slices of carrot also suggest the involvement of auxin and cytokinin in the differentiation of TEs. In a medium containing only auxin the carrot cvs Kuroda-gosum and Kintoki, roots of which contain zeatin, differentiated TEs in light as well as in the dark. In the

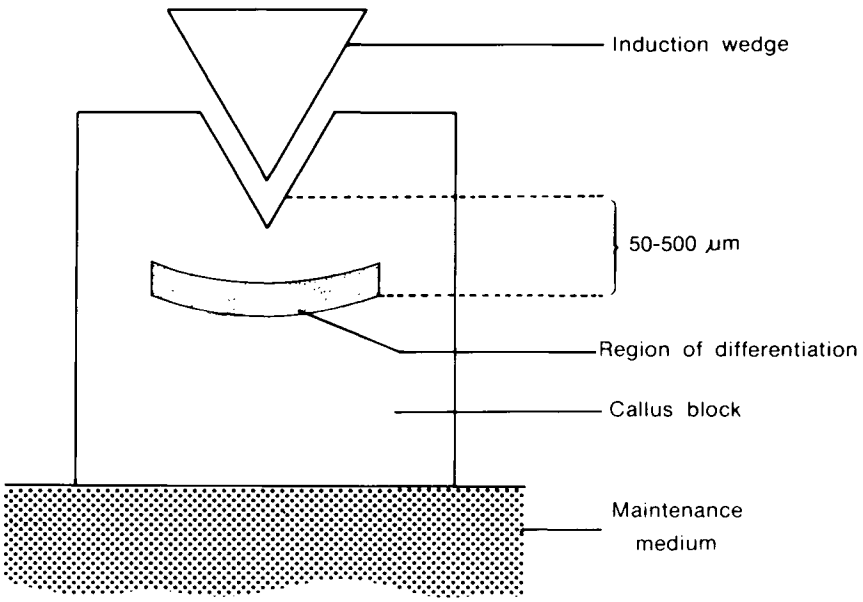


Fig. 5.2. Diagram to show the induction of vascularization in a block of *Phaseolus* callus by inserting an agar-block wedge containing auxin and sucrose (after Jeffs and Northcote, 1967).

same medium the carrot cvs Ogata-sanzun and Hakkaido-gosum, roots of which lack zeatin, formed TEs only in light. Light induces the synthesis of zeatin in Hakkaido-gosum (Mizuno and Komamine, 1978), suggesting thereby a positive role of cytokinin in xylogenesis in these systems.

In mesophyll cell cultures of *Zinnia*, maximum differentiation of TEs occurs within a narrow range of both auxin and cytokinin, suggesting that the absolute concentration of the two hormones in the culture medium is more important than auxin/cytokinin ratio (Fukuda and Komamine, 1980a; Church and Galston, 1988; Lin and Northcote, 1990). In this system the differentiation of TEs with optimum hormonal treatment (0.1 mg l^{-1} NAA and 0.1–1 mg l^{-1} BAP) is inhibited or delayed by the inhibitors of both auxin and cytokinin synthesis (Church and Galston, 1988). Whereas cytokinin is required only for a brief period in the early stage of differentiation, auxin must be present until the last stage of differentiation (Fukuda and Komamine, 1985; Church and Galston, 1988). In contrast, for TE differentiation in tuber discs of *Helianthus* exogenous cytokinin was required for 2 days while exogenous auxin was required for less than 1 day (Phillips, 1987).

A stimulatory interaction between auxin and gibberellin for xylem differentiation has been reported by Roberts and Fosket (1966), Bornman (1974), and Gautheret (1966).

(ii) *Sucrose.* The effect of auxin on vascular tissue differentiation seems to be closely dependent on the presence of sugar (Jacobs, 1952; Fosket and Roberts, 1964). The relative amounts of xylem and phloem formed in callus pieces of *Syringa* (Wetmore and Rier, 1963) and *Phaseolus vulgaris* (Jeffs and Northcote, 1967) could be changed by varying the sucrose concentration in the presence of a low concentration of auxin. In *Syringa* if the agar applied to the cavity at the top of the callus contained 0.05 mg l^{-1} IAA and 1% sucrose, only a few xylem elements appeared in the callus. Keeping the auxin concentration constant and raising the sucrose level to 2% favoured better xylem formation with little or no phloem. With 2.5–3.5% sucrose, both xylem and phloem differentiated, and with 4% sucrose, the vascular tissue formed was phloem with little or no xylem. Unlike *Syringa*, suspension cultures of *Parthenocissus tricuspidata* showed an increase in xylem elements with an increase in sucrose concentration up to 8%. It should, however, be noted that for xylogenesis in *Parthenocissus*, sucrose is hardly effective up to a concentration of 1.5% which is around optimal for *Syringa*.

Jeffs and Northcote (1967) tested a variety of sugars in the presence of an auxin and observed that besides sucrose, the disaccharides maltose and trehalose were effective in stimulating TE differentiation in *Phaseolus* callus. Glucose, fructose and other monosaccharides were non-stimulatory. The authors have expressed the view that sucrose may be acting almost like a hormone in this category of differentiation.

(iii) *Calcium.* Recent studies using *Zinnia* system have highlighted the importance of calcium in TE differentiation. Roberts and Haigler (1990) observed that calcium deprivation or application of calcium channel blockers or calmodulin antagonists inhibited TE differentiation. Whereas calmodulin antagonists were effective only when added at the beginning of culture, calcium channel blockers inhibited TE differentiation when added at any time between 0 and 48 h of culture. These results indicate the involvement of at least two calcium regulated events in TE differentiation.

Based on the fluorescence of chlortetracycline (CTC), an antibiotic that fluoresces in the presence of membrane bound Ca^{2+}, Roberts and Haigler (1990) recorded an increase in the concentration of Ca^{2+} in the mesophyll cells prior to visible wall thickening. CTC fluorescence was initially distributed evenly throughout the cytoplasm but during secon-

dary wall deposition it got localized between secondary wall thickenings and was associated with plasma membrane. Finally, the fluorescence became punctate, probably due to breakdown of the plasma membrane.

(iv) *Physical and physiological factors*. Very little attention has been paid to the effect of physical factors on vascular differentiation. In *Helianthus* there is no differentiation of vascular elements at a temperature below 17°C, and within the range of 17–31°C an increase in temperature enhances xylem formation (Gautheret, 1961). Light is reported to stimulate wound vessel differentiation in *Coleus* (Fosket, 1968).

Wound stress is reported to be another physical factor essential for the induction of TEs in *Zinnia* (Church and Galston, 1989). In leaf disc cultures very few mesophyll cells differentiate into TEs. However, peeling off the epidermis brings about considerable enhancement in the number of TEs formed. This is not due to better contact of cells with the medium because infiltration of the medium into leaf tissue did not substitute peeling (Fukuda, 1989). The stress effect could be due to ethylene production which has been implicated to play a positive role in the differentiation of TEs in lettuce explant cultures (Miller et al., 1984; Miller and Roberts, 1984). Even in *Zinnia* the inhibitors of ethylene synthesis caused blockage of TE differentiation (Fukuda, 1989).

Cells harvested from old leaves of *Zinnia* divide in culture but do not differentiate into TEs in a medium that induces the cells derived from younger leaves to differentiate into TEs (Iwasaki et al., 1986, 1988). Direct differentiation of cells into TEs in *Helianthus tuberosus* occurs only in the explants taken from immature tubers. This capacity declines with the age of tuber, and in mature tuber's TE differentiation occurs only after cell proliferation (Phillips, 1981). Another observation demonstrating the importance of the physiological condition of cells in TE differentiation is the higher frequency differentiation of TEs in the cultures of cells isolated mechanically than those obtained by enzymatic maceration of the tissue (Fukuda and Komamine, 1985).

5.2.2. Cell cycle and TE differentiation

Several workers had reported that a cell must divide before the differentiation of TE can occur (Fosket, 1968; Torrey, 1971; Torrey et al., 1971). This conclusion is based on some in vitro observations (Fosket, 1968, 1970; Fosket and Torrey, 1969; Torrey and Fosket, 1970; Tucker et al., 1986) and is supported by the in vivo situation. Procambium, from which the primary xylem and phloem are derived, usually exhibits continued

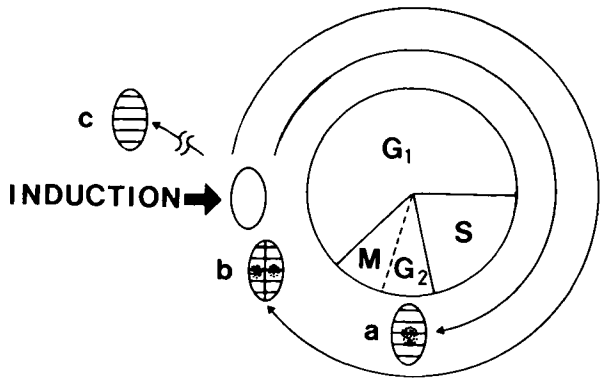


Fig. 5.3. Diagram showing relationship between Tracheary element (TE) differentiation and cell cycle in the cultures of isolated mesophyll cells of *Zinnia elegans*. Hormonal induction of TE differentiation occurs in the G_1 phase but the cell may get out of the cell cycle to differentiate at different stages in the cell cycle. The induced cell may differentiate into TE without progressing further along the cell cycle (c), it may pass through the S phase and differentiate in the G_2 phase (a), or it may undergo mitosis and both the daughter cells differentiate into TE (b) (reprinted with permission from Fukuda and Komamine, 1981, Physiol. Plant., 52: 423–430).

divisions. This is also true of the secondary vascular tissue which is contributed by the meristematic cells of vascular cambium.

The chemical factors (auxin, cytokinin, sugar, etc.) reported to be involved in xylem differentiation are generally the same as those regulating cell division. This raises the question of whether in effecting TE formation these hormones act on the differentiation process per se or the preceding cell division. A related question was asked by Dodds (1979): 'Is cell cycle activity necessary for xylem cell differentiation?'

BUdR (an inhibitor of DNA synthesis), at 10^{-5} M, completely suppressed xylem differentiation in coleus stem explants (Fosket, 1968), pea root explants (Shininger, 1975), Jerusalem artichoke tuber explants and lettuce pith tissue (Dodds, 1979). Malawer and Phillips (1979) furnished additional evidence to support the idea that differentiation of xylem elements is preceded by cell division. They added tritiated thymidine to the medium in which tissues of Jerusalem artichoke were cultured and noted that if tritiated thymidine was present throughout the culture period (48 h) all the xylem cells were labelled. Furthermore, silver-grain counting revealed that the xylem cells in culture had undergone three rounds of DNA synthesis.

Recent studies have clearly established that cell division is not always a prerequisite for TE differentiation. In cell cultures of *Centaurea cyanus*,

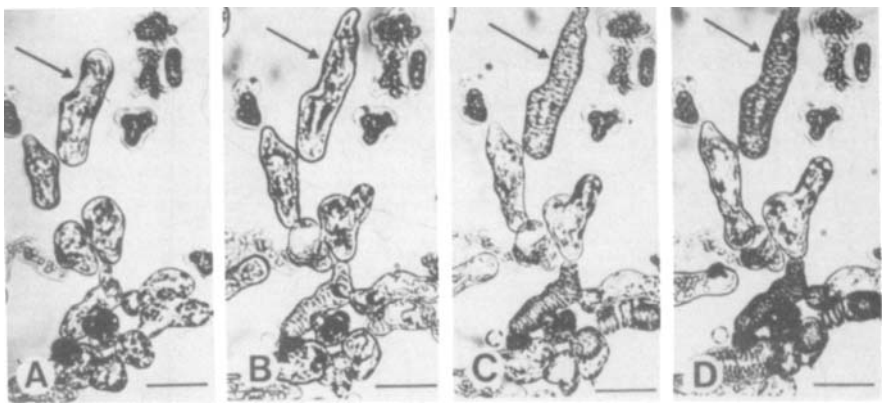


Fig. 5.4. Time-lapse pictures to show the process of tracheary element differentiation from single cells, isolated from mesophyll of *Zinnia elegans*, without cell division. The pictures taken at 48 h (A), 71 h (B), 77 h (C), and 96 h (D) after culture (from Fukuda and Komamine, 1980).

raised by Bergmann's technique of cell plating, Torrey (1975) observed that some of the single parenchymatous cells differentiated directly into TE without a preceding cell division. Since these cells were taken from fast-growing suspension cultures it could be argued that the cells directly forming TEs were derived from a recent cell division. More convincing evidence against the assumption of the need of a cell division for xylogenesis was provided by Kohlenbach and Schmidt (1975) and Fukuda and Komamine (1980b). Kohlenbach and Schmidt reported that the mechanically isolated quiescent mesophyll cells of *Zinnia* differentiated into TEs after a period of extension growth but without a cell division. Even mesophyll protoplasts of this species exhibited direct differentiation into TEs (Kohlenbach and Schopke, 1981).

Through serial observations, microdensitometry and autoradiography, Fukuda and Komamine (1980a, 1981) confirmed that in *Zinnia* the majority of TEs (60%) differentiated directly from cells in the G_1 phase (Figs. 5.3 and 5.4); the differentiation required neither the replication of total genomic DNA nor cell division. However, various inhibitors of DNA synthesis cause complete inhibition of TE differentiation (Fukuda and Komamine, 1981). It has been suggested that some kind of a minor repair type DNA synthesis is essential for cytodifferentiation but not complete genomic DNA replication during S phase of the cell cycle (Sugiyama and Komamine, 1990).

Most of the observations included above are based on gross morphology of the vascular elements. Very little, if any, is known about the structural and functional aspects of the vascular elements formed in cell and tissue

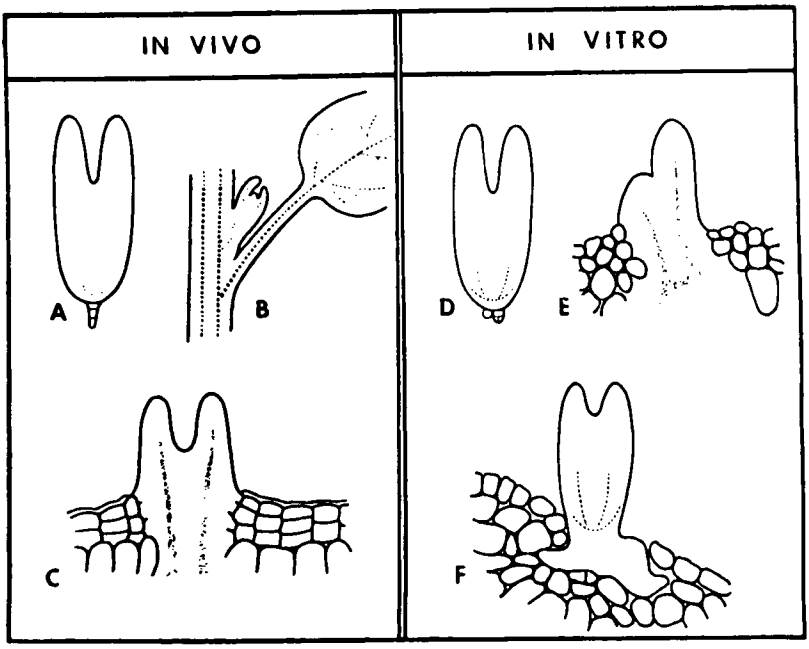


Fig. 5.5. Diagrams to show difference of anatomy in the basal ends of embryos (A,D,F) and shoot buds (B,C,E) under in vivo and in vitro conditions. The stippled areas represent the vascular traces (after Haccius, 1978).

cultures. Most probably these vascular elements are non-functional (Torrey et al., 1971).

5.3. ORGANOGENIC DIFFERENTIATION

For a considerable time the totipotency of somatic cells has been exploited in vegetative propagation of plant species. In nature, stem, leaf, and root pieces of several taxa are able to differentiate shoots and roots leading to the establishment of new individuals (Dore, 1965). In vitro studies have revealed that this potential is not restricted to only some species. Most plants provided with appropriate conditions would differentiate shoot buds and roots from somatic as well as reproductive tissues.

Whole plant regeneration from cultured cells may occur either through shoot-bud differentiation or somatic embryogenesis. A shoot bud and an embryo are distinguishable on the basis of recognizable morphological differences between the two (see Fig. 5.5) (Haccius, 1978). The former is a monopolar structure. It develops procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explant (see Fig. 5.5B,C,E). On the other hand, an

embryo is a bipolar structure with a closed radicular end (see Fig. 5.5A,D,F). It has no vascular connection with the maternal callus tissue or the cultured explant.

Plant regeneration from isolated cells, protoplasts or unorganized callus is generally more difficult than that from intact explants such as cotyledons, hypocotyl segments and immature embryos. With the advent of techniques to insert alien genes into cells of intact explants (see Sections 14.2.1 and 14.2.4) success in genetic engineering of plants no longer depends on the arduous step of plant regeneration from isolated protoplasts. The regenerants obtained through de novo differentiation of shoot buds or somatic embryos directly from the explants also exhibit genetic variability suitable for somaclonal variant selection (see Chapter 9). Therefore, during the last decade considerable attention has been paid to optimize protocols for in vitro organogenic and embryogenic differentiation directly from immature embryos and seedling explants.

Most of the recent reports of in vitro plant regeneration deal with somatic embryogenesis, as it is potentially more useful than organogenesis for plant propagation (see Sections 6.8, 6.12) and is proving to be an ideal system to investigate cellular basis of differentiation in higher plants (see Section 6.4). This chapter deals with some aspects of shoot-bud differentiation in vitro. Regeneration of plants via somatic embryogenesis is discussed in Chapter 6. Loss of morphogenic potential in long-term cultures, practical applications of cellular totipotency, and concluding remarks on the subject are considered at the end of Chapter 6.

5.3.1. Factors affecting shoot-bud differentiation

(i) *Chemical factors.* Shoot-bud differentiation in cultured tissues is known since the earliest publications in the field of plant tissue culture. White (1939b) reported the development of shoot buds in tissue cultures of tobacco maintained in liquid medium. In 1944 Skoog confirmed this observation. However, a systematic approach to shoot/root induction in vitro started after Skoog and co-workers (Skoog and Tsui, 1948; Skoog, 1954, 1955; Skoog and Miller, 1957) demonstrated that in tobacco the differentiation of the two organs can be induced more or less at will by manipulation of the balance of IAA and adenine/kinetin in the culture medium (see Fig. 5.6). The first indication of the phenomenon of chemical control of organ formation in tobacco tissue cultures was published in 1948 by Skoog and Tsui. A comprehensive summary of this and subsequent work on the subject by Skoog's group was presented in the classic paper by Skoog and Miller in 1957, and updated by Skoog in 1971. On the basis of their observations, Skoog and Miller (1957) rejected the concept

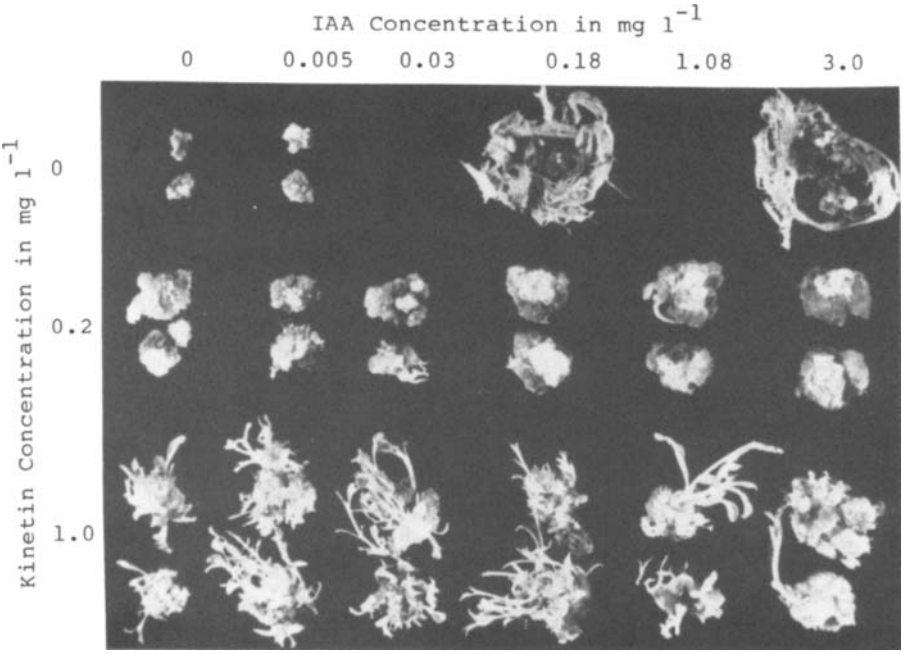


Fig. 5.6. Organogenesis in tobacco ('Wisconsin No. 38') callus. Effect of increasing IAA concentrations at different kinetin levels and in the presence of casein hydrolysate (3 mg l^{-1}) on growth and organ formation in tobacco callus cultured on semi-solid White's medium. Age of cultures: 62 days. Note root formation in the absence of kinetin and in the presence of 0.18–3.0 mg l^{-1} IAA and shoot formation in the presence of 1.0 mg l^{-1} kinetin, particularly with IAA concentration in the range of 0.005–0.18 mg l^{-1} (from Skoog and Miller, 1957).

of specific organ-forming substances (Rhizocalines and Caulocalines) proposed by Went (1938), and regarded organ formation to be determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.

In tobacco the presence of adenine or kinetin in the medium leads to the promotion of shoot bud differentiation and development. Kinetin is 30 000 times more potent than adenine (Skoog, 1971). The shoot-forming effect of adenine and kinetin is modified by other components in the medium, particularly IAA and NAA. Auxins inhibit bud formation. As low as 5 μM IAA is enough to completely suppress the spontaneous differentiation of buds in tobacco. When used in combination with kinetin or adenine, auxin counteracts their bud forming effect. Skoog and Miller (1957) and Skoog (1971) estimated that 15 000 molecules of adenine or 2 molecules of kinetin are required to offset the inhibitory effect of 1 molecule of IAA on shoot-bud differentiation. In combination, a relatively higher concentration of IAA favours cell proliferation and root differen-

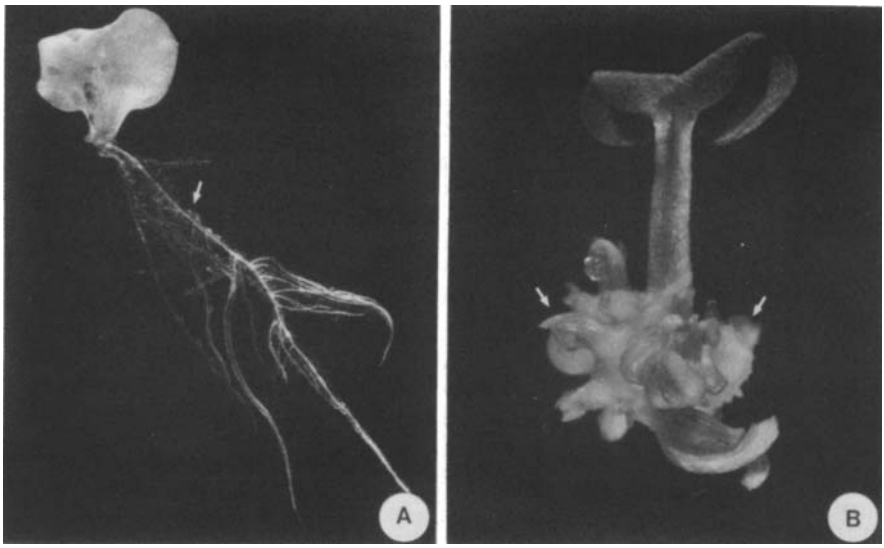


Fig. 5.7. Differentiation of roots and shoots from excised cotyledons of *Brassica juncea*. (A) On MS medium only roots (arrow marked) are formed at the cut end of the petiole. (B) On MS supplemented with 5×10^{-6} BAP multiple shoot buds differentiate from the cut end of the petiole. On this medium the petiole also elongates (after Bhojwani and Sharma, 1989).

tiation whereas relatively higher levels of adenine or kinetin promote bud differentiation. Thus, root-shoot differentiation is a function of quantitative interaction between IAA and kinetin. Despite the elegant demonstration of hormonal control of organ formation in tobacco and its applicability to several other plants, there are exceptions to the qualitative (shoot buds versus roots) differentiation based on exogenous auxin/cytokinin ratio. This may be due to: (i) the degree of cell sensitivity towards growth regulators due to the origin of the explant, (ii) the endogenous levels of active growth regulator molecules, (iii) their uptake, (iv) their degree of glycosylation and hydrolysis, (v) the type of auxin and cytokinin used, (vi) their mode of action or (vii) the activity of auxin and cytokinin oxidases (Tran Thanh Van and Trinh, 1990).

Single-cell tissue clones of *Convolvulus* differentiated shoot buds in complete absence of a growth regulator in the medium (Earle and Torrey, 1965). The addition of either IAA or kinetin promoted bud formation. Whereas IAA was promotive only at a very low level (10^{-7} M), kinetin was so up to a concentration of 10^{-5} M. The highest frequency of bud differentiation occurred with a combination of IAA (10^{-7} M) and kinetin (10^{-5} M).

Unlike tobacco and *Convolvulus*, some tissues are characterized by a complete lack of bud formation in the absence of exogenous growth regu-

lators [*Scurrula pulverulenta* (Bhojwani and Johri, 1970); *Dendrophthoe falcata* (Nag and Johri, 1971); *Taxillus vestitus* (Nag and Johri, 1971); *Lactuca sativa* (Doerschug and Miller, 1967); *Brassica juncea* (Sharma et al., 1990)]. In cotyledon cultures of *B. juncea* BAP alone induced shoot bud differentiation from the petiolar cut end; in the absence of BAP or any other hormone only roots were formed at the same site (Fig. 5.7). In some other systems a cytokinin is effective for shoot bud induction only in the presence of an auxin (Doerschug and Miller, 1967) or adenine (Nitsch and Nitsch, 1967).

Besides kinetin, several other cytokinins, viz., BAP, 2ip, SD 8339, thidiazuron and zeatin have been tested for shoot-bud induction in tissue cultures. Of these, BAP has proved most effective and has been used most widely. The number of vegetative buds per thin cell layer explants of tobacco was five times greater in the presence of CPU (a urea derivative cytokinin related to thidiazuron) than with kinetin (Tran Thanh Van and Trinh, 1990).

In most of the cereals, callus tissue exhibits organogenesis when it is transferred from a medium containing 2,4-D to a medium lacking it or having IAA or NAA in its place. Whether the tissue would form shoots or roots, however, depends on the innate capacity of the tissue; an effective exogenous control to stimulate shoot-bud formation selectively is almost unknown. Indeed, in these plants root formation is more common than shoot-bud differentiation. A two-step process of organogenic differentiation also occurs in alfalfa (Saunders and Bingham, 1972; Walker et al., 1978, 1979). Callus is initiated and multiplied on a medium containing 2,4-D and kinetin ('induction medium'). Organogenesis occurs when pieces of tissue from such calli are transferred to a hormone-free medium ('regeneration medium'). Unlike cereals, in alfalfa (*Medicago sativa*) the type of organ formed in the regeneration medium can be controlled by manipulating the ratio of the two hormones in the induction medium; a higher 2,4-D to kinetin ratio favours shoot formation, whereas a higher kinetin to 2,4-D ratio supports root differentiation (Walker et al., 1979). The hormone ratio in the induction medium during the last 4 days is critical in determining the nature of the organ formed.

Gibberellin inhibits shoot-bud differentiation in tobacco (Murashige, 1961, 1964; Thorpe and Murashige, 1970), *Plumbago indica* (Nitsch and Nitsch, 1967), *Begonia* (Heide, 1969) and rice (Maeda, 1978). In tobacco, exposure of the differentiating callus, in the dark, to GA_3 for a period as short as 30–60 min reduced shoot bud differentiation (Thorpe and Meier, 1973) and after 48 h of GA_3 treatment no meristemoids or shoot buds were left (Thorpe and Meier, 1973, 1975). Gibberellin was most effective

at the stage of meristemoid formation. Once shoot buds were formed GA_3 did not inhibit their further development. Complete inhibition by GA_3 occurred only in the dark (Thorpe and Meier, 1973).

Tobacco tissue contains gibberellin-like substances (Lance et al., 1976b) and is capable of metabolizing exogenous gibberellin (Lance et al., 1976a). Thorpe (1978) has suggested the involvement of gibberellin in bud formation in tobacco. The inhibition by exogenous gibberellin is probably because the tissue synthesizes the hormone in quantities optimal for the organogenic process. According to this theory, in *Chrysanthemum* (Earle and Langhans, 1974a) and *Arabidopsis* (Negrutiu et al., 1978a,b), where application of gibberellin promotes budding, the endogenous level of the hormone must be suboptimal. On the other hand, in sweet potato the promotion of bud formation by abscisic acid may be explained on the basis of supraoptimal quantities of endogenous gibberellins (Yamaguchi and Nakajima, 1974). At a non-toxic level (10^{-6} M) abscisic acid partially overcomes the repression of bud formation induced by GA_3 in tobacco (Thorpe and Meier, 1973).

Kumar et al. (1987) examined the role of ethylene in shoot bud differentiation in cotyledon cultures of *Pinus radiata*. The shoot forming explants produced considerable amounts of ethylene and carbon dioxide, and the frequency of shoot bud formation could be correlated with the concentration of the two gases inside the culture vial. Maximum number of buds per explant were formed when the flask contained 5–8 μl l^{-1} of C_2H_4 and 10% CO_2 in the head space, during the first 15 days of culture. Removal of these gases from the culture vessel completely stopped organogenesis. On the other hand, excessive accumulation of the gases beyond 15 days caused partial dedifferentiation of shoot buds.

In some of the recalcitrant tissues shoot formation could be induced or promoted by applying unconventional substances such as abscisic acid (Yamaguchi and Nakajima, 1974; Shepard, 1980), 2,3,5-triiodobenzoic acid (0.02 μM; Cassells, 1979), kanamycin (2.5–20 μM; Owens, 1979), and auxin synthesis inhibitors (0.01–0.1 mg l^{-1} 7-aza-indole or 5-hydroxy nitrobenzylbromide; Kochba and Spiegel-Roy, 1977a). Direct and rapid (within 2 weeks) formation of shoot buds in thin cell layer explants of *Beta vulgaris* was induced by TIBA (Tran Thanh Van and Trinh, 1990). Addition of raw powder of *Panax pseudo-ginseng* or *Panax ginseng* to culture medium, in addition to auxin and cytokinin, doubled the regeneration frequency in the cultures of cotyledons and hypocotyl of *Brassica oleracea* ssp. *italica* (Hui and Zee, 1980). Virtually all our knowledge of the role of phytohormones in differentiation is based on in vitro studies involving manipulation of exogenously applied hormones. It poses a fundamental question: Whether the exogenous phytohormones act directly

on the target cells to induce organogenic differentiation or indirectly by setting up conditions which allow some intrinsic programme to be initiated (Torrey, 1966). Transformation of plant cells with cloned T-DNA genes specific for the synthesis of auxin (*iaah* and *iaam*) or cytokinin (*ipt*) enhance the endogenous level of the respective hormone, which is associated with root/shoot differentiation in a manner similar to the effect of exogenous auxins and cytokinins (Owens and Smigocki, 1990). Furthermore, exogenous hormones can reverse the T-DNA induced morphogenesis, suggesting that the hormones play direct role in organogenesis (Inze et al., 1984).

(ii) *Electrical stimulation.* Organogenic and embryogenic (see Section 6.3.7) differentiation in tissue cultures can be markedly enhanced by the application of weak electric current. Rathore and Goldsworthy (1985a) reported 70% increase in tobacco callus growth by the application of weak electric current (1 μA) in such a way that the callus was made negative and the medium positive. This stimulation occurred only on IAA-containing medium (Rathore and Goldsworthy, 1985b). On the callusing medium, which normally does not favour any caulogenesis, some greening and shoot bud differentiation occurred after the electric treatment. On shoot differentiation medium, the application of microampere current to the callus caused 5-fold stimulation of shoot bud differentiation as compared to the control (Rathore and Goldsworthy, 1985c). The shoot buds first appeared in the most negative region of the callus irrespective of the polarity of the current. The electric stimulation of caulogenesis affected both the number of cultures forming shoot buds and the number of buds per culture.

The callus derived from mature embryos of wheat, which only formed roots, was induced to form several shoots by exposure to electrical treatments (Rathore and Goldsworthy, 1985c).

(iii) *Explant.* Whereas in some plants, such as tobacco, almost all parts are amenable to in vitro plant regeneration, in others this potential is restricted to only certain tissues. In plants where different explants respond, some may be more regenerative than the others. In *Crotalaria juncea* (Ramawat et al., 1977) and *Glycine* (Kameya and Widholm, 1981) the hypocotyl exhibits higher potentiality for shoot formation than the root segments. Similarly, in *Lactuca sativa* (Doerschug and Miller, 1967) and *B. juncea* (Sharma, 1987) cotyledon was the best explant for plant regeneration.

The regenerability of an explant is influenced by several factors, such as the organ from which it is derived, the physiological state of the ex-

plant and its size. Orientation of the explant on the medium and the inoculation density may also affect shoot bud differentiation.

The physiological status of an explant is affected by the age of the donor plant which has a direct bearing on the regenerability of the explant. The use of young and meristematic tissues has, in many cases, enabled raising of regenerative cultures when mature and differentiated explants failed to show such a response. This is especially true for cereals and tree species. Wernicke and Brettell (1980) demonstrated that in *Sorghum bicolor* the regeneration capacity is restricted to the two youngest leaves and the basal part of the third leaf. The number of shoots per culture and the percent cultures of tissue peels from hypocotyl of *Psophocarpus tetragonolobus* showed a decline with increasing age of the seedlings. In *Brassica juncea* 5-day-old seedlings provided most regenerative cotyledons (Sharma et al., 1990). The cotyledons from seedlings older than 10 days did not form shoots at all. In *Pinus radiata* the cotyledons lose the potential to form adventitious shoot buds 3 days after germination which coincides with the complete depletion of lipid in the cells of the cotyledons. In contrast, in *P. gerardiana* the cotyledons derived from ungerminated seeds show higher potential to form shoot buds (Banerji and Bhojwani, unpublished).

Preparation of explants is also important. In cotyledon cultures of *B. juncea*, shoot buds or roots, depending on the culture medium, are formed only at the cut end of the petiole (Fig. 5.7). The lamina lacks this potential. However, the presence of laminar tissue is essential for the petioler cells to exhibit totipotency. Therefore, the ideal explant to achieve regeneration is the lamina together with a short (1 mm) petiole. This is also true for *B. oleracea* (Lazzeri and Dunwell, 1986; Horeau et al., 1988).

Orientation of the explant on the medium proved to be a critical factor for organogenic differentiation in cotyledon cultures of *B. juncea* (Sharma et al., 1990). Planting the cotyledons with their abaxial surface in contact with the medium and the petiolar cut end embedded in the medium gave best response. The explants in which, due to expansion and curling of the lamina, the petiole lost contact with the medium within 3–5 days after culture failed to form roots or shoots. Similarly, the frequency of shoot formation in the cultures of thin layer explants of *B. napus* when epidermis was in contact with the medium was only 13% as against 40% regeneration when the cortical cells were touching the medium (Klimaszewska and Keller, 1985). In *Cunninghamia lanceolata* the explants plated horizontally on the medium produced three times more shoots than those planted vertically (Bigot and Engelmann, 1987).

(iv) *Genotype*: Plant regeneration was once thought to be primarily dependent on the concentration of phytohormones in the medium (Skoog and Miller, 1957). However, it is now well established that for in vitro differentiation the genotype of the plant plays an equally, if not more, critical role as the growth regulators. Indeed the success in obtaining regeneration in leguminous species, once regarded as a recalcitrant group (Bhojwani et al., 1977a), has been mainly due to shift in the emphasis from media selection to genotype selection (Bhojwani and Mukhopadhyay, 1986).

Genotype specificity to regeneration has been reported in a number of plants. Genetic variation for regeneration occurs between varieties and, in outbreeding species, even within varieties. Different cultivars of alfalfa exhibited variation in regeneration capacity when subjected to the same culture regime (Saunders and Bingham, 1972). In tomato intervarietal differences were observed regarding the percentage of rhizogenesis, ability to regenerate shoots and the number of shoots regenerated (Padmanabhan et al., 1974; Kurtz and Lineberger, 1983).

Dietert et al. (1982) reported that in *Brassica* species of the U's triangle inter-cultivar differences for organogenic potentiality were as great as inter-species variation. Intraspecific variation for regeneration in tissue cultures of *B. oleracea* was also observed by Lazzeri and Dunwell (1984), Murata and Orton (1987) and Horeau et al. (1988).

An overall survey of the literature reveals that among the three monogenomic species of the U's triangle of *Brassica* (U, 1935), *B. oleracea* (CC) is the most regenerative and *B. campestris* (AA) the least (Lazzeri and Dunwell, 1984; Chopra et al., 1986; Glimelius and Ottosson, 1983; Narasimhulu and Chopra, 1988; Jourdan and Earle, 1989). Glimelius and Ottosson (1983) cultured the protoplasts of *B. campestris*, *B. juncea*, *B. napus* and *B. oleracea* and succeeded in obtaining calli in all species but regeneration of shoots occurred only in *B. oleracea* and *B. napus*. Similar results were reported by Lu et al. (1982). It seems regeneration genes in *B. napus* have been contributed by *B. oleracea* (Murata and Orton, 1987; Narasimhulu and Chopra, 1988; Jourdan and Earle, 1989). This argument is supported by the fact that all the seedling explants of *B. carinata*, the other amphidiploid species which has received *B. oleracea* genome, have shown regeneration of shoots (Jaiswal et al., 1987), unlike *B. juncea* (George and Rao, 1980; Sharma, 1987) which is the amphidiploid species between *B. nigra* and *B. campestris*.

The studies of Jourdan et al. (1985), Robertson and Earle (1986) and Jourdan and Earle (1989) suggest that both nuclear and cytoplasmic genes affect the frequency of plant regeneration in protoplast cultures

of *B. oleracea*. The alloplasmic lines of *B. oleracea* with Ogura R_1 male sterile cytoplasm show an overall lower regeneration than those with normal *Brassica* cytoplasm (Jourdan and Earle, 1989). Narasimhulu et al. (1988) noted significant cytoplasmic influence on regenerability in *B. carinata* synthesized from reciprocal crosses between *B. oleracea* and *B. nigra*.

Several workers have independently demonstrated that regeneration in wheat is genetically controlled. Rode et al. (1988) suggested that mitochondrial genes are involved in differentiation. However, Mathias and Fukui (1986) have shown that a specific chromosome, whose substitution greatly reduces the capacity of the cultures to regenerate, controls morphogenesis in vitro. According to Galiba et al. (1986) regeneration is controlled primarily by the genes on 7B, 7D and 1D chromosomes but genes on some other chromosomes may also be involved. If the genes controlling regeneration are identified and mapped then there is the exciting possibility of the transfer of such genes to recalcitrant species.

The genotypically selected regenerating lines do not exhibit stringent culture requirements and display regeneration ability on a wide range of media (Keyes et al., 1980; Bhojwani and White, 1982; Kurtz and Lineberger, 1983).

(v) *Physical factors*. White (1939b) reported that in a solid medium the tissue cultures of *Nicotiana glauca* × *N. langsdorffii* grew in a completely unorganized state, but in the liquid medium of otherwise identical composition it formed leafy shoot buds. This report was confirmed by Skoog (1944), but Dougall and Shimbayashi (1960) observed extensive bud formation in tobacco cultures grown on medium solidified with 1% agar and negligible bud differentiation in tissues grown on the surface of liquid medium of the same composition. A striking alteration in the morphogenic pattern with change in agar concentration in the medium occurred in thin tissue peels of tobacco (Tran Thanh Van and Trinh, 1978). With 1% agar only flowers were formed. With lowering of agar concentration the frequency of flower formation dropped and vegetative bud differentiation occurred. In liquid medium the tissue exhibited only callusing and vegetative bud formation.

For shoot differentiation in callus cultures derived from mesophyll protoplasts of a dihaploid cultivar of potato it was essential to maintain the osmotic pressure between 200 and 400 mmol by adding 0.2–0.3 M mannitol. Without this, the calli did not exhibit greening which must precede shoot initiation (Shepard et al., 1980). In rice callus cultures, enhanced osmolarity of the medium, achieved by the addition of NaCl (1.5%) (Binh and Heszky, 1990) or sorbitol or mannitol (3%) (Kavi Kishore and Reddy,

1986), not only improved the frequency of regeneration in primary cultures but also helped maintaining it in long-term cultures (see Section 6.11). Even dehydration of rice callus by placing it on dry filter paper inside a sealed petriplate promoted regeneration frequency (Tsukahara and Hirosawa, 1992). Under the experimental conditions the water content of callus dropped around 50% within 1 h and remained relatively constant thereafter. However, the regeneration frequency peaked (45% as compared to 5% in the untreated control) after 24 h of dehydration treatment.

High light intensity has been shown to be inhibitory for shoot-bud formation in tobacco (Skoog, 1944; Thorpe and Murashige, 1970). Callus of *Pelargonium hortorum* differentiated shoots only under alternating light and dark periods (15–16 h day proved best). Callus maintained under continuous light remained whitish and did not exhibit organogenesis (Pillai, 1968). The quality of light also influences organogenic differentiation (Weis and Jaffe, 1969; Bagga et al., 1985). Blue light promoted shoot-bud differentiation whereas red light stimulated rooting in tobacco (Letouze and Beauchesne; cited in Narayanaswamy, 1977). The observations of Bagga et al. (1985) suggest the involvement of phytochrome in shoot induction. Calli of *Brassica oleracea* grown in dark for 20 days formed shoot buds 12 days after transfer to light while those shifted to light after 12 days of growth in dark differentiated shoots within 9 days. The calli given red light treatment for 5 min followed by 24 h of dark for 5 continuous days produced shoots within 2 days of growth in light, and the response was much more intense. Infrared radiation nullified the effect of red light.

Skoog (1944) studied the effect of a range of temperature (5–33°C) on tobacco callus growth and differentiation. Growth of the callus increased with rise in temperature up to 33°C, but for shoot-bud differentiation 18°C was optimum; no bud formation occurred at 33°C. Shoot-bud initiation in the cultures of hypocotyl segments of *Linum usitatissimum* is, however, better at higher temperatures (30°C) (Murray et al., 1977).

5.3.2. Induction of organogenic differentiation

Organogenic differentiation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, is a multistep process. A series of intracellular events, collectively called induction, occur before the appearance of a morphologically recognisable organ. The cells induced to form a specific organ in the presence of appropriate hormonal combination would continue to develop into that organ even if the inductive hormones are removed. Thus, induction leads to irreversible com-

mitment of cells to follow a particular developmental pathway. For example, in cotyledon cultures of *Brassica juncea* BAP induces shoot bud differentiation at the cut end of the petiole, and in the absence of BAP only roots are formed at the same site (Fig. 5.7) (Sharma et al., 1990). The cotyledons transferred to basal medium after 11 days of incubation on BAP-containing medium form only shoots and no roots. Similarly, the cotyledons lose the potentiality to form shoots on BAP-medium if they are pre-cultured on BAP-free medium for longer than 7 days.

Leaf explants of *Convolvulus arvensis* form only shoots on MS + 7 mg l^{-1} 2ip + 0.05 mg l^{-1} IAA (SIM), only roots on MS + 12 mg l^{-1} IBA (RIM) and only callus on MS + 0.3 mg l^{-1} kinetin + 3 mg l^{-1} IAA (CIM). Root or shoot bud formation is preceded by slight callusing. The leaf explants are induced to form shoots on SIM within 10–14 days and after this period the destiny of the cells is not changed even if the leaf pieces are transferred to RIM or CIM (Christianson and Warnick, 1983, 1984). The induction process involves two major steps. During the first 3–5 days on the induction medium the cells acquire competence to respond to the inductive conditions (Fig. 5.8). At this stage the cells are plastic in terms of their morphogenic potential and can form roots or shoots depending on the medium to which they are exposed. Under the continued action of SIM the competent cells become committed to form shoots. This irreversible commitment of the cells, which is achieved after 10–12 days on SIM is referred to as determination. The competence to respond to SIM can be acquired even on RIM or CIM. Interestingly, some of the genotypes of *C. arvensis* which exhibited poor or no shoot formation when directly cultured on SIM could be induced to form large number of shoots by pre-culture on RIM for 3–5 days followed by transfer to SIM (Christianson and Warnick, 1985). Probably these genotypes were blocked in the acquisition of competence to respond to SIM which could be achieved on RIM. Christianson and Warnick (1984) have demonstrated that the period between the acquisition of competence and the determination is composed of several sub-stages sensitive to various substances. It includes a stage sensitive to salicylate, followed by a stage sensitive to TIBA, which is followed, in turn, by a stage sensitive to sorbitol. However, the significance of these sub-stages is not clear.

5.3.3. Ontogeny of shoot buds

Under the conditions favouring unorganized growth, the meristems in a callus are random and scattered. Transfer of the tissue to conditions supporting organized growth leads first to the appearance of localized clusters of cambium-like cells. These meristemoids (also termed 'nodules'

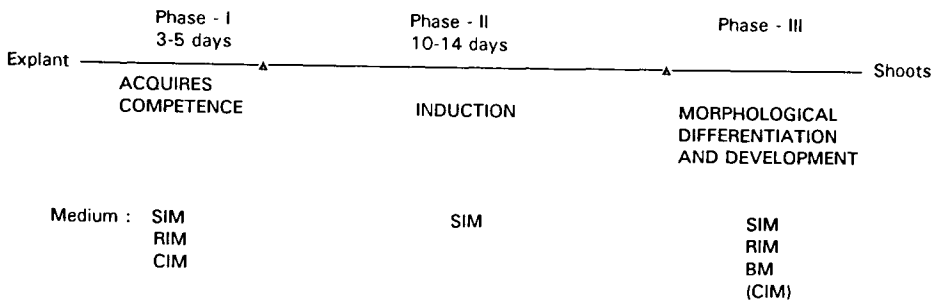


Fig. 5.8. Scheme of events in the process of in vitro shoot differentiation from leaf discs of *Convolvulus arvensis*. The cells acquire competence for shoot bud induction after 3–5 days of culture on shoot induction medium (SIM), root induction medium (RIM) or callus induction medium (CIM). The competent cells become determined to form shoot if maintained on SIM for another 10–14 days. Thereafter the cells continue to develop into shoot, irrespective of the culture medium. If SIM is replaced by RIM during the induction period the cells would form roots instead of shoots. BM, basal medium (adapted from Christianson and Warnick, 1984, 1985).

or 'growing centres'), which may become vascularized due to the appearance of tracheidal cells in the centre, are the site for organ formation in the callus (Ross et al., 1973). Initially the meristemoids are plastic, and can form roots or shoots (Torrey, 1966). Generally roots appear endogenously, whereas shoots originate exogenously. However, there are several examples of endogenous differentiation of shoots (Buvat, 1944; Bonnett and Torrey, 1966).

Steward et al. (1958) described the histological development of whole plants from carrot tissue cultures. in suspension cultures nodules with a central nest of tracheidal elements surrounded by cambium-like cells are formed. This is followed by the development of roots from the central cells of the nodules. When such rooted nodules are transferred to a semi-solid medium a shoot bud appears at another locus far removed from the root. Gradually a vascular connection develops between the root and shoot and a whole plant is formed. A somewhat different pattern of plant regeneration from cell colonies of *Convolvulus* was reported by Earle and Torrey (1965b). These authors emphasized that shoot-bud differentiation occurred independent of vascular tissue; buds often developed in the nodules completely lacking mature vascular elements (see also Torrey, 1966).

Excised cotyledons of *Pinus radiata* cultured on BAP-free medium rapidly elongate and the anticlinal divisions which occur throughout the explant at the time of culture completely cease after 2 days in culture (Villalobos et al., 1985). However, in the presence of BAP the cotyledons do not elongate and cell divisions become restricted to the epidermal and sub-epidermal cell layers on the side in contact with the medium. Six to

eight celled organised 'promeristemoids', arising from a single sub-epidermal cell as a result of both anticlinal and periclinal division, can be seen by day 5. The cells within each promeristemoid are tightly packed together with little or no intercellular spaces but prominent plasmodesmata are present within each promeristemoid. By day 10 the cotyledon surface becomes nodular due to the development of promeristemoids into meristemoids which give rise to shoot primordia by day 21. A similar pattern of shoot bud differentiation also occurs in cotyledon cultures of *P. gerardiana* (Banerji and Bhojwani, unpublished).

Thorpe and Murashige (1970) examined histochemically the changing status of nucleic acid, protein and carbohydrate in differentiating and non-differentiating calli of tobacco. The two tissues did not exhibit much difference in the level of DNA per cell, but RNA and protein contents were higher in the shoot-forming regions of the calli. The difference in the starch content of the two types of tissues was especially remarkable. The intracellular accumulation of starch has been ascribed a positive role in the process of shoot-bud differentiation. This conclusion is based on the following observations: (a) heavy accumulation of starch occurs only in the shoot-forming tissues; (b) no meristemoids are formed in the regions lacking heavy deposition of starch; (c) the accumulation of starch precedes any observable organized development and reaches the maximum level in 11-day-old cultures, which is 3 days before the appearance of meristemoids and shoot formation; and (d) gibberellin, which inhibits shoot formation, prevents starch accumulation reaching a threshold level required for shoot-bud differentiation by decreasing starch synthesis and increasing starch degradation (Thorpe and Meier, 1975). It has been suggested that starch, together with the free sugars in the medium, may be serving as the source of energy during meristemoid and shoot-bud differentiation, which are high energy-requiring processes. Similar conclusions are drawn from the biochemical studies of shoot bud differentiation from excised cotyledons of *Pinus radiata* (Thorpe, 1990, 1993). The shoot forming layer of the cotyledons showed elevated level of respiration, increased concentration of several enzymes, including acid phosphatase, ATPase, and succinate dehydrogenase (Patel and Thorpe, 1984), enhanced amino acid synthesis, and depletion of lipids and soluble sugars (Biondi and Thorpe, 1982).

5.3.4. Totipotency of epidermal cells

Flax (*Linum usitatissimum*) is the classic example of shoot-bud development from the intact hypocotyl (Crooke, 1933; Link and Eggers, 1946). The origin of these buds has been ascribed to single epidermal cells (Link

and Eggers, 1946). Other species reported to form shoot buds/embryos from superficial cell layers of stem in cultures are *Ranunculus sceleratus* (Konar and Nataraja, 1965), *Daucus carota* (Kato and Takeuchi, 1966; Kato, 1968), *Exocarpus cupressiformis* (Bhojwani, 1969a), *Torenia fournieri* (Bajaj, 1972; Chlyah, 1974). *Nicotiana tabacum* (Tran Thanh Van, 1973a,b) and *Brassica napus* (Thomas et al., 1976).

In very young seedlings of flax, epidermal cells all along the length of the hypocotyl are capable of forming shoot buds, but in the seedlings older than 15 days this potential is restricted to the basal half of the hypocotyl. In an intact seedling, decapitation results in the development of numerous buds from the hypocotyl, but only one of them grows into a full shoot (Link and Eggers, 1946). On the other hand, in cultures a 15 mm long hypocotyl segment may develop 160–170 potential plants (Murray et al., 1977). In cultures some shoots also develop from sub-epidermal cells.

Exclusively epidermal peels generally do not survive in culture (Chlyah et al., 1975) or give a poor response (Tran Thanh Van and Trinh, 1978). Nevertheless, in the cultures of thin superficial peels (one to seven layers) from stem and leaf the epidermal cells divide and elicit their totipotency (Kato, 1968; Tran Thanh Van and Trinh, 1990). It is interesting that in the cultures of thin surface peels from stem, epidermal cells can be induced to develop directly into a root or a shoot, or even a fertile flower (see Fig. 5.9) at will (Tran Thanh Van, 1973a,b; Tran Thanh Van et al., 1974a; Tran Thanh Van and Trinh, 1990). The advantages of such a system are: (a) it may allow direct observation of the changes in a single cell leading to different types of organogenic differentiation, and (b) since the explant lacks vascular tissue and cambium, and the amount of other parental tissues is reduced to a bare minimum, it carries very little or no influence of endogenous growth substances.

The type of buds formed in the cultures of thin tissue peels taken from the stem of a flowering plant of *Nicotiana tabacum* varies with the region of the stem from which the peel is derived (see Fig. 5.10) (Tran Thanh Van, 1973b). Under certain culture conditions the peels from floral branches produce only flower buds, while those from the basal part of the plant form only vegetative buds. The explants derived from the middle portion bear both types of buds in different proportions, depending on their distance from the base of the plant. Within the inflorescence, peels from the basal region give a better response compared to those from the terminal portion. The number of peels from basal portions of the inflorescence that form flower buds is also influenced by the physiological stage of the donor plant. For example, 100% of the tissue pieces from plants bearing green fruits exhibited flower formation. If the donor plant had reached mature fruit stage the response was slightly poor (85% explants

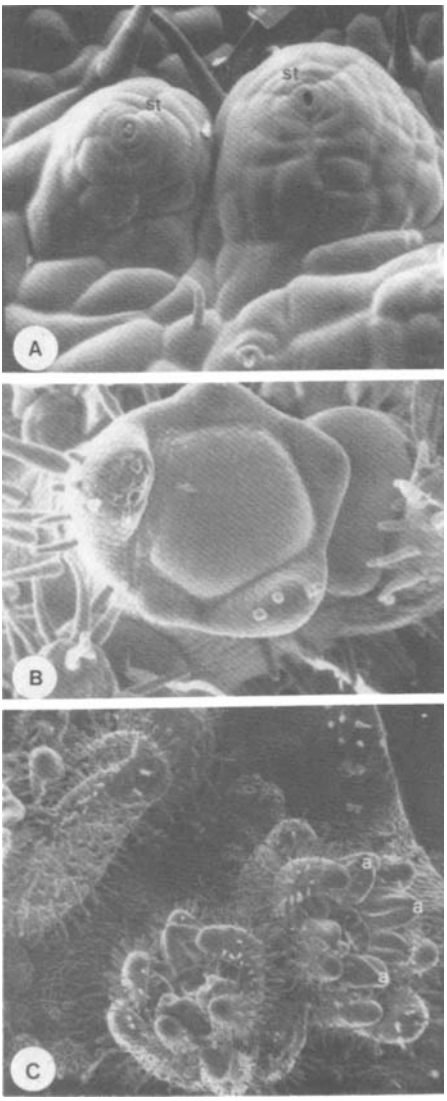


Fig. 5.9. Scanning electron micrographs to show direct differentiation of floral buds from somatic cells in the cultures of thin cell layers of *Nicotiana tabacum*. (A) Some protuberances can be seen just under stomata (st). (B) Sepal primordia have differentiated. (C) Direct and de novo formation of flowers 10–18 days after culture (a, anther) (after Tran Thanh Van, 1977).

formed buds). No flower buds were formed if the parent plant bore only flowers but no fruit.

The peels from floral branches of tobacco formed flower buds only if the medium contained kinetin and IAA at an equimolar concentration of 10^{-6} M (the absolute concentration of the hormones is important, not

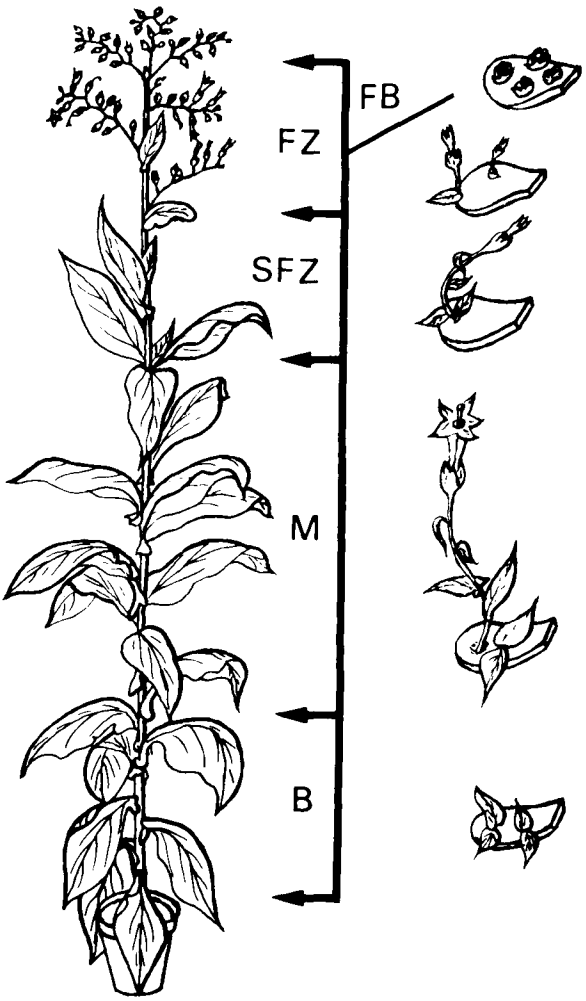


Fig. 5.10. Diagram showing capacity of thin cell layers excised from different levels of a flowering plant of tobacco to form vegetative or floral bud in culture. B, base; M, middle zone; SFZ, sub-floral zone; FZ, floral zone; FB, floral branches) (after Tran Thanh Van, 1973b).

their ratio of 1:1) together with 2–3% sucrose. An increase in kinetin concentration to 10^{-5} M without affecting the IAA concentration completely suppressed flower bud formation; instead vegetative buds appeared. With further alteration of the hormonal balance in the medium it was possible to shift the morphogenesis in favour of rooting or callusing (see Table 5.2). This is another good example of hormonal control of organ differentiation. Under ideal conditions 7–30 flower buds developed on a single explant measuring 1 × 0.4 cm.

TABLE 5.2

Optimal conditions for different types of differentiation in small explants from floral branches of tobacco[a]

Type of neoformation	Glucose ($g\ l^{-1}$)	Growth substances	Auxin/ cytokinin ratio	Other favourable conditions
Floral buds	30	IBA 10^{-6} M Kinetin 10^{-6} M	1.0	Light; terminal bud in green fruit stage
Vegetative buds	30	IBA 10^{-6} M Kinetin 10^{-5} M	0.1	Light
Roots	10	IBA 10^{-5} M Kinetin 10^{-7} M	100.0	Darkness; terminal bud in mature fruit stage
Callus	30	IBA 3×10^{-6} M Kinetin 10^{-7} M	50.0	–

[a]After Tran Thanh Van et al. (1974b) and Tran Thanh Van and Trinh (1990).

The flowers formed in cultures of thin tissue peels were normal; they formed viable gametes and set fertile seeds. Interestingly, epidermal peels from even male sterile plants of tobacco developed several fertile flowers (Tran Thanh Van and Trinh, 1978). Androgenic plants could be raised by culturing anthers from such flowers. By taking epidermal peels from these dihaploids ($2n = 24$) and culturing the anthers from flowers produced from them, and repeating this cycle once more, Tran Thanh Van (1977) could obtain some hypohaploids with less than six chromosomes (see Fig. 5.11). This could not be achieved with seed-grown plants.

To date controlled organogenesis in cultured thin-layer peels has been achieved with *Nautilocalyx lynchei* (Tran Thanh Van, 1973a), *Cichorium intybus* (Nguyen, 1975), *Nicotiana tabacum* (Tran Thanh Van, 1973a,b; Tran Thanh Van et al., 1974b), *Sesbania* (Tran Thanh Van and Trinh, 1990), *Torenia fournieri* (Chlyah, 1974), and *Bryophyllum daigremontianum* (Bigot, 1976).

5.3.5. Totipotency of crown-gall cells

A typical crown-gall tumour cell is characterized, both in the host and

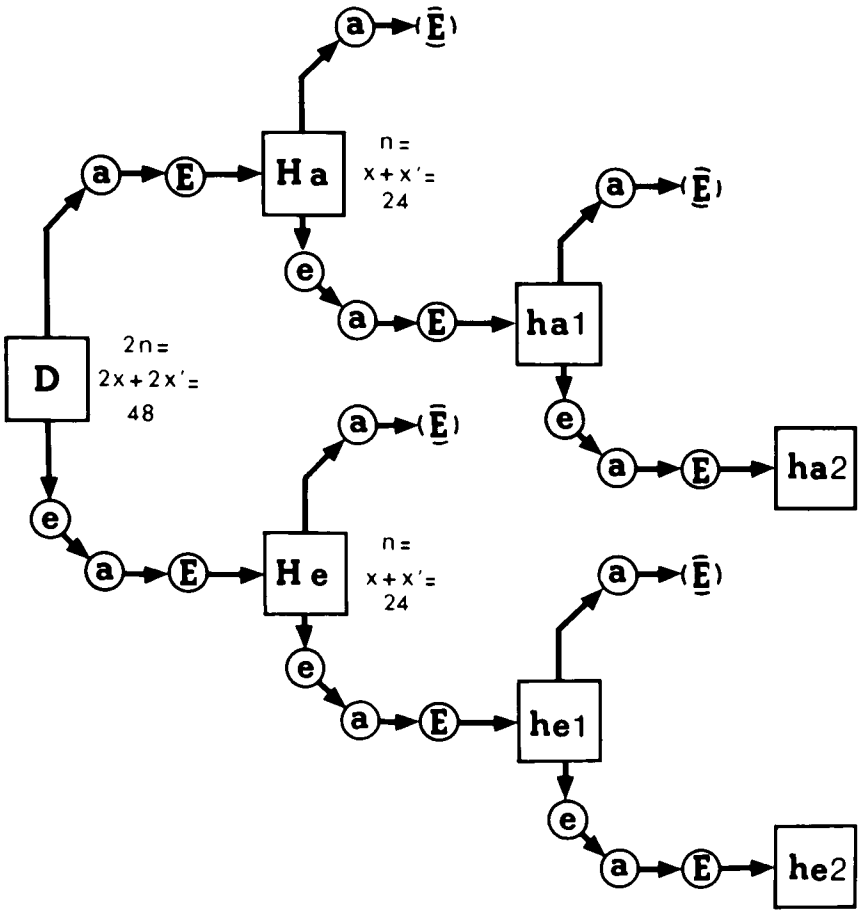


Fig. 5.11. Diagrammatic summary of androgenic embryo (E) development in the cultures of anthers (a) from flower buds formed normally on the plant and those differentiated in the cultures of thin cell layers (e). Anthers from parent plants do not form pollen embryos (E) beyond the dihaploid stage ($n = 2x = 24$) whereas those from the flowers differentiated directly from thin cell layers yield haploids (ha 1, he 1) and hypohaploids (ha 2, he 2) (after Tran Thanh Van and Trinh, 1978).

in culture, by a capacity for unlimited growth independent of exogenous hormones. It shows a complete lack of organogenic differentiation and is, therefore, considered to have permanently lost the totipotentiality of the parent cells.

In some plant species the crown-gall bacterium (*Agrobacterium tumefaciens*) induces a special type of tumour, called teratoma, the cells of which possess a pronounced capacity to differentiate shoot buds and leaves when they are in the host tissue or grown in cultures for unlimited

periods. However, the shoots formed by these cells are abnormal morphologically and in their growth form. In tobacco completely normal shoots could be recovered from teratoma shoots (Braun, 1959; Braun and Wood, 1976). Braun (1959) cultured pieces of crown-gall teratoma tissues in shake cultures, picked up individual cells from them and raised single-cell tissue clones using the nurse-culture technique of Muir et al. (1954). Before the calli started to differentiate shoot-buds pieces of tissue were taken from them and grafted onto the cut ends of stems of healthy tobacco plants from which axillary buds had been removed. In successful grafts the tumour tissue developed some highly abnormal shoot buds. Tips (3–5 mm long) of the teratoma shoots from the grafts were excised and grafted onto another healthy plant. Through a series of similar grafts of tips of teratoma shoots onto normal plants, Braun (1959) obtained actively growing shoots which appeared structurally, histologically, and functionally normal (Braun and Wood, 1976). The recovery, although complete and persistent, was not irreversible. It was a case of suppression of the neoplastic condition of the cells. If the restraints of organization were removed, and pieces of sporophytic tissues from vegetative or reproductive organs of the recovered shoots cultured, they reverted to tumour characteristics, such as auxin autonomy (Braun and Wood, 1976). Nevertheless, somatic tissues of plants raised from seeds formed by the recovered teratoma shoots did not revert back to the neoplastic growth (Turgeon et al., 1976). Even the androgenic haploids obtained by culturing anthers from the recovered shoots exhibited permanent and irreversible loss of the neoplastic condition, suggesting that the stable recovery occurred during the meiotic process (Turgeon et al., 1976). Yang et al. (1980) demonstrated that the recovered plants looking normal but still exhibiting tumorous trait when recultured carried the T-DNA, whereas the F_1 plants, which had completely lost the tumour trait, lacked it.

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Chapter 6

Somatic Embryogenesis

6.1. INTRODUCTION

The act of fertilization triggers the egg cell (called the zygote after fertilization) to divide and develop into an embryo (the process of embryo development is called embryogenesis). However, fertilization is not always essential to stimulate the egg to undergo embryogenesis. As happens in parthenogenesis, the pollination stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development. Moreover, it is not the monopoly of the egg to form an embryo. Any cell of the female gametophyte (embryo sac), or even that of the sporophytic tissue around the embryo sac may give rise to an embryo. In several species of *Citrus* and *Mangifera* the development of adventive embryos from nucellar cells is a normal feature. However, the nucellar embryos attain maturity only if they are pushed into the embryo sac at an early stage of development, or else they fail to mature. In nature there is no instance of ex-ovulo embryo development (Bhojwani and Bhatnagar, 1990). These in vivo observations would suggest that for their growth and development embryos require a special physical and chemical environment available only inside the 'magic bath' of the embryo sac.

During the last three decades considerable information has accumulated to establish the embryogenic potential of somatic plant cells, and there has been an explosion in the number of species that form somatic embryos (SEs). Based on the recent spectacular development in cell and tissue culture of higher plants it would be fair to say that any cell, in which irreversible differentiation has not proceeded too far, will, if placed in an appropriate medium, develop in an embryo-like way and produce a complete plant. The whole complex sexual apparatus is, therefore, not an essential prerequisite for cells to acquire embryonic properties. The events occurring in the ovule after fertilization thus provide only a special case of embryogeny. For detailed recent reviews on in vitro somatic embryogenesis refer to Ammirato (1989), Carman (1990), Gray and Purohit (1991), Michaux-Ferriere and Schwendiman (1992), Zimmerman (1993), de Jong et al. (1993) and Emons (1994).

Embryos formed in cultures have been variously designated as accessory embryos, adventive embryos, embryoids, and supernumerary em-

bryos. In this chapter the embryos formed in cultures have been referred to as somatic embryos (SEs) or simply embryos.

6.2. SOME EXAMPLES OF SOMATIC EMBRYOGENESIS

The first observations of in vitro somatic embryogenesis were made in *Daucus carota* (Reinert, 1958, 1959; Steward et al., 1958). Ever since, this species has been widely used to investigate various aspects of in vitro somatic embryogenesis (Terzi et al., 1985; Molle et al., 1993; Zimmerman, 1993). Other plants in which this phenomenon has been studied in some detail are *Citrus* sp. (Rangaswamy, 1961; Sabharwal, 1963; Rangan et al., 1968; Kochba and Spiegel-Roy, 1977b; Tisserat and Murashige, 1977; Gavish et al., 1991, 1992), *Coffea* sp. (Monaco et al., 1977; Sondahl et al., 1979a,b; Sharp et al., 1980; Nakamura et al., 1992), *Macleaya cordata* (Kohlenbach, 1977), *Medicago* sp. (Redenbaugh and Walker, 1990, McKersie et al., 1993), *Ranunculus sceleratus* (Konar and Nataraja, 1969; Konar et al., 1972; Thomas et al., 1972), and *Zea mays* (Emons and Kieft, 1991; Songstad et al., 1992; Emons, 1994).

In *Ranunculus sceleratus* various floral parts (including anthers) as well as somatic tissues proliferate to form a callus on a medium containing coconut milk (10%) with or without IAA. Within 3 weeks numerous embryos appear on the callus (Fig. 6.1) (Konar and Nataraja, 1969). The embryos originate from the peripheral as well as deep-seated cells of the callus (Fig. 6.1H,I). Embryo differentiation also occurs in suspension cultures raised from these calli. The SEs germinate in situ or when they are excised and planted individually on a fresh semi-solid medium. A specially interesting feature is the development of a fresh crop of embryos from the stem surface of these plantlets (Figs. 6.2 and 6.3) (Konar and Nataraja, 1965, 1969). The number of adventive embryos formed per plantlet varied from 5 to 50. Light microscopic (Konar and Nataraja, 1965) and ultrastructural studies (Konar et al., 1972) revealed that the stem embryos originated from single epidermal cells (Fig. 6.3) through stages reminiscent of in vivo zygotic embryogeny in this species. Direct embryogenesis from intact epidermal cells also occurs in the cultures of hypocotyledonary segments and their superficial peels in carrot (Kato and Takeuchi, 1966; Kato, 1968).

Kohlenbach (1965) and Lang and Kohlenbach (1975, 1978) demonstrated the ability of mechanically isolated, fully differentiated mesophyll cells of *Macleaya cordata* to yield an embryogenic callus (Fig. 6.4). On a medium containing 2,4-D and kinetin at an equimolar concentration of 5 μM the individual mesophyll cells divided to form tissues which could be continuously multiplied on this medium. A reduction in kinetin con-

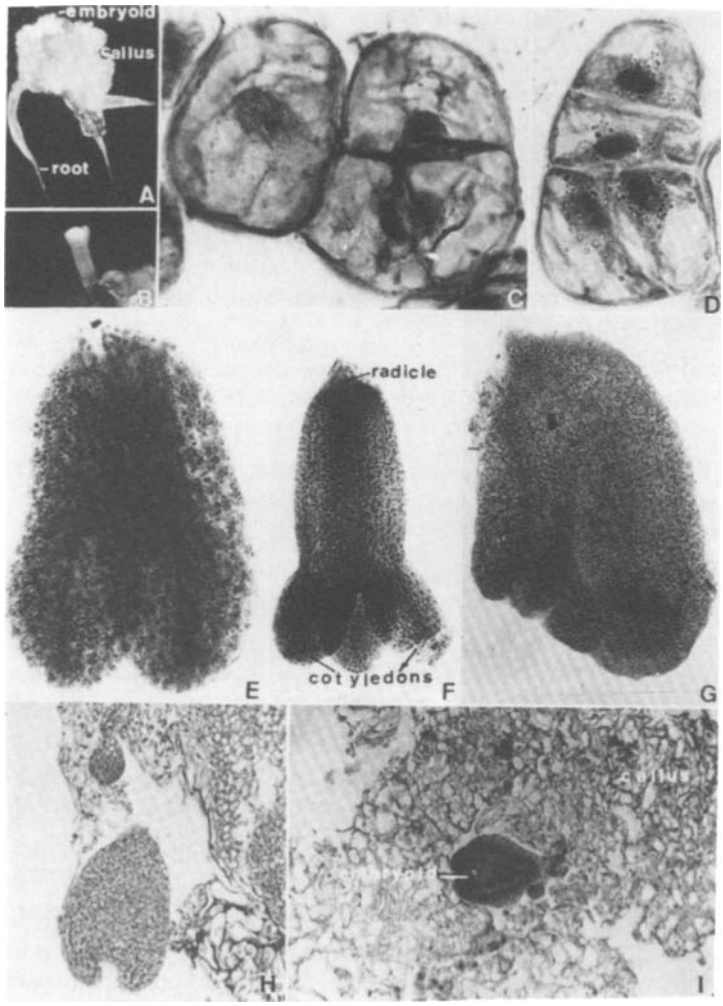


Fig. 6.1. Somatic embryogenesis in the cultures of floral buds of *Ranunculus sceleratus*. (A) Callused explant bearing roots and an aggregate of embryos. (B) An embryo magnified from A. (C,D) Squash preparation of callus, showing early stages in embryo development. (E–G) Whole mounts of mature di-, tricotyledonous and twin embryos. (H) Section through a portion of callus showing globular and heart-shaped embryos. (I) An embryo differentiated deep within the callus (after Konar and Nataraja, 1969).

centration to 1 μM and replacement of 2,4-D by 2 μM IAA or 0.01 μM NAA supported shoot-bud and embryo development from the callus. The embryogenic callus selected on the medium containing 0.1 μM NAA and 1 μM kinetin formed embryos at high frequency upon transfer to a basal medium completely lacking in growth hormones. The embryogenic po-

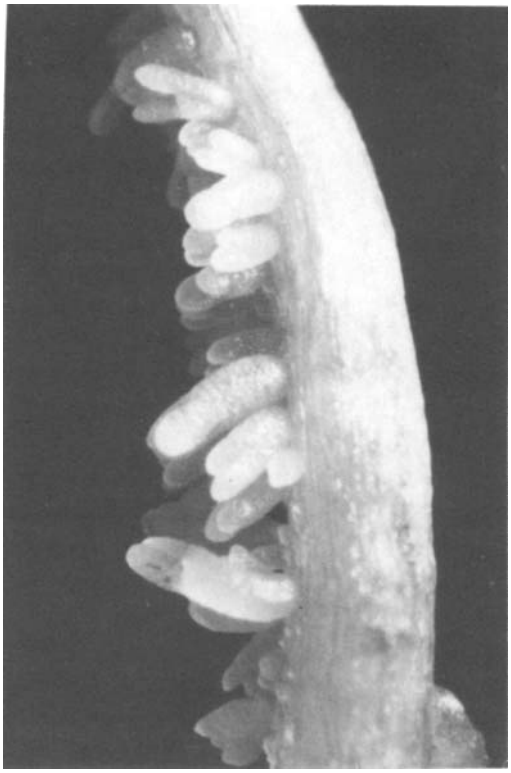


Fig. 6.2. Enlarged view of a portion of stem of an in vitro developed plantlet of *Ranunculus sceleratus*, bearing a large number of adventive embryos (after Konar and Nataraja, 1969).

tential of the callus could be further improved by enriching the medium with reduced nitrogen.

In the embryological texts (Maheshwari, 1950; Bhojwani and Bhatnagar, 1990), *Citrus* is commonly cited as an example of natural polyembryony (seeds with more than one embryo). In several species of this genus the nucellar tissue forms 1–40 adventive embryos per seed (Furusato et al., 1957) of which many attain maturity and form plantlets following seed germination.

The embryogenic potential of *Citrus* nucellar cells is also maintained in cultures. The embryos may arise directly from the nucellar cells (Rangan et al., 1968) or from the callus derived from them. In the latter case embryogenesis occurs in repeated subcultures (Kochba and Spiegel-Roy, 1977b). Nucelli from some monoembryonate (seeds bearing only the zygotic embryo) species of *Citrus* also exhibit somatic embryogenesis in cultures (Rangan et al., 1968).

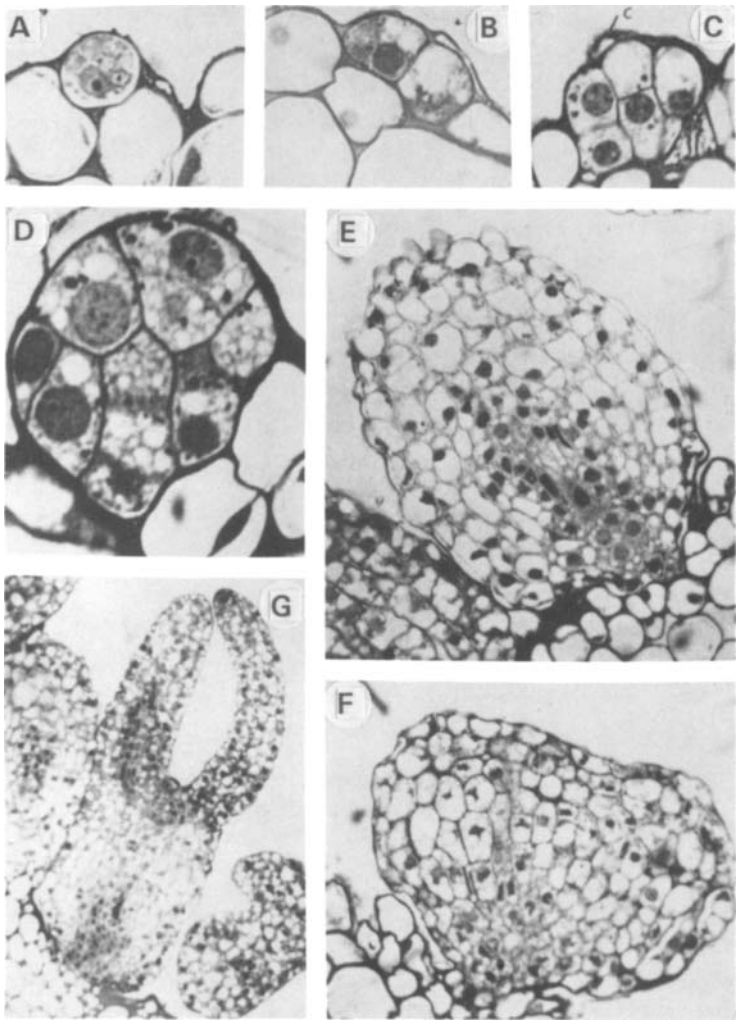


Fig. 6.3. Light micrographs showing stages in embryo development from epidermal cells of the stem of *Ranunculus sceleratus* plantlets. (A,B) Showing a single and a pair of cytoplasm-rich epidermal cells, respectively. (C) Two 2-celled proembryos and a single cytoplasm-rich epidermal cell surrounded by a layer of cuticle (c). (D) A young embryo. (E) Late globular proembryo showing central core of meristematic cells. (F) Heart-shaped proembryo. (G) Cotyledonous embryo, with root and shoot apices (after Konar et al., 1972).

Rangaswamy (1961) and Sabharwal (1963), working with *Citrus microcarpa* and *C. reticulata*, respectively, concluded that the nucellus only from the micropylar end of the ovule is capable of forming embryos in cultures, as it happens in nature. They also emphasized that nucelli from only post-fertilized ovules would form embryos in culture. Button and

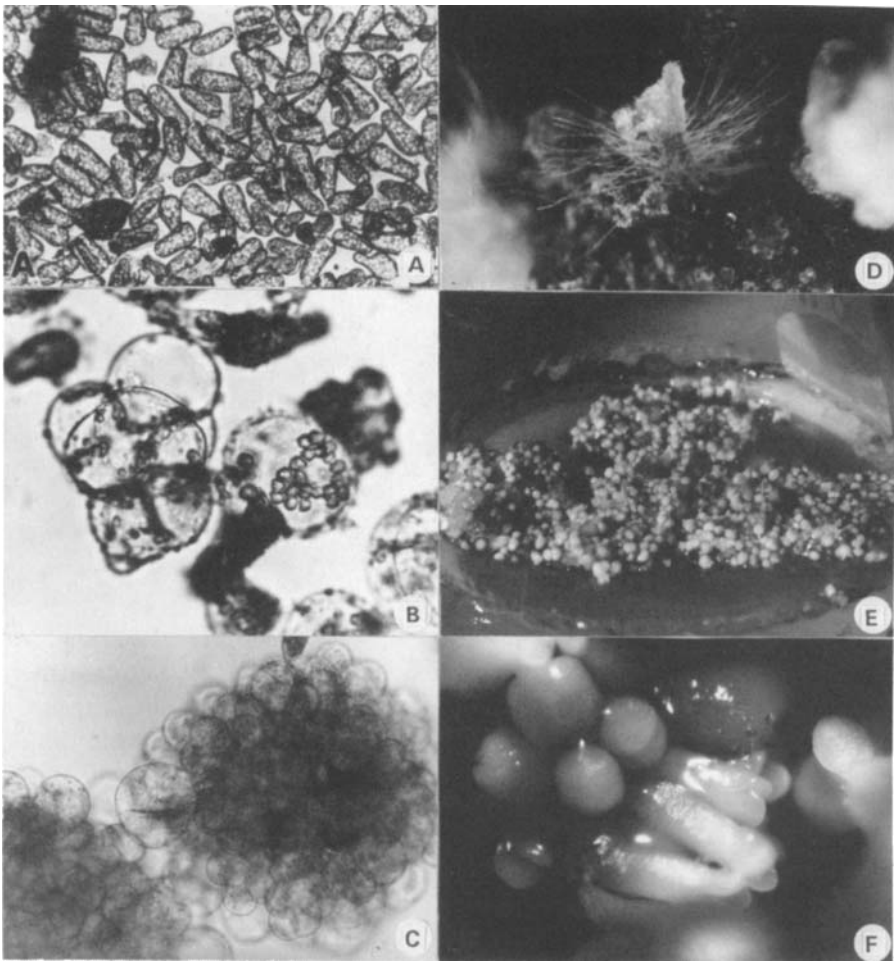


Fig. 6.4. Somatic embryogenesis in the cultures of isolated mesophyll cells of *Macleaya cordata*. (A) A suspension of mechanically isolated mesophyll cells. (B) A small cell colony arising from a palisade cell. (C) A larger colony derived from a mesophyll cell. (D) Root differentiation from a callus. (E) Embryogenic callus formed by a mesophyll cell. (F) A portion enlarged from (E) to show embryos in different stages of development. (A)–(C) After Kohlenbach (1966); (D) after Kohlenbach (1967); (E),(F) after Lang and Kohlenbach (1975) courtesy of Kohlenbach, Germany.

Bornman (1971), however, demonstrated the occurrence of embryogenesis in the cultures of nucellus from pre-fertilized ovules of Washington Navel orange. The non-essentiality of fertilization was confirmed by Kochba and Spiegel-Roy (1973). The embryogenic potential of nucellar cells from the chalazal end was demonstrated by Wakana and Uemoto (1988). Embryogenic calli have also been raised from single cells (Button and Botha,

1975) and protoplasts (Kunitake et al., 1991) enzymatically isolated from the nucellar callus of *C. sinensis*. Of the various supplements to basal medium, malt extract (500 mg l^{-1}) has proved to be most promotive for embryogeny in nucellus cultures of mono-embryonate (Rangan et al., 1968) as well as polyembryonate (Kochba and Spiegel-Roy, 1973) species of *Citrus*.

6.3. FACTORS AFFECTING SOMATIC EMBRYOGENESIS

Conventionally, somatic embryogenesis is regarded as a two step process: 'induction of embryogenesis' and 'embryo development', both requiring different culture conditions. Recently, the third step of 'embryo maturation' has been identified, during which the embryo is prepared for germination. The factors controlling this final step of embryogenesis are discussed in Section 6.5.

6.3.1. Explant

The success in obtaining regenerating cultures of several plant species which were once regarded recalcitrant, such as cereals, grain legumes and forest tree species, has been possible largely due to a shift in emphasis from media manipulation to explant selection. Immature zygotic embryos have proved to be the best explant to raise embryogenic cultures of these plants. In the cultures of embryonic explants SEs may arise directly (Fig. 6.5) or after slight callusing. Cotyledons from SEs of soybean gave considerably higher embryogenic response then those from zygotic embryos (Liu et al., 1992).

In cereals, zygotic embryos exhibit the potential to form SEs only during shortly after histogenesis and prior to embryo maturation (Williams and Maheswaran, 1986), which corresponds to a period from 11–14 days post-anthesis (DPA) in *Triticum aestivum*. During this period embryogenic callus is readily induced from the tissue of the scutellum (Magnusson and Bornman, 1985; Carman et al., 1988). The loss of competence to form somatic embryos 14 days after anthesis is correlated with rapid accumulation of storage proteins within the scutellum (Negbi, 1984). Qureshi et al. (1989) could extend the developmental phase of wheat embryos suitable for somatic embryogenesis from 11–14 DPA to 11–25 DPA by incubating older embryos in ABA-containing medium.

The most successful starting material to date for the initiation of embryogenic cultures of coniferous species has been immature zygotic embryos (Bornman, 1993). In these cases embryogenic tissue arises from cells in the suspensor region of the zygotic embryo. In *Larix* the SEs de-

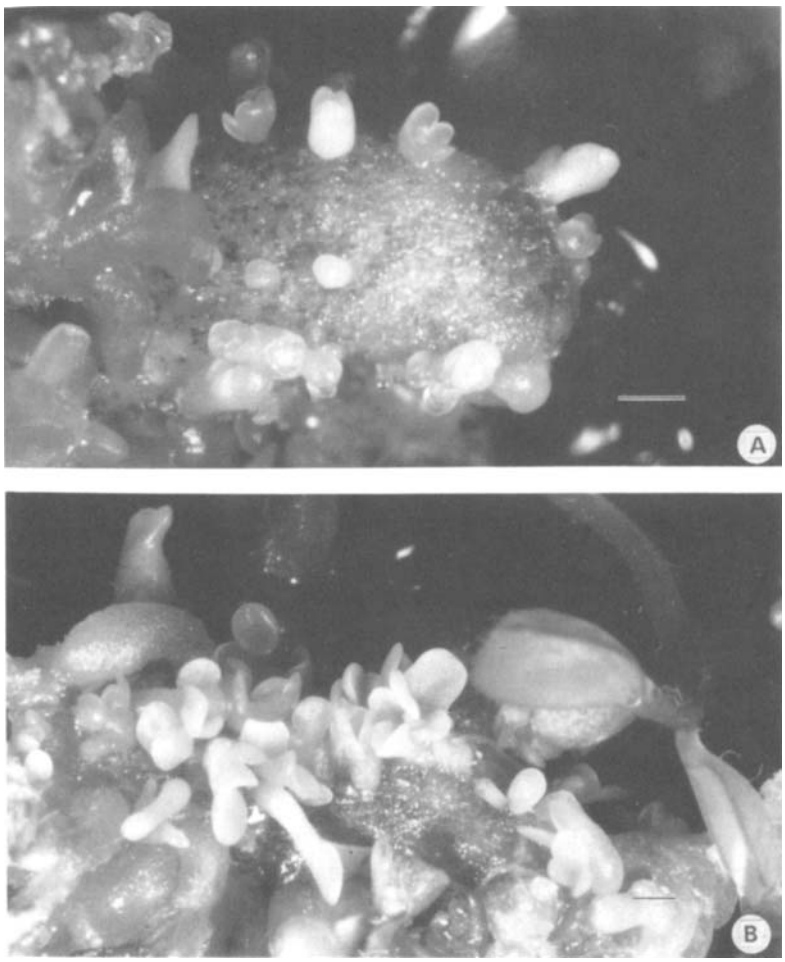


Fig. 6.5. Direct embryogenesis from somatic embryos of alfalfa. (A) Globular to heart shape embryos developing on a cotyledon. (B) Cotyledonary stage embryos formed on the hypocotyl of an older somatic embryo (after Merkle et al., 1990).

velop from the secondary suspensor (Klimaszewska, 1989; Aderkas von et al., 1991).

For the establishment of embryogenic cultures of alfalfa petiole sections from 2–3 youngest fully expanded leaves are suitable. Flowering or stress of any kind to the donor plants dramatically reduces both the quality and quantity of the SEs (McKersie et al., 1989). In orchardgrass the two innermost leaves exhibit somatic embryogenesis in the presence of dicamba. The segments from the most basal portion of these leaves form highly embryogenic callus. The tendency to form callus decreases

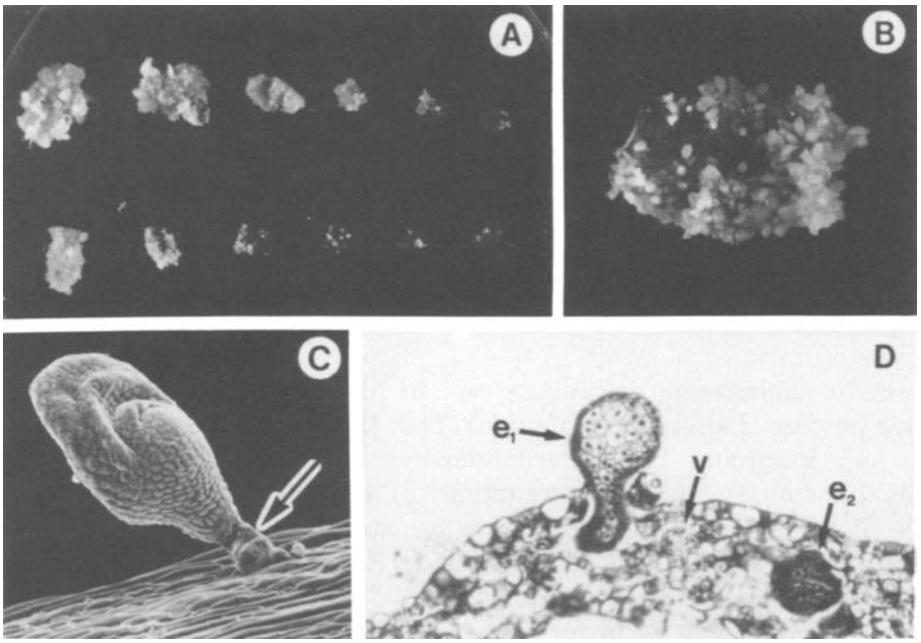


Fig. 6.6. Somatic embryogenesis in leaf cultures of *Dactylus glomerata*, on SH medium containing 30 μM dicamba. (A) 6-week-old cultures of the youngest enlarged leaf (top row) and the 2nd youngest enlarged leaf (bottom row). Cultures of the various segments, from the base to the distal end of a leaf are arranged sequentially from left to right. The basal segment produces embryogenic callus and it progressively changes to direct embryogenesis response towards the distal segment. (B) A leaf segment bearing numerous somatic embryos. (C) Scanning electromicrograph of a well-developed embryo arising directly from a leaf segment; the embryo is attached to the leaf by a suspensor (arrow marked). (D) Cross section of a leaf showing a very young embryo (e_1) produced from the leaf surface and a younger embryo (e_2) embedded in the mesophyll tissue; v, vascular bundle. Reprinted with permission from B.V. Conger et al., 1983, Science, 221: 850–851; © 1983 American Association for the Advancement of Science.

and the incidence of direct embryogenesis from the mesophyll cells increases with increase in the distance of the explant from the base of the leaf (Conger et al., 1983).

6.3.2. Genotype

Genotypic effect on somatic embryogenesis occurs as for regeneration via shoot bud differentiation (Section 5.3.1). Of the 500 varieties of rice screened by Kamiya et al. (1988) 19 showed 65–100% embryogenesis, 41 showed 35–64% embryogenesis and the remaining 440 cultivars were less efficient.

Genotypic variations could be due to endogenous levels of hormones (Carman, 1990). Carman and Campbell (1990) cultured the spike-bearing culms of a recalcitrant cultivar of *Triticum aestivum* in media containing high levels of zeatin. Immature embryos (12 DPA) from such spikes were less prone to produce embryogenic cultures and more prone to germination. ABA and IAA had a similar effect. Elimination of hormones caused a tenfold increase in the embryogenic response. The ovules of highly embryogenic lines of *Zea mays* contained 16–20 times less auxin and 10–15% less cytokinin than those of non-embryogenic and poorly embryogenic lines (Carnes and Wright, 1988). The optimum concentration of 2,4-D required for the formation of embryogenic callus in rice varied with the cultivar (Kamiya et al., 1988).

Somatic embryogenesis/caulogenesis in alfalfa is a genetically controlled process (Reisch and Bingham, 1980; Brown and Atanassov, 1985; Kris and Bingham, 1988; Hernandez-Fernandez and Christie, 1989; Kielly and Bowley, 1992). Most cultivars of alfalfa contain genotypes capable of regenerating in cultures; on an average the regeneration frequency is 10% but some cultivars, such as Rangelander, exhibit a much higher frequency (Brown and Atanassov, 1985). Inheritance studies have shown that the capacity to regenerate plants in alfalfa is controlled by two dominant genes (Reisch and Bingham, 1980; Hernandez-Ferandez and Christie, 1989; Kielly and Bowley, 1992). Somatic embryogenesis in orchardgrass is also shown to be a heritable dominant trait (Gavin et al., 1989). Highly regenerating genotypes of alfalfa have been produced using conventional breeding approach, suggesting that it is possible to genetically combine regeneration capacity with agronomic performance. A highly regenerating tetraploid line 'Regan-s' (67% regeneration) was produced by crossing two poorly responding parents 'Du Puits' (10% regeneration) and 'Sarnac' (14% regeneration) followed by recurrent selection (Bingham et al., 1975; Reisch and Bingham, 1980). McCoy and Bingham (1977) selected a diploid line of alfalfa 'HG2' by chromosome manipulation and breeding which showed even greater regeneration ability (96%) than 'Regan'.

Cytoplasmic factors have also been implicated in the control of somatic embryogenesis (Tomes and Smith, 1985; Rode et al., 1988; Ou et al., 1989; Peng and Hodges, 1989; Willman et al., 1989). In immature zygotic embryo cultures of Chinese spring wheat non-embryogenic callus can arise from embryogenic callus. Rode et al. (1988) observed a loss of an 8-kb mitochondrial DNA segment in the non-embryogenic cells, which could play a special role in the ability of dedifferentiated cells to regenerate.

6.3.3. Growth regulators

Auxin. All the well studied somatic embryogenic systems, such as alfalfa, carrot, celery, coffee, orchardgrass, and most of the cereals, require a synthetic auxin for the induction of somatic embryogenesis followed by transfer to an auxin-free medium for embryo differentiation. 2,4-D has been the most commonly used auxin for the induction of somatic embryogenesis. However, Kamada et al. (1989) and Smith and Krikorian (1990b) succeeded in establishing embryogenic cultures of carrot without a growth regulator. Whereas Kamada et al. achieved it with high concentration of sucrose, Smith and Krikorian managed it by manipulating the pH of the medium. At pH 4 embryogenic clumps continued to proliferate without the appearance of embryos. Embryos developed when the pH was increased to 5.6.

Generally, the embryogenic cultures of carrot are initiated and multiplied in a medium containing 2,4-D in the range of 0.5–1 mg l^{-1}. On such a medium ('proliferation medium') callus differentiates localized groups of meristematic cells called 'proembryogenic masses' (PEMs). In repeated subcultures on the proliferation medium the ECs continue to multiply without the appearance of embryos (Fig. 6.7). However, if the PEMs are transferred to a medium with a very low level of auxin (0.01–0.1 mg l^{-1}) or no auxin at all ('embryo development medium'; ED-medium), they develop into embryos (Fig. 6.7). The presence of an auxin in the proliferation medium seems essential for the tissue to develop embryos in the ED medium. The tissue maintained continuously in auxin-free medium would not form embryos. Therefore, the proliferation medium is regarded as the 'induction medium' for somatic embryogenesis (Sung and Okimoto, 1981) and each PEM an unorganized embryo (Kohlenbach, 1978).

All the major species of cereals and grasses have been reported to regenerate plants in vitro via somatic embryogenesis (Vasil and Vasil, 1986). Among the large number of growth regulators tested, 2,4-D is by far the most effective for producing embryogenic cultures. The cultures are initiated on a medium containing 1–2.5 mg l^{-1} 2,4-D and the embryo development generally occurs when the concentration of 2,4-D is reduced to 5–10% of the initial concentration. Orchardgrass (*Dactylis glomerata*) is an exception, where 30 μM dicamba (3,6-dichloro-*o*-anisic acid) has been reported to give best response (Hanning and Conger, 1982; Gray et al., 1984, 1993). 2-(2,4-Dichlorophenoxy) propionic acid was found to be the most effective auxin for the induction of somatic embryogenesis in alfalfa (Stuart and McCall, 1989, cited in Redenbaugh and Walker, 1990; Slade et al., 1989).

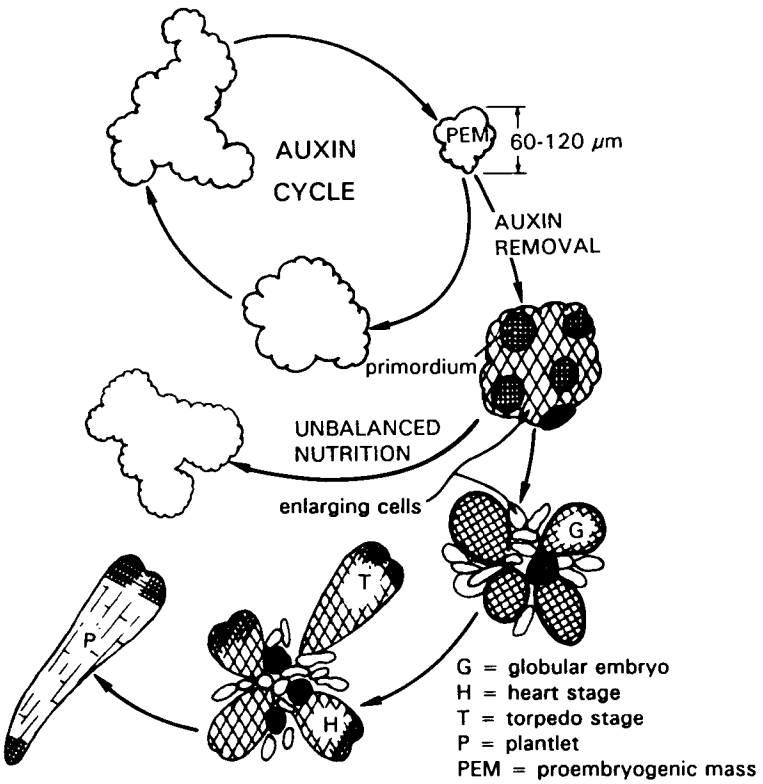


Fig. 6.7. Diagrammatic representation of in vitro embryogenesis in suspension cultures of wild carrot (after Wetherell, 1978).

The importance of auxin in embryogenesis is also suggested by the detailed work on habituated callus tissues of *Citrus sinensis* (Spiegel-Roy and Kochba, 1973; Kochba and Button, 1974; Kochba and Spiegel-Roy, 1977a,b,c). Originally, the nucellar callus of *C. sinensis* required IAA and kinetin for its growth and embryo differentiation (Kochba et al., 1972). In repeated subcultures the callus showed a gradual decline of embryogenic potential, and after about 2 years some tissue lines appeared which were phytohormone autonomous. In these habituated tissues the presence of as low as 0.001 mg l^{-1} of IAA in the medium inhibited embryogenesis. On the other hand, any treatment which checked auxin concentration in the cells, such as auxin synthesis inhibitors (2-hydroxy-5-nitro-benzyl bromide or 7-aza-indole) (Kochba and Spiegel-Roy, 1977a) and irradiation (Kochba and Spiegel-Roy, 1977c) significantly improved embryo differentiation. Irradiation is known to break down auxin (Chourey et al., 1973).

In the tissues exposed to irradiation levels higher than 16 kR the auxin, which otherwise inhibited embryo formation, turned out to be promotive. All these observations suggest that a high level of endogenous auxin was responsible for the decline in the embryogenic potential of the habituated *Citrus* callus. Prolonged duration of subculture (ranging from 6 to 14 weeks) and sucrose starvation in the preceding passage also considerably promoted embryo formation in the habituated *Citrus* callus. These studies as well as those of Fujimura and Komamine (1979a) suggest that a minimal level of endogenous or exogenous auxin is necessary for in vitro somatic embryogenesis.

Wochok and Wetherell (1971) have suggested that the 2,4-D-induced suppression of embryo development may be mediated through endogenous ethylene production. 2-Chlorophosphonic acid (Ethephon), which releases ethylene in plant tissues, also suppresses the development of mature SEs without an appreciable reduction in the growth and multiplication of the PEMs in suspension cultures of carrot (Wochok and Wetherell, 1971). Moreover, it has been shown that the auxin-grown tissue cultures of carrot produce more ethylene than auxin-free cultures (Huang, 1971). High ethylene content would result in enhanced activity of cellulase or pectinase or both, causing breakdown of the clumps before polarity is established in the proembryos for further organized development (Wochok and Wetherell, 1971). Thus, in 2,4-D medium tissue multiplication goes on but mature embryos do not appear.

Cytokinin. Halperin (1970) reported that the presence of BAP in the proliferation medium may promote cell division, but it inhibits the embryogenic potential of carrot cultures. This may be due to selective stimulation of multiplication of non-embryogenic cell-components of the cultures. However, some workers have recorded a promotive effect of cytokinins on embryogenesis. In carrot itself, Fujimura and Komamine (1975) observed that whereas BAP and kinetin were inhibitory for embryogenesis, zeatin at a concentration of 0.1 μM promoted the process. Zeatin was especially promotive when supplied to the embryogenic callus during days 3 and 4 after their transfer from the proliferation medium to the ED medium (Fujimura and Komamine, 1980). Embryogenesis could be induced in coffee in the presence of a single cytokinin (Yasuda et al., 1985). The induction medium for alfalfa contains kinetin (5 μm) in addition to 2,4-D (22.6–45.2 μm) (Meijer and Brown, 1987). The relative concentrations of the two growth regulators in the induction medium determines the type of morphogenic differentiation after transfer to hormone-free medium. Whereas high 2,4-D to kinetin favours embryo/shoot differentiation the reverse ratio favours rooting.

Others. Gibberellin inhibits somatic embryogenesis (Halperin, 1970). IAA, ABA and GA_3 have been reported to suppress embryogenesis in carrot (Fujimura and Komamine, 1975) and *Citrus* (Tisserat and Murashige, 1977). In this context it is interesting to note that the ovules of monoembryonate *Citrus medica* contain significantly higher levels of IAA, ABA and GA_3 than those of polyembryonate *C. reticulata* (Tisserat and Murashige, 1977).

6.3.4. Nitrogen source

The form of nitrogen in the medium significantly affects in vitro embryogenesis. Halperin and Wetherell (1965) reported that in the cultures of wild carrot raised from petiolar segments, embryo development occurred only if the medium contained some amount of reduced nitrogen. The calli initiated on a medium with KNO_3 as the sole source of nitrogen failed to form embryos upon removal of auxin. However, the addition of a small amount (5 mM) of nitrogen in the form of NH_4Cl in the presence of 55 mM KNO_3 allowed embryo development. Halperin and Wetherell also demonstrated that the presence of reduced nitrogen was critical only in the induction medium. Meijer and Brown (1987) found an absolute requirement for ammonium during induction and differentiation of SEs in alfalfa, with 5 mM being optimum for induction and 10–20 mM optimum for differentiation of embryos. White's (lacking NH_4^+) and SH (with 2.6 mM NH_4^+) media are non-inductive for somatic embryogenesis in carrot (Tazawa and Reinert, 1969) and orchardgrass (Trigiano et al., 1992), respectively. Addition of 2.5–3 mM NH_4^+ makes these media inductive. In orchardgrass the number of embryos formed in the presence of optimum concentration of NH_4^+ (12.5 mM) was substantially higher than that with any other form of reduced nitrogen but the embryos were of poor quality (Trigiano et al., 1992).

The suspension cultures of orchardgrass maintained on a medium containing the salts of SH medium, 88 mM sucrose, 5.5 mM inositol, 15 μM thiamine·HCl and 30 μM dicamba (SH-30) exhibited sustained proliferation of cell masses that differentiated only root primordia. The addition of 3 g l^{-1} of casein hydrolysate (CH) to the established cultures stimulated rapid development of SEs which matured to the germinable stage directly in the liquid medium. Substitution of CH with individual amino acids did not support the production of germinable embryos. However, a combination of proline and serine/threonine, at their optimal concentration of 12.5 mM, proved even superior to CH for embryo production, particularly with respect to the quality of SEs (Tregiano and Conger, 1987; Trigiano et al., 1992). The embryos formed in the presence of proline and serine/

threonine were not attached to cell aggregates as opposed to the CH-supplemented medium where the embryos were embedded in large cell masses. The embryos formed on amino acid containing medium showed higher percentage of conversion (84% versus 69%), considerably less incidence of precocious germination (14% versus 46%), and had a smoother epidermis as against the irregular surface of the embryos developed in the presence of CH (Trigiano et al., 1992). The yield of alfalfa SEs was also considerably improved when amino acids such as proline, alanine, arginine and glutamine were added to the callus maintenance medium, resulting in up to 100 times more embryo production (see Redenbaugh et al., 1991a). Organic acids such as K-citrate, K-malate or K-tartrate applied during the last subculture of callus growth increased the number of embryos formed, improved the quality of SEs in terms of conversion frequencies and enhanced accumulation of seed storage proteins.

6.3.5. Polyamines

There is some evidence to suggest that polyamines are required for embryo development in vivo and in vitro (Altman et al., 1990; Mengoli and Bagni, 1992). Nagl (1990) observed rapid translocation of putrescine through the funiculus and suspensor in *Phaseolus*. Nucellus of polyembryonate cultivars of mango contain significantly higher levels of the three polyamines, putrescine, spermidine and spermine, than those of monoembryonate cultivars (Litz and Schaffer, 1987). Increase in the endogenous level of polyamines (Montague et al., 1978) and the enzymes for their biosynthesis (Fienberg et al., 1984) concomitant with the induction of somatic embryogenesis in carrot and the suppression of somatic embryogenesis by the inhibitors of polyamine biosynthesis (Minocha et al., 1990; Altman et al., 1990) suggest the involvement of polyamines in somatic embryogenesis. Globular embryos of celery showed 37-fold higher polyamine content than the plantlets; of the three polyamines studied (putrescine, spermidine and spermine) putrescine showed the most dramatic increase (6-fold) (Altman et al., 1990). Similarly, embryogenic cultures of mango showed substantial increase in the level of putrescine (Litz et al., 1993). However, the increase in the cultures of monoembryonate variety was almost double that in the cultures of polyembryonate variety. It was even higher in the non-embryogenic cultures. The role of the observed changes in polyamine content and biosynthesis and their causal relationship to somatic embryogenesis remains to be established.

6.3.6. Oxygen concentration

Oxygen tension has been shown to promote embryogenic development in cultures (Carman, 1990). Kessel et al. (1977) reported that the amount of dissolved oxygen (DO_2) in the medium should be below the critical level of 1.5 mg l^{-1} to allow embryo development in carrot. Higher levels of DO_2 favoured rooting. The need for reduced DO_2 could be substituted by the addition of ATP to the medium, suggesting that, probably, oxygen tension enhanced the level of cellular ATP.

Similarly, immature embryos of *Triticum aestivum*, on callus induction medium for 28 days, in 8% O_2 produced about 3600 SE g^{-1} of scutellar tissue which was 6 times higher than when cultures were incubated at atmospheric level (21%) of oxygen (Carman, 1988). Incubation in a low O_2 environment also reduced the amount of 2,4-D required to initiate embryogenic callus. With low 2,4-D concentration (1 μM), under atmospheric O_2 level a large number of SEs were converted into unipolar root structures. This abnormality is considerably reduced at low O_2 level (Carman, 1990). Low O_2 level also significantly decreases precocious germination of SEs and the frequency of abnormal scutellar enlargement. In contrast to these observations, in alfalfa somatic embryogenesis was better in DO_2 higher than 70% (Stuart et al., 1987); no embryos were formed in 21% DO_2. Recently, the findings of Nishimura et al. (1993) have contradicted those of Kessel et al. (1977). They have reported that at DO_2 concentration of 88% or more 394 SE ml^{-1} were formed as against 19 embryos ml^{-1} at a DO_2 concentration of 18%.

6.3.7. Electrical stimulation

Very young (spherical) SEs of carrot exhibit electric gradient along the future longitudinal axis (Brawley et al., 1984). Rathore and Goldsworthy (1985c) reported stimulation of shoot bud differentiation in tobacco and wheat callus cultures by exposure to mild electric field (see Section 5.3.1). Exposure of freshly isolated mesophyll protoplasts to an electric field (0.02 V DC current for 20 h) considerably promoted the embryogenic response in alfalfa (Dijak et al., 1986). The pattern of embryogenesis in protoplast cultures of alfalfa varies with the genotype (Dijak and Brown, 1987). While the cultivars 'Rambler' and 'RegenS' form embryos via a callus phase, 'Rangelander' exhibits almost direct embryogenesis. Exposure of 'Rangelander' protoplasts to a low voltage electric field induced direct embryogenesis in a 100% preparations as against 40% preparations of the untreated controls. The number of embryos per plate was en-

hanced from 76 to 116. Even the cv. 'RegenS' protoplasts exposed to electric field showed direct embryogenesis (Dijak et al., 1986).

The electric stimulus seems to promote the differentiation of organised structures (shoots/embryos) by affecting cell polarity through changes in the organization of microtubules (Dijak and Simmonds, 1988; de Jong et al., 1993). Two days after culture the treated alfalfa protoplasts contained more microtubules, which were thinner and more branched than the untreated control cells. In the untreated cells the microtubules were arranged in parallel strands whereas in the treated cells they occurred as a disordered network. Another striking effect of electric treatment was the induction of an asymmetric first division (47%) coupled with a relatively short period of cell expansion, resulting in spherical structures composed of many small irregularly shaped cells and a few large ones. In contrast, the untreated cells underwent a symmetrical first division, and this and subsequent divisions were followed by a period of cell expansion, such that the colonies formed consisted of cells of similar size.

6.3.8. Selective subculture

Multicellular explants are generally heterogeneous in terms of the morphogenic potential of its constituent cells. Only a small proportion of these cells are able to express their cellular totipotency under a set of culture conditions. Therefore, the calli derived from such explants are also heterogeneous. Sometime the embryogenic/organogenic portions of the callus are distinct from the non-morphogenic tissue on the basis of their morphological appearance and it is essential to make artistic subcultures to establish regenerating tissue cultures.

In cereals, irrespective of the explant used, two types of calli are formed: (1) white, off-white or pale yellow, compact and often nodular and (2) soft, granular and transluscent. Of these, only the first type of calli exhibit embryogenic differentiation (Vasil and Vasil, 1991). Finer (1988) classified the cotyledon callus of *Gossypium hirsutum* on the basis of their colour as green, yellow, white, brown and red. Only yellow callus yielded embryogenic cultures. Maintenance of embryogenic cultures of conifers involves subculture, at extended intervals, of carefully selected, morphogenically distinct embryogenic tissue (Bornman, 1993). Selective or artistic subculture has also been practised in producing embryogenic cultures of *Daucus carota* (Nomura and Komamine, 1985), *Zea mays* (Duncan et al., 1985), *Sorghum bicolor* (Bhaskaran and Smith, 1988), *Hordeum vulgare* (Mohanty and Ghosh, 1988) and *Picea abies* (Jain et al., 1988).

Calli derived from seedling explants of *Cucumis melo* developed green nodular structures after 3–4 weeks of culture. If subcultured along with the whole callus the nodules continued unorganized proliferation. However, if the nodular structures were carefully isolated from the rest of the callus and cultured separately they produced multiple shoot buds (Kathal et al., 1986).

6.3.9. Other factors

Brown et al. (1976) reported that high potassium (20 mM) is necessary for embryogenesis in wild carrot. Mathias and Boyd (1986) observed a 68% increase in the embryogenic response of the cultures of immature embryos of wheat in the presence of the antibiotic cefotaxime. This affect also occurred in barley (Mathias and Makasa, 1987). A promotion of somatic embryogenesis in maize was induced by ethylene action inhibitors, including $AgNO_3$ (Songstad et al., 1988). In a recalcitrant inbred line of maize treatment of ovules (3 DAP) in vivo or in vitro with high levels of dicamba increased embryogenic response from approx. 1% to 30% (Duncan et al., 1989).

Tisserat and Murashige (1977) demonstrated that the ovules of monoembryonate *Citrus medica* synthesize and release certain volatile and non-volatile substances which can inhibit in vitro somatic embryogenesis in co-cultured nucellar tissue of polyembryonate *C. reticulata*. Ethanol is one of the volatile inhibitors. When applied at a concentration equal to that produced by the ovules of *C. medica*, it markedly inhibited embryogenesis in carrot cultures. The non-volatile component of the inhibitors has been identified with IAA, ABA, and GA_3.

6.4. INDUCTION AND DEVELOPMENT

Differentiation of somatic embryos involves reactivation of cells to divide and, depending on the nature of the explant and the culture conditions, the embryos may arise directly from the cells of the explant or after several cycles of cell divisions. In the latter case the embryogenic differentiation is preceded by a phase of unorganised growth. Direct embryogenesis generally occurs in the cultures of immature embryos (Maheswaran and Williams, 1986; Merkle et al., 1990). Plantlets of carrot (Terzi and Lo Schiavo, 1990) and buttercup (Konar and Nataraja, 1969) regenerated via somatic embryogenesis also form embryos directly from the epidermal cells of the stem (Fig. 6.2) but the plantlets derived from zygotic embryos of these species do not exhibit this potentiality. The explants capable of direct embryogenesis seem to carry competent or 'pre-

embryogenic determined cells' (PEDCs). In other cases the embryogenic competence is acquired following exposure to suitable treatments. Once induced, the 'induced embryogenic determined cells' (IEDCs) are functionally equivalent to PEDCs. Both can be maintained and multiplied in the embryonic state under appropriate conditions.

6.4.1. Induction

An auxin, particularly 2,4-D, is generally necessary to induce embryogenesis in plants such as carrot, alfalfa and cereals. However, the requirement of exogenous auxin for the induction of somatic embryogenesis depends on the nature of the explant used. For example, petiole explants (Ammirato, 1985), hypocotyl explants (Kamada and Harada, 1979) and single cells isolated from established suspension cultures (Nomura and Komamine, 1985) of carrot required exposure to 2,4-D for 1,2 or 7 days, respectively, to acquire competence to form embryos on ED medium (devoid of 2,4-D). Microcalli of alfalfa required even a shorter pulse (a few minutes to a few hours) of relatively high concentration (100 μM) of 2,4-D to produce embryos in ED medium (Dudits et al., 1991). An important phenomenon associated with the induction of somatic embryogenesis is the change of cellular polarity.

Polarity. Several observations support the hypothesis that plant growth regulators and other treatments employed for the induction of embryogenesis do this by altering the cell polarity and promotion of subsequent asymmetric division (de Jong et al., 1993). On a cytokinin-containing medium the immature embryos of white clover develop several adventive embryos directly from the hypocotyl epidermis. The first cytological sign of the induction of embryogenic cells is a shift from the normal anticlinal divisions in the epidermis to periclinal or oblique divisions (Maheswaran and Williams, 1985). Following stimulation by auxin, asymmetric cell divisions were frequently observed in leaf protoplast cultures of an embryogenic cultivar of alfalfa; the protoplasts from non-embryogenic cultivar divided symmetrically (Bogre et al., 1990; Dudits et al., 1991). In carrot, the first division of single suspension cells was asymmetric (Backs-Hüsemann and Reinert, 1970; Komamine et al., 1990) and only the small daughter cell ultimately developed into embryo (Fig. 6.8). Since the root pole of the SE is always oriented towards the larger cell the polarity of the entire SE is already determined prior to the first division of an embryogenic cell. The positive effect of pH (Smith and Krikorian, 1990b) and electric field (Dijack et al., 1986) on the induction of embryogenesis appears to be due to their effect on cell polarity (see Section 6.3.7).

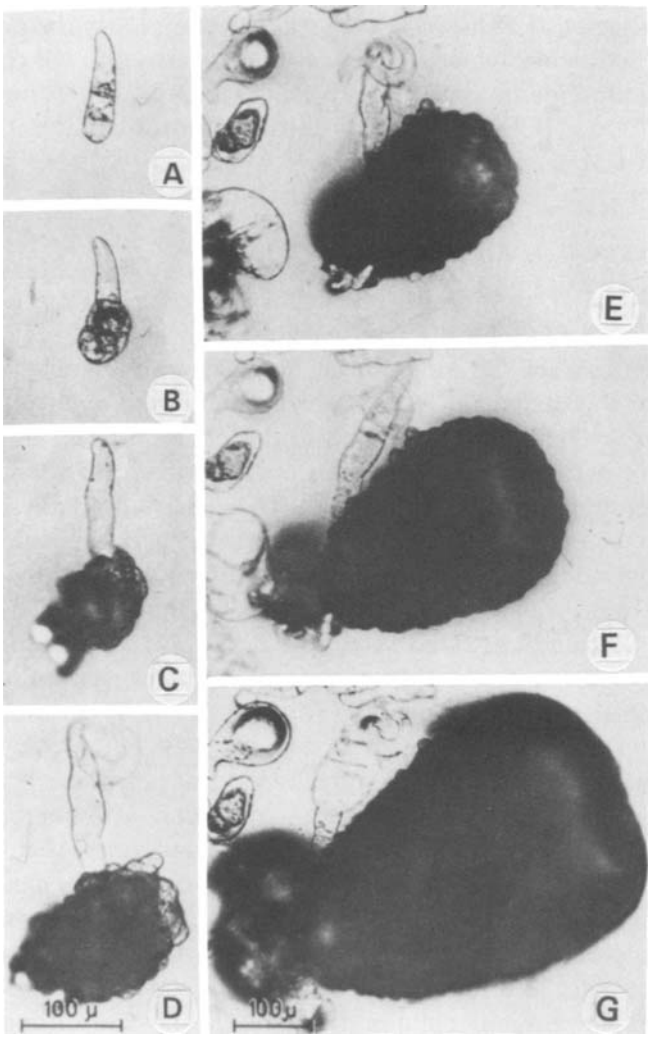


Fig. 6.8. Serial stages in embryo formation by an isolated single cell from tissue culture of *Daucus carota*. The cell first divides by an unequal division forming a large vacuolated cell and a small richly cytoplasmic cell (A). The latter, by a series of divisions, gives rise to a tissue mass from which differentiate embryos. (A)–(G) 4, 8, 15, 17, 19, 21 and 23 days after cell isolation, respectively (after Backs-Hüsemann and Reinert, 1970).

6.4.2. Development

The usual strategy to start an embryogenic suspension culture of carrot is to expose explants to a high concentration of 2,4-D. After reinitiation of cell division and a period of cell proliferation in the presence of the auxin embryogenic cells are released into the medium (de Vries et al.,

1988). These cells are in the form of clusters of cytoplasmic cells (PEMs). The suspension cultures also contain single cells of two types: (1) small and cytoplasmic, and (ii) long and vacuolate. The former type of cells may give rise to PEMs (Komamine et al., 1990) but most of the PEMs are derived from pre-existing PEMs (de Vries et al., 1988; Emons et al., 1992). The PEMs comprise embryogenic cells, which are small (400–800 μm^3), angular, connected with the adjacent cells by many plasmodesmatas (2–4 per μm^2), have several small vacuoles (30% of cell volume), many starch grains (5–25 per cell), high density of ribosomes, numerous profiles of rough endoplasmic reticulum, spherosome-like vesicles, high dehydrogenase activity, many exocytosis configurations on the plasmalemma (0.6 per μm^2) and polylamellate walls at all wall facets (McWilliam et al., 1974; Emons et al., 1992; Emons, 1994). The embryogenic cells are held together by non-embryogenic cells which are larger (1000–3000 μm^3), rounded, have intercellular spaces, fewer plasmodesmata (0.1–1 per m^2), larger vacuoles (80% of cell volume), few starch grains (1–2 per cell), low population of ribosomes, very few endoplasmic reticulum profiles, little or no spherosome-like vesicles, low dehydrogenase activity and sometimes fewer exocytosis configurations.

Besides inducing embryogenesis, auxin also causes cell elongation and disruption of formerly adhering cells. Consequently, with the continued presence of the auxin non-embryogenic cells of the PEMs elongate leading to breakdown of the PEMs. The released embryogenic cell clusters, which are also referred to as 'pre-globular embryos' or 'globules' (Emons, 1994), develop into a new PEMs and the non-embryogenic cells of the disintegrated PEMs further elongate to form non-embryogenic component of the suspension. Thus, in the presence of 2,4-D this cycle of PEM or pro-embryo proliferation continues (Fig. 6.7) as long as cytoplasmic cells remain present and their daughter cells adhere. However, if auxin concentration is too high or subculturing is too often the population of small embryogenic cells drops because of their disruption and elongation, and the embryogenic potential of the culture is eventually lost. The embryogenic cells secrete certain proteins into the culture medium which not only help in maintaining the embryogenic potential of the cultures by restricting cell elongation in the presence of 2,4-D but also induce the appearance of small embryogenic cells in previously non-embryogenic cultures (Kreuger and Van Holst, 1993).

When embryogenic cultures of carrot are transferred to auxin-free medium, the disruption of cells from each other stops and the globules develop into globular embryos. In this process the first differentiation step is the formation of a protoderm outside the globule. The globular embryos then continue further development and form typical embryos. In

maize, globular and later stage embryos develop in the presence of 2,4-D but the differentiation of protoderm occurs only after transfer to 2,4-D-free medium (Emons, 1994). ABA stimulates epidermis development.

After embryo induction the role of auxin changes in that the embryos start to synthesize their own auxin (Michalczuk et al., 1992a,b). Several studies have shown that proper polar transport of auxin is a pre-requisite for normal embryogenesis beyond the globular stage (Schiavone and Cooke, 1987; Liu et al., 1993b).

SEs formed on the surface of the callus have intrinsic polarity because they are attached to the callus cells at one end. The future root of such SEs is always oriented towards the callus centre. In maize, attachment of SEs to callus is very important. Only such embryos which are attached to the callus until the formation of proper shoot meristem develop into complete embryos. Those grown from cell clusters in liquid medium do not form a proper shoot meristem (Emons and Kieft, 1991). In carrot which easily forms complete embryos in liquid medium, the globular embryos exhibit electric polarity in the form of ionic current; there is influx of potassium ions at the future plumular end and efflux of hydrogen ions at the radicular end (Brawley et al., 1984). The PEMs of carrot also show polarity in the distribution of calcium-calmodulin complex before morphological polarity is visible (Timmers et al., 1989). The concentration of activated calmodulin was higher in the region of the root pole. Polarity can also be induced by attachment of cells to a substrate. The production of embryos in liquid cultures of sweet potato was improved by anchoring PEMs to alginate beads to maintain a physiological polarity (Chee and Cantliffe, 1989).

6.4.3. Molecular aspects

Somatic embryogenesis has been studied extensively from morphological and physiological viewpoints but the molecular mechanism that determines and controls the process is not well understood. It has been known for some time that changes in gene expression occur when the somatic cells embark on embryogenic development, as evinced by the synthesis of embryo-specific proteins (Sung and Okimoto, 1981), increased organic decarboxylase activity (Montague et al., 1979), increased enzyme activities in the pyrimidine pathway (Ashihara et al., 1981) and the development of a capacity to inactivate cyclohexamide (Sung et al., 1981). During the past 5 years or so several molecular markers indicating the transition of somatic cells into embryogenic cells have been recognised, and about 21 'embryo specific' or 'embryo enhanced' genes have been cloned from somatic embryos (Zimmerman, 1993).

According to de Vries et al. (1988) cells competent to form embryos do not exist in the carrot explants. They acquire the competence in 19 days after the initiation of cultures in a medium containing 2,4-D. Most of the embryogenic capacity is acquired after 50 days of culture initiation and it reaches a maximum after 75 days. The acquisition of embryogenic potential in fresh cultures is considerably accelerated by supplementing the medium with cell-free medium conditioned by established embryogenic cultures. Analysis of the conditioned medium has revealed that a number of extracellular proteins (EPs) are excreted by the embryogenic cells into the medium which can act as molecular markers to distinguish between embryogenic and non-embryogenic cultures. Some of these proteins are also shown to play an important role in the induction and development of SEs. These studies pertain, almost exclusively, to carrot suspension cultures.

Carrot EP2 gene, which encodes a secretory lipid transfer protein, is not an embryo specific gene but its expression is enhanced during embryogenesis in vivo and in vitro (Sterk et al., 1991; Meijer and Hendriks, cited in de Jong et al., 1993). It is expressed in the peripheral cells of PEMs and the protoderm of SEs. The EP2 gene product is probably involved in chitin synthesis (Sterk et al., 1991; Meijer and Hendriks, cited in de Jong et al., 1993).

Another extracellular protein (EP3) purified from the conditioned medium and shown to play an important role in normal development of SEs has been identified as a glycosylated acidic endochitinase (de Jong et al., 1992). A temperature sensitive mutant of carrot (tsll) exhibits normal embryogenesis at the permissive temperature of 24°C but at the restrictive temperature of 32°C SEs develop only up to the globule stage due to the failure of correct protoderm formation (Lo Schiavo et al., 1988, 1990). Properly glycosylated endochitinase seems to be involved in inducing normal protoderm formation.

Cell wall associated arabinogalactan proteins (AGPs) occur in many plant tissues but their type varies with the tissue. Cell cultures of carrot secrete a characteristic set of AGPs into the medium (Stacey et al., 1990) and the composition of this set changes with the age of the culture. After 7 weeks of culture initiation, as the cultures are becoming embryogenic, the AGP profile shows a single peak (R_f 0.6) which is similar to the AGP pattern of carrot seeds. After 3 months, when the cultures are highly embryogenic, the AGP pattern changes to two peaks (R_f 0.4 and R_f 0.6). In cultures the AGPs are found associated with only small, richly cytoplasmic cells which are capable of forming embryos but not with the PEMs, suggesting that the appearance of characteristic AGPs marks the transition in the formation of embryogenic cells (Pennell et al., 1992).

However, the number of cells showing the presence of specific AGPs is far more than the number of embryogenic cells, suggesting that only a few cells in the transitional phase develop into embryos (up to 2%; see de Vries et al., 1988). Recently, Kreuger and Van Holst (1993) have shown that the addition of specific AGPs from carrot seeds or embryogenic cultures to the medium at a very low concentration (10–100 μM) enhanced the frequency of PEMs from 30% (control) to 80% and restored the embryogenic potential of old cultures which had lost it.

An important determinant of embryogenic potential of carrot cultures is the sustenance of small, richly cytoplasmic cells. Arrest of the SEs at the globular stage by tunicamycin, a glycosylation inhibitor, is associated with cell elongation, a characteristic feature of non-embryogenic cultures (Cordewener et al., 1991). A 38 kDa peroxidase purified from embryogenic cultures inhibited cell elongation in the presence of tunicamycin. A commercial preparation of horseradish peroxidase also promoted normal embryo development in the presence of the drug as long as its enzymatic properties remained intact. Since tunicamycin causes the cells of very young SEs to enlarge, van Engelen and de Vries (1992) have suggested that the function of peroxidases is to ensure that the cells of early proembryos remain small.

6.5. MATURATION OF SOMATIC EMBRYOS

Although somatic embryogenesis has been reported for several crop species, the quality of SEs with regard to their germinability or 'conversion' into plants has been generally very poor. As poor as 3–5% conversion has been observed in many cases. This is because the apparently normal looking SEs are actually incomplete in their development. Unlike seed embryos, the SEs normally do not go through the final phase of embryogenesis, called 'embryo maturation', which is characterised by the accumulation of embryo specific reserve food materials and proteins which impart desiccation tolerance to the embryos; embryo size does not increase during this phase.

ABA, which prevents precocious germination and promotes normal development of embryos by suppression of secondary embryogenesis and pluricotyledony (Ammirato, 1974, 1983) is reported to promote embryo maturation in several species. A number of other factors, such as temperature shock, osmotic stress, nutrient deprivation and high density inoculum, can substitute for ABA, presumably by inducing the embryos to synthesize the hormone. ABA is known to trigger the expression of genes which normally express during the drying-down phase of seeds (Dure et al., 1981). Probably the products of these genes impart desiccation toler-

ance to the embryos (Florin et al., 1993). ABA has been shown to increase desiccation tolerance in SEs of carrot (Kitto and Janick, 1985), celery (Kim and Janick, 1989) and soybean (Obendorf and Slawinska, 1988).

Senaratna et al. (1989, 1990) were able to confer desiccation tolerance on alfalfa SEs by treating them with ABA at the torpedo to cotyledonary stages. Over 60% of the ABA treated embryos survived desiccation to 10–15% moisture and converted to plantlets when placed on moist filter paper or sown directly onto sterile soil. Moreover, the vigour of these plants was greater than that of the plantlets derived from non-desiccated embryos. Fujii et al. (1989) also found that embryo maturation with ABA (optimum at 5 μM) was essential for high soil conversion (50–64%) of alfalfa SEs. According to these authors the effect of ABA was due to accumulation of embryo specific reserves such as carbohydrates. Janick et al. (1993) reported that treatment of celery SEs with 1 μM ABA and 5 mM proline enhanced their desiccation survival from 13 to 84%. Fujii et al. (1993) achieved high conversion frequencies of the SEs of this species by adding ABA (30 μM) and mannitol (4%) to the maturation medium. SEs of interior spruce required much higher concentration (40–60 μM) of ABA to completely stop precocious germination and promote the formation of mature embryos that appeared similar to mature zygotic embryos (Roberts et al., 1990a, 1993). The mature SEs contained twice the level of 22, 24, 33 and 41 kDa proteins as in the zygotic embryos of these plants.

Buccheim et al. (1989) observed that the conversion of soybean SEs was increased from 50% to 96% when matured in the presence of 10% sucrose. Similarly, SEs of maize required a maturation phase in a medium with a high sucrose concentration, resulting in the formation of the typical storage organ (scutellum) of this species (Emons and Kieft, 1993). During maturation starch accumulated in the scutellar cells and the formation of lignin was suppressed as in the zygotic embryos (Emons et al., 1993). Apart from starch, the SEs also possessed proglobulin (Emons and Thijssen, 1993; cited in Emons, 1994), the protein which in zygotic embryos is transiently present and modified to globulin (Belanger and Kriz, 1989).

Soybean SEs desiccated in empty petri plates until they shrivelled to 40–50% of their volume rapidly imbibed water following transfer to medium and germinated with at least seven times the frequency of non-desiccated embryos (Hammatt and Davey, 1987). Similarly, Parrott et al. (1988) found that only a few (0–5%) soybean SEs from several genotypes germinated without dehydration treatment. After dehydration up to 100% of the embryos germinated. Somatic embryos of red oak germinated and produced shoot only after a period of dehydration treatment with

osmotically active sugars (Gingas and Lineberger, 1988). Gradual drying of the alfalfa SEs with progressive and linear loss of water give better response and sometimes improves the embryo quality as compared to uncontrolled drying. A series of relative humidities (RH) are generated in desiccators over saturated salt solutions of NaCl (78% RH), NH_4NO_3 (63% RH), $Ca(NO_3)_2$ (51% RH) and K_2CO_3 (43% RH). The embryos in a petri plate, without a nutrient, are equilibrated at each humidity for 1 day and then air-dried to a final moisture of 10–15% (McKersie and Bowley, 1993).

6.6. SOMATIC EMBRYOS VERSUS ZYGOTIC EMBRYOS

In the classical embryology of angiosperms the early segmentation pattern of the zygote and its derivatives has been used as a key to the classification of embryogeny which, in turn, is employed as a taxonomic character (Bhojwani and Bhatnagar, 1990). This is based on the assumption that the sequence of early divisions in the zygotic embryogeny is fixed for a plant. However, the published evidence for the sequence of early development of SEs corresponding to that followed during zygotic embryogeny does not appear very satisfactory. It should not be surprising if the SEs, which originate from superficial cells of calli or PEMs and develop under conditions very different from those experienced by zygotic embryos, do not follow a fixed pattern of early segmentation (McWilliam et al., 1974). In nature the adventive embryos do not follow the sequence of early divisions as strictly as do the zygotic embryos. Even the zygotic embryos often exhibit deviation from the normal pattern of development (Borthwick, 1931). Irrespective of the early mode of development, the zygotic embryos and SEs share similar gross ontogenies, with both typically passing through globular, torpedo and cotyledonary stages of dicots and gymnosperms, and globular, scutellar and coleoptilar stages of monocots (Gray and Purohit, 1991). The SEs also accumulate seed-specific storage reserves and proteins characteristic of the same species, although in less amounts than the zygotic embryos (Kim and Janick, 1990; Stuart et al., 1988).

Reports about the occurrence of a distinct suspensor in SEs are inconsistent. In some cases a few-celled suspensor-like structure, connecting the embryo proper to the parent callus tissue, has been reported (Konar et al., 1972; McWilliam et al., 1974) but in most cases a distinct suspensor is not present (Emons and De Does, 1993; Ho and Vasil, 1983). Even where present it may not be functional, as in the seed embryos (Raghavan, 1976a). Other abnormalities exhibited by somatic embryos are a double or triple vascular system caused by polar transport of auxins

(Chee and Cantliffe, 1989) and non-development of shoot (rubber plant) or root (Palms) meristems (Michaux-Ferriere and Schwendiman, 1992). Unlike the zygotic embryos, the SEs generally lack a dormant phase and often show secondary embryogenesis and pluricotyledony. Some of these abnormalities can be corrected by the application of a low concentration (0.1–1.0 μM) of ABA (Ammirato, 1974).

6.7. SYNCHRONIZATION OF EMBRYO DEVELOPMENT

Generally, the differentiation of SEs in solid or liquid medium is highly asynchronous which adversely affects the germination phase. Since synchronous embryo maturation is extremely important with regard to the artificial seed technology, several approaches have been tried to achieve it. Of these, physical separation of embryogenic stages and use of growth regulators to physiologically synchronize development have proved most effective.

Fujimura and Komamine (1979b) achieved synchronization of carrot embryogenic cultures by careful selection of 3–10 celled clusters from the proliferation medium. Fractionation of embryos of different stages by filtration of suspension through meshes of different sizes (Fig. 6.9) or by gradient centrifugation is a very effective approach to collect embryos of the right stage. Recent development of the image analysis technique for counting SEs could make way for computer assisted sorting methods (Harrell and Cantliffe, 1991).

In carrot, the 50–100 μm fraction obtained by filtration of embryogenic suspension allowed the best synchronization of embryo development and improved the quality of embryos (Molle et al., 1993). Such cultures gave maximum singulation rate (>95%). Rinsing the fraction to eliminate intracellular 2,4-D before transfer to 2,4-D-free medium enhanced the embryo development response. The 1400 μm fraction of celery SEs, obtained by filtration, showed the best growth and synchronization (Altman et al., 1990).

The second most effective method to achieve synchronous development of somatic embryos is the use of substances that would induce reversible cessation of embryo development at a particular stage. Currently, ABA at low concentration is the most satisfactory substance for the purpose. In carrot it inhibits root growth and enriches suspension with torpedo shaped embryos, which are under 2 mm in length (Molle et al., 1993). In celery, ABA had no effect on embryo maturation but culture of globular embryos for 1 week on ED medium supplemented with 0.3 μM of ABA followed by transfer to ED medium without ABA increased embryo synchronization (Altman et al., 1990).

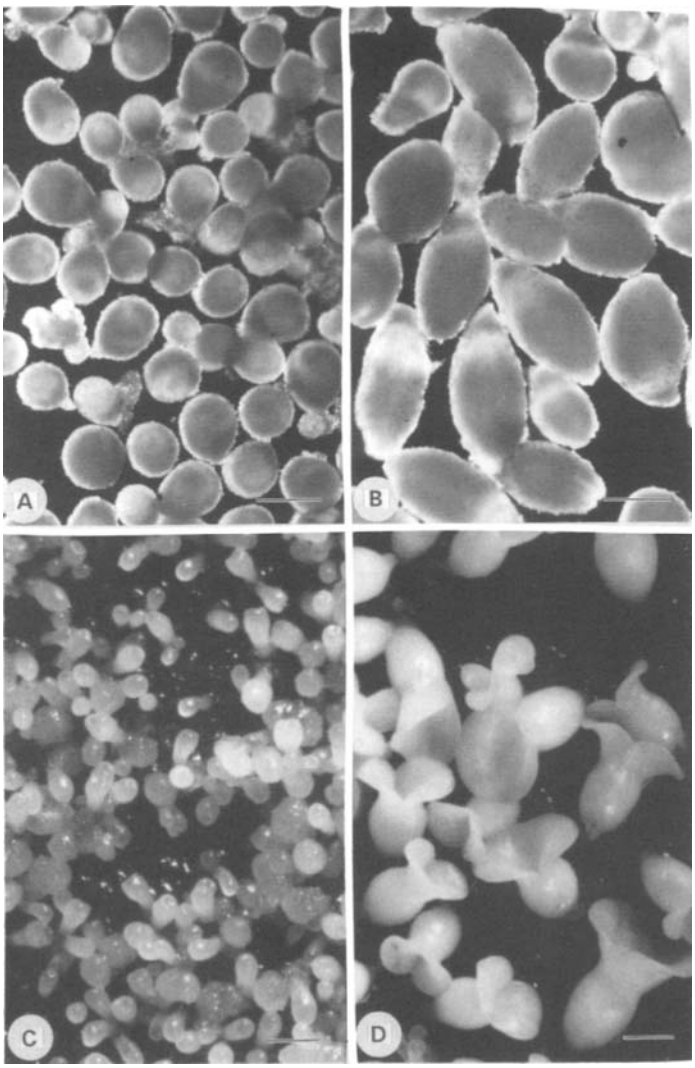


Fig. 6.9. Synchronous development of somatic embryos of yellow-poplar; the cultures were initiated with 38–148 μm fraction of PEMs sieved out from suspension cultures. (A,B) Globular and torpedo shape embryos from 3- and 10-day-old cultures of PEMs in liquid ED medium, respectively. (C) Roughly synchronous population of SEs 6 days after culture on semi-solid ED medium. (D) Mature embryos 14 days after culture on medium as in (C) (after Merkle et al., 1990).

6.8. LARGE SCALE PRODUCTION OF SOMATIC EMBRYOS

As the multiplication of embryogenic cells and the subsequent development of SEs can occur in liquid medium, somatic embryogenesis offers a potential system for large scale plant propagation in automated biore-

actors, with low labour inputs. For mass production of SEs in bioreactors, callus is initiated on a semi-solid medium. Pieces of undifferentiated or embryogenic callus are transferred to liquid medium in small flasks and agitated on a shaker. After a few cycles of multiplication in flasks, the embryogenic suspension may be filtered through a sieve of suitable pore size and PEMs or globular embryos transferred to the bioreactor flask. SEs being individual propagules, a 2–5 l bioreactor with a production capacity of 10–100 $\times$ 10^3 embryos should be sufficient for commercial micropropagation (Preil, 1991; Denchev et al., 1992).

Most of the modern bioreactors are fitted with probes for measurement and control of temperature, agitator speed, pH, pO_2 and pCO_2, which not only allows cultivation of cells under highly controlled conditions but also enables precise analysis of interacting factors for cell growth and embryo development.

The first attempt to scale-up somatic embryogenesis was performed by Backs-Hüsemann and Reinert (1970) with carrot cells using a 20 l carboy, which resulted in the formation of only a few embryos. Over the last 25 years different types of stirred-tank bioreactors, originally designed for microbial cultures, have been tested for plant cell cultures (Panda et al., 1989; Taticek et al., 1991). However, a major problem in using such bioreactors for plant systems is the sheer damage caused to the cells which are relatively larger in size and possess a thinner wall than the microbes. Air-lift or bubble-column bioreactors reduce sheer damage but cause the undesirable formation of foam and callus growth above the surface of the medium. Vibration mixers, in which the medium is agitated by reciprocating vertical motion of a centrally mounted agitator shaft, provided with horizontally inserted discs, generate less sheer stress and minimize foam formation. Conical holes in the discs cause an upward or downward stream in various flow patterns (Preil, 1991).

For the production of poinsettia SEs, Preil (1991) used a round bottom 2 l bioreactor, in which stirring was achieved by vibrating plates (vibro mixer plates of 55 mm diameter) and bubble-free O_2 was supplied through a stabilized silicon tubing which was inserted as a spiral of 140 cm total tube length. A vibro stirring system proved to be suitable for gentle agitation that did not cause any cell damage. The poinsettia plants derived from the bioreactor-raised SEs exhibited high genetic stability (98%).

In bioreactors, embryogenesis is highly asynchronous. In order to achieve synchrony, Molle et al. (1993) modified the bioreactor (Fig. 6.10), fitted with a stirrer and air sparger, by adding a side tube provided with nylon filters to collect embryos of specific stages which could be immedi-

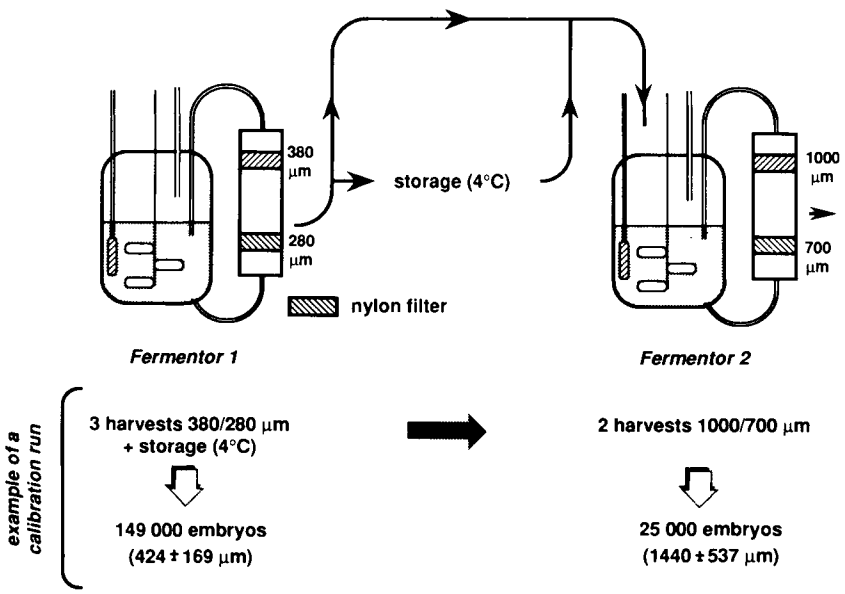


Fig. 6.10. Schematic representation of a stirred-tank bioreactor (= fermentor) system designed for synchronized development of somatic embryos of carrot. Embryogenic cultures are initiated in Fermentor 1 and the suspension is regularly passed, by highly pressurized air, through the two nylon filters of different pore size (380 μm and 280 μm) fixed in the side arm. The filtrate of the 280 μm pore size filter, containing globular embryos or PEMs, are sent back into Fermentor 1 and the heart and young torpedo shaped embryos, collected on 280 μm filter, are either used as such or transferred to Fermentor 2 for further synchronous development. Harvests from Fermentor 1 can also be stored at 4°C and transferred together to Fermentor 2 for synchronization of the maturation phase. Reprinted with permission from F. Molle et al., 1993. In: Synseeds, edited by K. Redenbaugh; © CRC Press.

ately transferred to another bioreactor or a semi solid-medium for further development.

Only a few articles on plant propagation in bioreactors have been published so far which does not allow a critical assessment of the progress achieved in the area. Different types of bioreactors have been used for the production of alfalfa SEs. In the air-lift bioreactor used by Chen et al. (1987) no embryos were formed. Stuart et al. (1987) achieved high yields of alfalfa SEs from air-lift bioreactor but the conversion rates of these embryos were disappointingly low (2–3%). Bioreactor raised poinsettia embryos showed normal DNA content but the plantlets derived from them differed from the donor cultivar in various characters (Preil, 1991). Even the production of a large number of normally formed embryos in this system was not reproducible. Thus, the present success in bioreactor production of SEs is far from a practical application.

6.9. SYNTHETIC SEEDS

Somatic embryogenesis is expected to be the only clonal propagation system economically viable for crops currently propagated by seeds (see also Section 16.3.3). However, it would require mechanical planting of SEs. Although suggestions have been made to use naked embryos for large scale planting, it would be desirable to convert them into 'synthetic seeds' or 'synseeds' by encapsulating in a protective covering (Fig. 6.11). The coating material of synseeds should have several qualities: (a) it must be non-damaging to the embryo, (b) the coating should be mild enough to protect the embryo and allow germination but it must be sufficiently durable for rough handling during manufacture, storage, transportation and planting, (c) the coat must contain nutrients, growth regulators, and other components necessary for germination, and (d) the artificial seeds should be transplantable using existing farm machinery. The success of synthetic seed technology would also depend on the quality of the SEs; uniform stage with reversible arrested growth and showing high rates of conversion on planting.

Currently two types of synthetic seeds are being developed: (1) desiccated and (2) hydrated. Of these, desiccated synthetic seeds, of course, would be closer to true seeds and, therefore, have greater potential.

6.9.1. Desiccated synthetic seeds

The first synthetic seeds produced by Kitto and Janick (1982) involved encapsulation of multiple carrot SEs followed by their desiccation. Of the various compounds tested for encapsulation of celery embryos, Kitto and Janick (1982, 1985a,b) selected polyoxyethylene (Polyox^r) which is readily soluble in water and dries to form a thin film, does not support growth of microorganism and is non-toxic to the embryos (Janick et al., 1993).

In the original protocol for encapsulation of carrot SEs with polyox, equal volumes of embryo suspension and a 5% (w/v) solution of polyox were mixed to give a final concentration of 2.5% polyox. The suspension was dispensed as 0.2 ml drops from a pipette on to Teflon sheets (dried suspension sticks to glass plate) and dried to wafers in a laminar flow hood. The drying time is based on the ability of wafer to separate from teflon plate (about 5 h). Embryo survival and conversion of seeds are determined by redissolving the wafers in embryogenic medium and culturing the rehydrated embryos. With the considerable improvement made, during the past decade, in the desiccation of SEs and singulation of SEs by selecting them visually or by density separation (Janick et al., 1989), the survival of coated celery SEs could be improved from 35% (Kim and

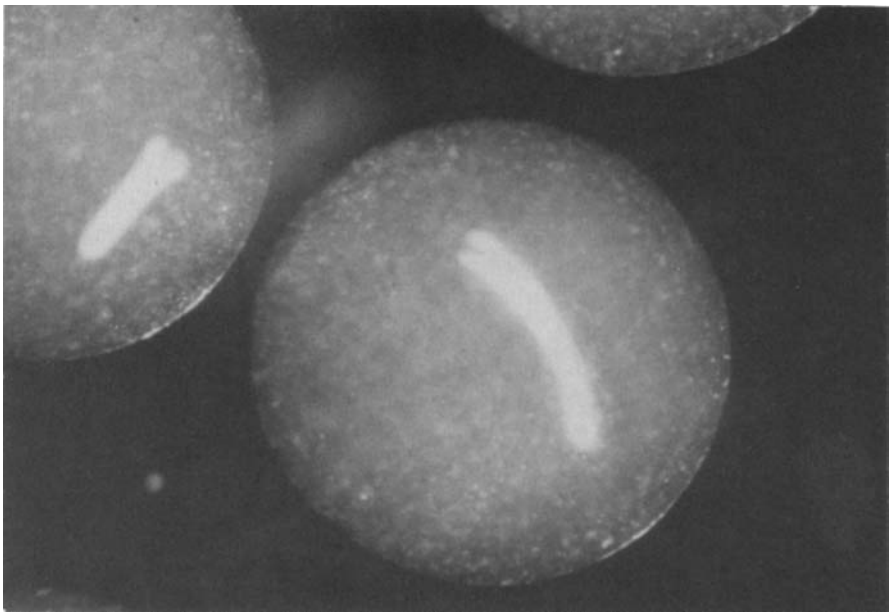


Fig. 6.11. Hydrated synthetic seeds of carrot, formed by encapsulation of somatic embryos in Ca-alginate. Reprinted with permission from F. Molle et al., 1993. In: Synseeds, edited by K. Redenbaugh; © CRC Press.

Janick, 1987) to 86% (Janick et al., 1989). SEs of alfalfa desiccated to 10–15% could be stored at room temperature for 1 year without a decline in their germinability (McKersie and Bowley, 1993). However, efficient coating and encapsulation methods for desiccated embryos are yet to be developed (Redenbaugh et al., 1991b).

6.9.2. Hydrated synthetic seeds

In 1984 Redenbaugh et al. developed a technique for encapsulation of single, hydrated SEs of alfalfa. Since then encapsulation in hydrogel remains to be the most studied method of artificial seed production (Redenbaugh and Walker, 1990; Redenbaugh et al., 1991a,b; McKersie and Bowley, 1993).

Several methods have been examined to produce hydrated artificial seeds of which Ca-alginate encapsulation has been most widely used. In practice, as described by Redenbaugh et al. (1991b) for alfalfa, SEs are mixed with 2% (w/v) solution of Na-alginate and dropped, using a plastic pipette, into a 100 mM solution of $Ca(NO_3)_2$. An ion exchange reaction occurs and sodium ions are replaced by calcium ions forming Ca-alginate.

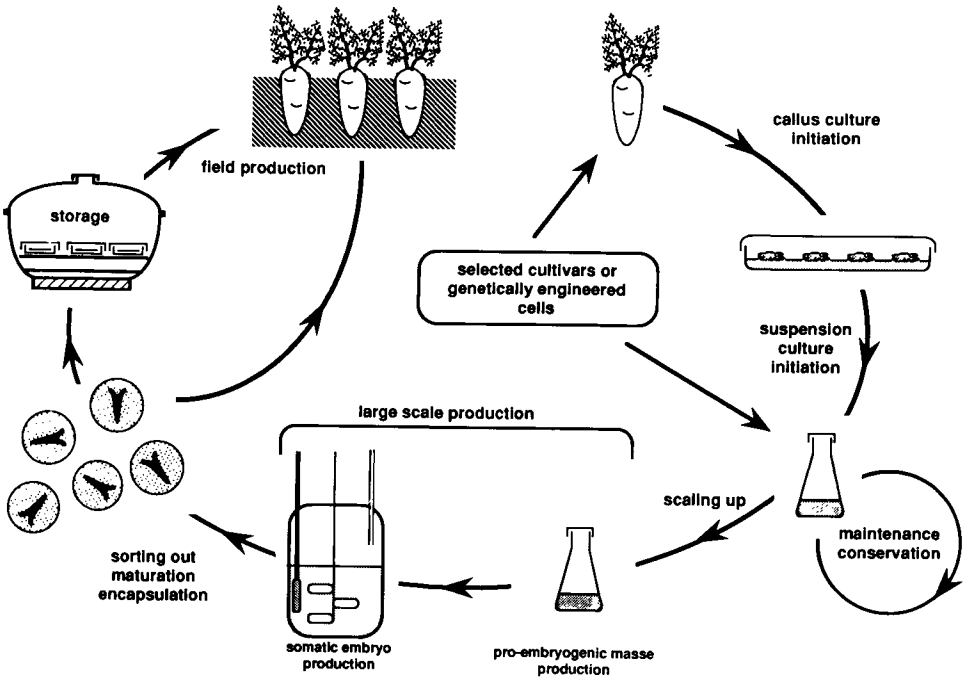


Fig. 6.12. A diagrammatic scheme for the production of hydrated synthetic seeds of carrot. Reprinted with permission from F. Molle et al., 1993. In: Synseeds, edited by K. Redenbaugh; © CRC Press.

Surface complexing begins immediately and the gelling is complete within 30 min. The size of the capsule could be controlled by varying the inside diameter of the pipette tip. Hardening of Ca-alginate gel can be modulated by varying the concentration of Na-alginate and Ca^{2+} ion and the duration of complexing.

Molle et al. (1993) found that for the production of synthetic seeds of carrot (Figs. 6.11 and 6.12) 1% Na-alginate solution, 50 mM Ca^{2+}, and 20–30 min of complexing were satisfactory. They have suggested the use of a dual nozzle pipette, in which the embryos flow through the inner pipette and the alginate solution through the outer pipette. As a result, the embryos are positioned in the heart of the beads for better protection.

Ca-alginate capsules are difficult to handle because they are very wet and tend to stick together slightly. In addition, Ca-alginate capsules lose water rapidly and dry down to a hard pellet within a few hours when exposed to the atmosphere. These problems can be offset by coating the capsules with Elvax 4260 (ethylene vinyl acetate acrylic acid terpolymer, DuPont, USA; Redenbaugh and Walker, 1990).

Onishi et al. (1994) and Sakamoto et al. (1995) have described a protocol for the production of synthetic seeds involving automation at the production and encapsulation stages. These authors have emphasized that high and uniform conversion of synthetic seeds under a practical sowing situation, such as nursery beds in a greenhouse or in the field, is an essential requirement for their use in clonal propagation of plants. For this, encapsulatable embryos should be of high quality. They observed that celery and carrot embryos produced in bioreactors showed almost 0% conversion under non-sterile, high humidity conditions in the glasshouse, which could be raised to 53–80% by three sequential treatments (Figs. 6.13 and 16.5): (1) *Promotion of embryo development*, by culturing the embryos, for 7 days, in a medium of high osmolarity (with 10% mannitol), under light (16 h photoperiod with 300 lx of illumination). It increased the size of embryos from 1–3 mm to 8 mm and chlorophyll content. (2) *Dehydration of embryos*, to reduce their water content from 95–99% to 80–90% by keeping them for 7 days, on 2–7 layers of filter paper, under a 16 h photoperiod and 14 μE m^{-2} s^{-1} irradiance. (3) *Post-dehydration culture*, on SH medium containing 2% sorbitol, 0.01 mg l^{-1} BAP and 0.01 mg l^{-1} GA_3, in air enriched with 2% CO_2, under 16 h photoperiod, at 20°C, for 14 days, to acquire autotrophic nature and reserve food. These authors also modified the bead quality by impregnating the gel beads with 3% sucrose microcapsules coated with a mixture of 8% Elvax 4260 and beeswax, and 0.1% Topsin M (Nippon Soda Co. Ltd., Japan) as the fungicide. The microcapsules release sucrose slowly over a period of 3–21 days at 20°C; at 4°C sucrose is not released. To facilitate the emergence of embryo during germination they made the gel capsule self-breaking under humid conditions. It involved rinsing the beads thoroughly with running tap water, followed by immersion in a 200 mM solution of KNO_3 for 60 min and, finally, desalting them by rinsing in running tap water for 40 min. Such synthetic seeds showed 50% conversion 2 weeks after sowing in a greenhouse.

Using the optimum embryogenic system and the alginate encapsulation process, the cost of a hydrated artificial seed of alfalfa has been calculated as 0.03 cents (Redenbaugh et al., 1991a). The true seeds of alfalfa retail for 0.09 cents (McKersie and Bowley, 1993).

Despite considerable research input into artificial seed production during the last 10 years, several major problems remain with regard to their commercialization. The first requirement for the practical application of artificial seed technology is the large scale production of high quality SEs which is at present a major limiting factor. Additional factors for poor germination of synthetic seeds is the lack of supply of nutrients, lack of sufficient oxygen, microbe invasion and mechanical damage of

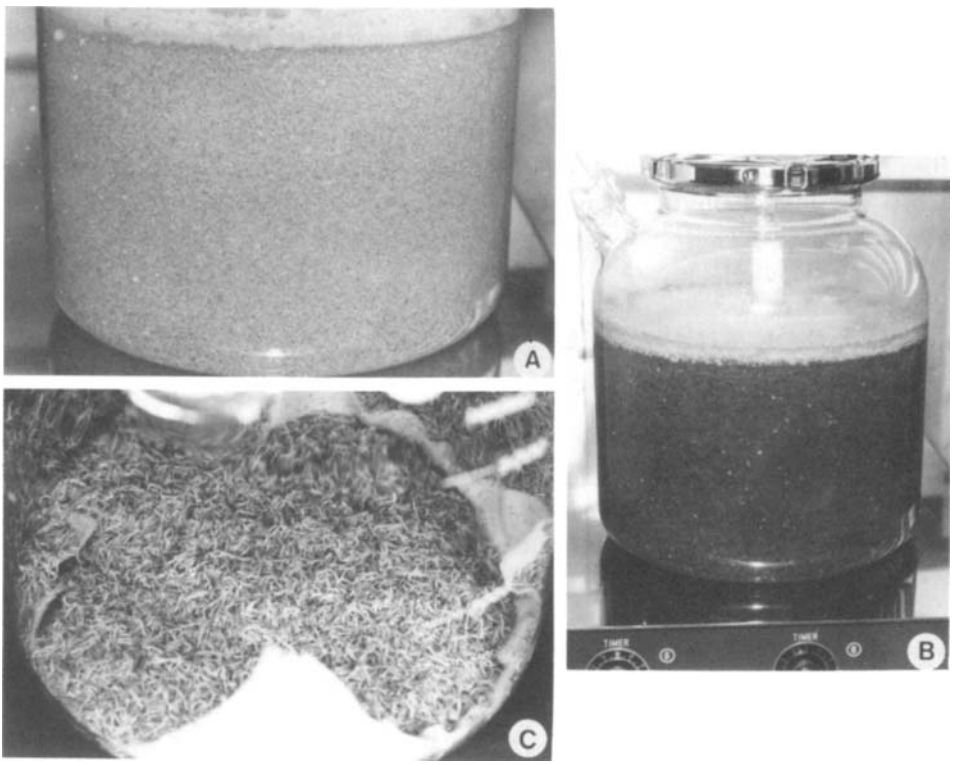


Fig. 6.13. Production of encapsulatable somatic embryos of carrot. (A) Somatic embryogenesis in 8 l bioreactor. (B) Promotion of embryo development in high osmoticum medium. (C) Desiccation of the embryos. Reprinted by permission of Kluwer from N. Onishi et al., 1994, Plant Cell Tissue Organ Culture, 39: 137–145.

SEs. Occurrence of a high level of somaclonal variation in tissue cultures is another aspect to be considered seriously while recommending the use of artificial seeds for clonal propagation.

6.10. SINGLE CELL TO PLANT

The technique of in vitro culture has reached a level of sophistication where plant tissues and organs can be broken down to fully viable single cells (see Chapter 4) and whole plants reconstituted from the individual cells via organogenesis or somatic embryogenesis. This was clearly demonstrated by Vasil and Hildebrandt (1965) (see Fig. 4.6) who mechanically isolated single cells from fresh suspension cultures of *Nicotiana tabacum* × *N. glutinosa* and cultured them individually in microchambers. The isolated cell, which could be constantly observed through the

microscope, underwent a series of divisions, forming a callus which later differentiated whole plants (Fig. 4.6). Kohlenbach (1965) and Lang and Kohlenbach (1975) raised whole plants starting from the mechanically isolated, highly mature mesophyll cells of *Macleaya cordata* (Fig. 6.4). Figure 6.8 shows embryo formation from an isolated cell of carrot. Isolated protoplasts represent the finest single cell system. There is an ever-increasing list of plant species where isolated protoplasts from cultured cells or mesophyll cells have been successfully cultured and whole plants regenerated from them.

6.11. LOSS OF MORPHOGENIC POTENTIAL IN LONG-TERM CULTURES

Callus and suspension cultures which are initially capable of organogenic and/or embryogenic differentiation often show a progressive decline and sometimes a complete loss of this morphogenic potential as they are maintained in culture through repeated subcultures (Wochok and Wetherell, 1972; Fridborg and Eriksson, 1975; Negrutiu and Jacobs, 1978; Molle et al., 1993). This is a serious limitation in the application of cellular totipotency for commercial propagation of plants.

The complex multicellular explants used to initiate cultures are highly heterogeneous with regard to the morphogenic potential of their constituent cells. Generally, only a small proportion of the cells are capable of yielding regenerative cultures; the remaining cells are non-totipotent. In long-term cultures, the number of non-totipotent cells may increase due to the well known cytological instability of the cultured cells (Chapter 9). If the non-totipotent cells are at a selective advantage for growth in the medium used, in repeated subcultures the population of non-totipotent cells increases and the totipotent component is gradually diluted out, resulting in non-morphogenic cultures. When a stage is reached that a culture does not contain any totipotent cell the restoration of morphogenesis would be impossible. However, if the culture carries a few totipotent cells which are unable to express their totipotency because of the inhibitory effect of the non-totipotent cells which are in predominance, it should be possible to restore cultures with morphogenic potential by altering the composition of the culture medium in such a way that it selectively supports the proliferation of the totipotent cells, or by raising single cell clones.

With an increase in culture duration, the embryogenic cultures of carrot showed variation in growth, decline in embryo quality and progressive loss of embryogenic potential (Molle et al., 1993). After 4–6 months the embryogenic potential was almost completely lost. By raising single

cell clones from such cultures, Coutos-Thevenot et al. (1990) could isolate some cell lines showing good embryogenic potential. Similarly, long-term cultures of rice, which had lost the potential for somatic embryogenesis, produced some calli with high embryogenic potential when grown on a medium containing high concentration of NaCl (1.5%) for 3 months (Binh and Heszky, 1990). The surviving embryogenic cells and all the plants regenerated from them were salt tolerant.

At least in some instances, the decline of morphogenic potential could be ascribed to the altered hormonal balance within the cells, or sensitivity of the cells to exogenous growth substances (Fridborg and Eriksson, 1975; Negrutiu and Jacobs, 1978). In such cases the cells stop differentiating organs and embryos without necessarily losing the potential to do so. Accordingly, in such cases it should be possible to restore this innate potential of the cells by modifying the exogenous treatments. Indeed, this has been achieved with certain tissues by giving cold treatment (Syono, 1965), or altering the composition of the medium (Wochok and Wetherell, 1972; Fridborg and Eriksson, 1975; Fridborg et al., 1978; Negrutiu and Jacobs, 1978; Drew, 1979).

The lost embryogenic potential of carrot cultures could be restored by adding 1–4% activated charcoal in the 'development medium' (Fridborg and Eriksson, 1975). The fact that incorporation of 1% charcoal to the 'proliferation medium' (with 2,4-D) supported the development of embryos in the presence of a comparatively high concentration of exogenous auxin strongly suggests that the loss of embryogenic potential in this system could have been due to the increased endogenous level of auxin. Another example suggesting a positive relationship between increased level of endogenous auxin and the loss of embryogenic potential in cells cultured for a long time is that of *Citrus* (see Section 6.3.3).

The embryogenic cultures of carrot are characterized by the presence of small, highly cytoplasmic cells. The loss of embryogenic potential in long-term cultures is associated with an increase in the population of elongated cells. Addition of specific arabinogalactan proteins from seeds or embryogenic cultures of carrot to the culture medium could restore the embryogenic potential of old cultures which had lost it (Kreuger and Van Holst, 1993).

6.12. PRACTICAL APPLICATIONS OF CELLULAR TOTIPOTENCY

Cellular totipotency is an important attribute of plant cells. It is of interest in basic as well as applied areas of plant sciences. The production of large numbers of haploids from microspores (Chapter 7) and the pos-

sibility of raising triploids from endosperm (Chapter 8) cells are the dramatic instances of the potential role of cellular totipotency in genetics and plant breeding. Improvement of crop plants through manipulations at the cellular level (somatic hybridization, mutation of isolated single cells, genetic transformation) is possible only if somatic cells are able to give rise to whole plants.

Citrus trees propagated from nucellar embryos are free of viruses as are the plants raised from zygotic embryos. The progeny from nucellar embryos is also a clone. Nucellar embryogeny is the only practical approach to raise virus-free clones of polyembryonate *Citrus* varieties in nature because shoot-tip culture in this genus has not been successful and the technique of shoot-tip grafting is very tedious (see Section 15.5). A few commercially important clones of *Citrus* are either monoembryonate or seedless (forming seeds only very rarely; e.g., Navel orange, Shamouti orange). For these cultivars there is no in vivo method to raise virus-free clones. However, it can be achieved by culturing their nucelli and inducing somatic embryogenesis artificially (Button, 1977).

A potentially very important application of cellular totipotency is in rapid multiplication of elite individuals of a wide range of plant species. Efforts are being made to standardize protocols for large scale production of SEs in bioreactors and their conversion into synthetic seeds. However, a major problem in introducing this technique for commercial clonal propagation of plants is the genetic instability of cells in long-term cultures (see Chapter 9). Until this phenomenon is fully understood and methods are developed to check it, a more conservative approach of shoot proliferation, which is comparatively slow but safe, is preferred for in vitro clonal multiplication of plants (for details see Chapter 16).

During the past century, higher plant embryogenesis has been studied intensively to describe morphological and anatomical changes that characterize embryogenic development (Natesh and Rao, 1984; Raghavan, 1986; Bhojwani and Bhatnagar, 1990). By contrast, very little is known about the biochemical, molecular and genetic regulation of embryogenesis because of the practical problem of accessibility to the zygotic embryo which develops under several cell layers. The progress in the field of somatic embryogenesis has been such that in some systems, particularly carrot (Terzi et al., 1985), gram quantities of practically pure fractions of embryos (histologically and biochemically similar to zygotic embryos) at different developmental stages can be obtained. Therefore, it has now become possible to apply the techniques of molecular biology to understand the cellular basis of induction and differentiation during embryogenesis. Some valuable information in this regard has already started to appear in the literature (Section 6.4.3).

6.13. CONCLUDING REMARKS

During the last 10 years the list of species reported to regenerate plants in tissue cultures has considerably enlarged, and it now includes many such species which were once regarded recalcitrant (Bhojwani and Razdan, 1983). A major factor for this success has been the change of emphasis from medium manipulation to explant and genotype selection (Sections 5.3.1, 6.3.1, 6.3.2). Most of the cereals, grain legumes, cotton, tree species (including conifers) etc. express cellular totipotency only in the cultures of embryonic explants. Older tissues of these plants have remained recalcitrant. Probably in these species cells lose their competence to respond to the inductive conditions very early during development. Recent studies suggest that regeneration in tissue cultures is a three step process (Section 5.3.2): (1) acquisition of competence, (2) induction and (3) development. Embryonic explants, which regenerate embryos directly without a callus phase, seem to carry the competent cells. In others the cells acquire competence on the induction medium. Rarely, as in some genotypes of *Convolvulus*, the first two steps require different treatments. We hardly know anything about the process of acquisition of competence which may be our handicap in achieving regeneration in hitherto recalcitrant taxa.

The induction of somatic embryogenesis is being examined at the molecular level. Several 'embryo specific' or 'embryo enhanced' genes have been isolated from embryogenic cultures of carrot and some molecular markers to distinguish between embryogenic and non-embryogenic cultures have been identified (Section 6.4.3). The information generated through such studies should enhance our manipulative power to induce somatic embryogenesis.

For a long time, the literature on somatic embryogenesis dealt with only the production of embryos. However, since the potential application of this system in commercial propagation of plants has been recognised, a new problem has come to the fore. The somatic embryos show very poor germination because of their physiological and biochemical immaturity. Therefore, a new step of 'embryo maturation', requiring special treatment, has been introduced in the protocols of somatic embryogenesis (Section 6.5). The prospects for commercialization of somatic embryogenesis also depends on the standardization of various parameters for large scale, synchronized production of SEs in bioreactors. Several novel bioreactors have been designed for embryogenic cultures but none has proved entirely satisfactory (Section 6.8). The genetic/physiological instability of cultured cells leading to loss of morphogenic potential in long-term cultures continues to be a major handicap in commercial exploitation of cellular totipotency for plant propagation.

APPENDIX 6.I

6.I.1. Protocol for inducing somatic embryogenesis in *Daucus carota* (after Smith and Street, 1974)

(a) Surface sterilize the seeds in 10% calcium hypochlorite for 15 min and, after washing three times in sterile distilled water, germinate them on sterilized moistened filter paper in petri dishes, in the dark, at 25°C.

(b) Cut 1 cm long segments of roots from 7-day-old seedlings and culture them individually on a semi-solid medium containing the inorganic salts of Murashige and Skoog's medium, organic constituents of White's medium, 100 mg l^{-1} myo-inositol, 0.2 mg l^{-1} kinetin (not essential), 0.1 mg l^{-1} 2,4-D, 2% sucrose, and 1% Difco bacto agar. Incubate the cultures in the dark.

(c) After 6–8 weeks transfer pieces of root calli (0.2 g fresh weight) to fresh medium of the original composition and maintain the cultures in light at 25°C. The tissues may be multiplied by subculturing every 4 weeks in a similar manner.

(d) After the first passage initiate suspension cultures by transferring ca. 0.2 g of callus tissue to a 200 ml Erlenmeyer flask containing 20–25 ml of liquid medium of the same composition as used for callus growth (without agar). Incubate the flasks on a horizontal rotary shaker at 100 rev. min^{-1}, in the light, at 25°C.

(e) Subculture the suspensions every 4 weeks by transferring 5 ml to 65 ml of fresh medium (1:13).

(f) To induce embryo development, transfer callus pieces or portions of suspension to 2,4-D-free medium of otherwise the same composition as used before.

(g) After 3–4 weeks the cultures contain numerous embryos in different stages of development.

6.I.2. Protocol for inducing somatic embryogenesis in *Citrus* sp. (after Tisserat and Murashige, 1977)

(a) Take a 6–8-week-old fruitlet of a local cultivar and surface-sterilize it with 1% sodium hypochlorite for 15–20 min. Follow the subsequent steps under aseptic conditions.

(b) Bisect the fruit and transfer the ovules to a sterile petri-dish.

(c) Excise nucellus tissue from the ovules using a dissecting microscope. Hold the chalazal region of the ovule with fine forceps. Give a shallow incision longitudinally through the integuments from

the chalazal region to the micropylar tip. Cut the ovule transversely into two halves. From the micropylar half, remove the integuments, endosperm and any embryo (especially zygotic), if present. Transfer the nucellar section to the culture vessel in a manner that its cut end is in contact with the medium.

(d) Use semi-solid medium containing inorganic salts of Murashige and Skoog's medium, 100 mg l^{-1} myo-inositol, 0.2 mg l^{-1} thiamine·HCl, 1 mg l^{-1} pyridoxine·HCl, 1 mg l^{-1} nicotinic acid, 4 mg l^{-1} glycine, 500 mg l^{-1} malt extract, 5% sucrose, and 1% Difco bacto agar.

(e) Incubate the cultures in 16 h light (1000 lx) at 27 ± 1°C.

(f) Within 4–6 weeks multiple embryos should develop from the callused nucellar tissue. To stimulate full plantlet development transfer the embryos to another medium which differs from the previous medium in having 1 mg l^{-1} GA_3, in place of malt extract.

6.I.3. Protocol for inducing somatic embryogenesis in *Coffea arabica* (after Sondahl and Sharp, 1977)

(a) Take mature leaves from plagiotrophic shoots and surface-sterilize them in 1% sodium hypochlorite solution for 15–30 min. Rinse three times in sterilized distilled water.

(b) Excise 7 mm^2 pieces from the lamina between the midrib and margin. Avoid the apical and basal portions. Cut the leaf in saline (half strength inorganic salts of Murashige and Skoog's medium, 10 mg l^{-1} thiamine·HCl, 86 mg l^{-1} *l*-cysteine·HCl, 99 mg l^{-1} myo-inositol)–sucrose (3%) solution.

(c) Transfer the leaf pieces to saline–sugar–agar (1%) medium in petri dishes. Place the pieces with their adaxial surface facing the medium. Store the plates in the dark.

(d) After 30 h, transfer the leaf pieces to jars containing the 'conditioning medium' (inorganic salts of Murashige and Skoog's medium, 10 mg l^{-1} thiamine·HCl, 86 mg l^{-1} *l*-cysteine·HCl, 99 mg l^{-1} myo-inositol, 4% sucrose, 4.5 mg l^{-1} kinetin, 1 mg l^{-1} 2,4-D, 1% Difco bacto agar). Store the cultures in the dark at 25 ± 1°C.

(e) After 40–45 days transfer the callused tissue formed by the leaf pieces to the 'induction medium' (as the conditioning medium in which the inorganic salts, except KNO_3, and sucrose have been reduced to half strength, and the hormonal composition changed to 0.6 mg l^{-1} kinetin + 0.1 mg l^{-1} NAA). Store these cultures in 12 h light (4600 lx) at 24–28°C. Low-frequency somatic embryogenesis occurs after 13–15 weeks and high-frequency SEs appear after another 3–6 weeks.

(f) The embryos develop into plantlets in situ, but to stimulate embryo germination excise the proembryogenic tissue after 4–6 weeks in the 'induction medium' and grow them in another medium which differs from the 'induction medium' in lacking agar and kinetin. Maintain the cultures in the light at 26°C. After 4–6 weeks transfer the torpedo-shaped embryos to saline–agar medium containing 0.5–1% sucrose, and continue keeping the cultures in the light.

6.I.4. Protocol for the production of SES of alfalfa and their desiccation for storage (after McKersie and Bowley, 1993)

(1) Take petiole segments from fully expanded 2–3 youngest leaves and culture them on Schenk and Hildebrandt medium (3% sucrose) containing 2,4-D (1 mg l^{-1}) and kinetin (0.2 mg l^{-1}) to induce callus and SE formation.
(2) After 14–21 days transfer the callus to liquid B_5 medium (3% sucrose; 1.5 g callus per 25 ml of medium).
(3) After 7 days sieve the suspension first through 500 μm nylon mesh and then 200 μm mesh.
(4) Spread the small clusters of PEMs collected on 200 μm mesh in a thin layer and place, with the screen, on to a hormone-free BOi2y (after Bingham et al., 1975. Crop Sci., 15: 519–721) containing 5% sucrose.
(5) After about 7 days, green heart-shaped embryos appear protruding from the bed of callus in a more or less synchronized stage of development.
(6) After 14 days, transfer the SEs to BOi2Y medium containing 40 mM glutamine and 2 μM ABA for the maturation of the embryos. During this phase deposition of storage protein occurs.
(7) After 10 days, transfer the embryos to BOi2Y containing 20 μM ABA which favours further deposition of storage reserves and acquisition of desiccation tolerance.
(8) After 10 days wash the embryos and dry slowly by transfer through atmosphere of progressively reduced RH, into air dryness over a period of 6 days. Such embryos, with 10–15% moisture content, can be stored for months at room temperature.

Chapter 7

Haploid Production

7.1. INTRODUCTION

The significance of haploids[1] in genetics and plant breeding has been realized for a long time. However, their exploitation remained restricted because of the extremely low frequency with which they occur in nature (usually 0.001–0.01%). Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from an unfertilized egg). Rarely, however, they reproduce the characters of the male parent alone, suggesting their origin through 'ovule androgenesis'[2] (embryo development inside the ovule by the activity of the male nucleus alone). In vivo occurrence of androgenic haploids has been reported in *Antirrhinum majus*, *Crepis tectorum*, *Hordeum bulbosum* x *H. vulgare*, *Nicotiana* and *Oenothera scabra*. Until 1964 the artificial production of haploids was attempted through: (a) distant hybridization, (b) delayed pollination, (c) application of irradiated pollen, (d) hormone treatments, and (e) temperature shocks. None of these methods, however, proved dependable. Therefore, the development of numerous pollen plantlets in anther cultures of *Datura innoxia*, first reported by two Indian scientists (Guha and Maheshwari, 1964, 1966), was a major breakthrough in haploid breeding of higher plants.

The technique of haploid production through anther culture ('anther androgenesis') has been extended successfully to numerous plant species, including many economically important plants, such as cereals and vegetable, oil and tree crops (see Table 7.1). To-date, 'anther androgenesis', henceforth referred to as simply androgenesis, has been reported in over 134 species and hybrids distributed within 25 families (see Table 7.1). During the last decade considerable success has been achieved with the induction of androgenesis in isolated pollen culture (see Section 7.2.2) and androgenic haploids have been used to breed new cultivars of crop plants (see Sections 7.9.1 and 7.9.2). This chapter describes the techniques of anther and pollen culture and the factors that influence in vitro androgenesis. The alternative in vitro methods of haploid production, viz.

[1] With reference to higher plants, haploids are defined as sporophytes with gametophytic chromosome constitution (Kimer and Riley, 1963).
[2] After Pandey (1973).

TABLE 7.1

Species for which androgenic haploids have been raised by anther culture (androgenic plants arise through direct pollen embryogenesis (E) or through pollen callusing (C))

Species	Mode of development	Reference
Annonaceae		
Annona squamosa	C	Nair et al (1983)
Apiaceae		
Daucus carota	E	Anderson et al. (1990)
Asteraceae		
Catharanthus tinctorius	C	Rajendra Prasad et al. (1991)
Gerbera jamesonii	C	Preil et al. (1977)
Helianthus annuus	E,C	Mezzarobba and Jonard (1986)
Brassicaceae		
Arabidopsis thaliana	C	Gresshoff and Doy (1972a), Scholl and Fieldmann (1990)
Brassica alba	C	Leelavathi et al. (1984)
B. campestris	E	Keller and Armstrong (1979)
B. carinata	E	Arora and Bhojwani (1988)
B. chinensis	?	Chung et al. (1978) cited in Hu (1978)
B. hirta	E	Klimaszweska and Keller (1983)
B. juncea	E	Sharma and Bhojwani (1985)
B. napus	E	Thomas and Wenzel (1975), Keller and Armstrong (1978)
B. nigra	E	Lichter (1989), Govil et al. (1986)
B. oleracea	C	Kameya and Hinata (1970)
B. oleracea × *B. alboglabra* (F_1)	C	Kameya and Hinata (1970)
B. pekinensis	?	Cited in Hu et al. (1978)
Raphanus sativus	E	Lichter (1989)
Caricaceae		
Carica papaya	E	Litz and Conover (1980)
Chenopodiaceae		
Beta vulgaris	?	Cited in Hu et al. (1978)
Convolvulaceae		
Pharbitis nil	C	Sangwan and Norreel (1975)
Euphorbiaceae		
Hevea brasiliensis	?	Chen et al. (1978)
Fabaceae		
Albizzia lebbeck	?	Gharyal et al. (1983)
Medicago denticulata	C	Zagorska et al. (1990)
M. sativa	?	Xu (1979)
Trifolium alexandrum	C	Mokhtarzadeh and Costantin (1978)

TABLE 7.1 (*continued*)

Species	Mode of development	Reference
Fagaceae		
Fagus sylvatica	C	Jorgensen (1991)
Quercus petraea	C	Jorgensen (1991)
Geraniaceae		
Pelargonium hortorum	C	Abo El-Nil and Hildebrandt (1973)
Gesneriaceae		
Saintpaulia ionantha	E	Hughes et al. (1975)
Hippocastanaceae		
Aesculus hippocastanum	E	Radojevic (1978)
A. carnea	E	Radojevic et al. (1989)
Liliaceae		
Asparagus officinalis	C	Pelletier et al. (1972), Dore (1974), Feng and Wolyn (1991)
Lilium longiflorum	C	Sharp et al. (1972)
Palmaceae		
Cocos nucifera	E	Thanh-Tuyen and De Guzman (1983)
Poaceae		
Aegilops caudata × *Ae. umbellata*	C	Kimata and Sakamoto (1972)
Agropyron intermedium	C	Yao et al. (1991)
Avena sativa	C	Rines (1983)
Bromus inermis	C	Saito et al. (1973)
Coix lacryma	?	Wang et al. (1980)
Hordeum vulgare	C	Clapham (1971, 1973) Mix et al. (1978)
Lolium multiflorum	C	Clapham (1971)
L. multiflorum × *Festuca arundinacea*	C	Nitzsche (1970)
Oryza sativa	C,E	Niizeki and Ono (1968), Guha et al. (1970), Iyer and Raina (1972)
O. perennis	C	Wakasa and Watanabe (1979)
Saccharum spontaneum	C	Fitch and Moore (1983)
S. cereale	C,E	Thomas et al. (1975), Wenzel et al. (1977)
Secale cereale × *S. vavilovii*	?	Friedt et al. (1983)
Setaria italica	C	Ban et al. (1971)
Sorghum bicolor	C	Rose et al. (1986)
Triticale	C	Wang et al. (1973), Sun et al. (1974)

TABLE 7.1 (*continued*)

Species	Mode of development	Reference
Triticum aestivum	C,E	Ouyang et al. (1973), Wang et al. (1973), Craig (1974), Schaeffer et al. (1979)
T. durum	?	Zhu et al. (1980)
T. vulgare × *Agropyron glaucum*	C	Wang et al. (1975a)
Zea mays	C,E	Miao et al. (1978), Brettell et al. (1981)
Primulaceae		
Cyclamen persicum	E	Ishizaka and Uematsu (1993)
Ranunculaceae		
Paeonia hybrida	E	Sunderland (1974)
Ranunculus asiaticus	E	Meynet and Duclos (1990)
Rosaceae		
Fragaria × *ananassa*	C	Niemirowicz-Szczytt (1990)
Malus pumila	E	Kubichi et al. (1975), Fei and Xue (1981)
Malus pumifolia	C	Fei and Xue (1981)
Rutaceae		
Citrus aurantifolia	E	Chaturvedi and Sharma (1985)
C. madurensis	E	Ling et al. (1988)
C. microcarpa	E	Chen et al. (1980b)
Poncirus trifoliata	E	Hidaka et al. (1979)
Salicaceae		
Populus alba × *P. simonii*	C	Lu and Liu (1990)
P. berolinensis	C	Lu and Liu (1990)
P. berolinensis × *P. pyramidalis*	C	Lu and Liu (1990)
P. canadensis × *P. koreara*	C	Lu and Liu (1990)
P. euphratica	C	Lu and Liu (1990)
P. harbinensis × *P. pyramidalis*	C	Lu and Liu (1990)
P. maximowiczii	C	Lu and Liu (1990)
P. nigra	C	Wang et al. (1975b)
P. pseudosimonii	C	Lu and Liu (1990)
P. pseudosimonii × *P. pyramidalis*	C	Lu and Liu (1990)
P. simonii	C	Lu and Liu (1990)
P. simonii × *P. nigra*	C	Anonymous (1975)
P. simonii × *P. pyramidalis*	C	Lu and Liu (1990)
P. ussuriensis	C	Anonymous (1975)
Sapindaceae		
Dimocarpus longana	C	Wei (1990)
Litchi chinensis	C	Fu (1990)
Scrophulariaceae		
Digitalis purpurea	C	Corduan and Spix (1975)

TABLE 7.1 (*continued*)

Species	Mode of development	Reference
Solanaceae		
Atropa belladonna	E	Zenkteler (1971), Rashid and Street (1973)
Capsicum annuum	C,E	Wang et al. (1973), Harn et al. (1975)
Datura innoxia	E	Guha and Maheshwari (1966), Nitsch (1972),
D. metel	C,E	Narayanaswamy and Chandy (1971), Iyer and Raina (1972)
D. meteloides	E	Nitsch (1972), Kohlenbach and Geier (1972)
D. muricata	E	Nitsch (1972)
D. stramonium	E	Guha and Maheshwari (1967)
D. wrightii	E	Kohlenbach and Geier (1972)
Hyoscyamus albus	E	Raghavan (1975)
H. muticus	?	Wernicke et al. (1979)
H. niger	E,C	Corduan (1975), Wernicke and Kohlenbach (1977), Raghavan (1978)
H. pusillus	E	Raghavan (1975)
Lycium halimifolium	E	Zenkteler (1972)
Lycopersicon esculentum	C	Sharp et al. (1971), Gresshoff and Doy (1972b)
Nicotiana alata	E	Nitsch (1969),
N. attenuata	E	Collins and Sunderland (1974)
N. clevelandii	E	Vyskot and Novak (1974)
N. glutinosa	E	Nitsch and Nitsch (1970), Nakamura and Itagaki (1973)
N. knightiana	E	Collins and Sunderland (1974)
N. langsdorffii	E	Durr and Fleck (1980)
N. otophora	E	Nitsch (1972), Nakamura and Itagaki (1973)
N. paniculata	E	Nakamura et al. (1974)
N. raimondii	E	Collins and Sunderland (1974)
N. rustica	E	Nitsch and Nitsch (1970); Nakamura and Itagaki (1973)
N. sanderae	E	Vyskot and Novak (1974)
N. sylvestris	E	Bourgin and Nitsch (1967), Nitsch and Nitsch (1970)
N. tabacum	E	Bourgin and Nitsch (1967), Sunderland and Wicks (1971)
Petunia axillaris	E	Engvild (1973), Swamy and Chacko (1973)

TABLE 7.1 (*continued*)

Species	Mode of development	Reference
P. axillaris × *P. hybrida*	C	Raquin and Pilet (1972)
P. hybrida	C	Wagner and Hess (1974), Sangwan and Norreel (1975)
P. violaceae	E	Gupta (1983)
Scopolia carniolica	E	Wernicke and Kohlenbach (1975)
S. lurida	E	Wernicke and Kohlenbach (1975)
S. physaloides	E	Wernicke and Kohlenbach (1975)
Solanum bulbocastanum	C,E	Irikura (1975)
S. carolinensis	E	Reynolds (1990)
S. chacoense	E	Cappadocia et al. (1984)
S. demissum	C,E	Irikura (1975)
S. dulcamara	C,E	Zenkteler (1973)
S. fendleri	C,E	Irikura (1975)
S. hjertingii	E	Irikura (1975)
S. melongena	C	Raina and Iyer (1973)
S. nigrum	C	Harn (1972), Irikura (1975)
S. phureja	E	Irikura (1975)
S. polytrichon	C,E	Irikura (1975)
S. stenotomum	E	Irikura (1975)
S. stoloniferum	E	Irikura (1975)
S. surattense	C	Sinha et al. (1979)
S. tuberosum	C,E	Dunwell and Sunderland (1973), Sopory et al. (1978)
S. tuberosum × *S. chacoense*	E	Cappadocia et al. (1984)
S. verrucosum	C,E	Irikura and Sakaguchi (1972)
S. verrucosum × *S. chacoense*	E	Irikura (1975)
S. verrucosum × *S. tuberosum*	E	Irikura (1975)
Theaceae		
Camellia sinensis	C	Chen and Liao (1990)
Vitaceae		
Vitis vinifera	E	Zhou and Li (1981)

gynogenesis and distant hybridization followed by embryo culture are also described. Finally, the practical importance of haploidy in higher plants and the current limitations of anther and pollen culture are discussed.

7.2. THE TECHNIQUES

7.2.1. Anther culture

The experimental plants for anther and pollen culture should ideally

be grown under controlled conditions of temperature, light and humidity, and anthers should be taken from young plants. Generally, with increasing age of the donor plants the androgenic response declines and abnormalities appear. In plants grown under controlled environmental conditions it may be possible to draw a rough correlation between the stage of pollen development and certain visible morphological features of the bud, such as the length of the corolla tube, emergence of corolla from the calyx, and the like. These external markers can be used for selecting buds of approximately the required stage. However, the correlation is never absolute and, therefore, it is always necessary to crush one of the anthers from each bud to assess the exact stage of pollen development.

The selected buds are surface sterilized with a suitable disinfectant (see Chapter 2). Anthers along with their filaments are excised under aseptic conditions and placed on a sterilized petri-plate. One of the anthers is crushed in acetocarmine to test the stage of pollen development and if it is found to be of the correct stage the anthers of the remaining stamens are gently detached from their filaments, without injuring the anthers, and placed horizontally on the medium (anther culture). In *Brassica oleracea* even a part of the filament left attached caused a reduction in the androgenic response by about 30%, although the overall average number of productive anthers was not affected (Arnison et al., 1990).

When dealing with plants having minute flowers, such as *Asparagus*, *Brassica* and *Trifolium*, it may be necessary to use a stereoscopic microscope for dissecting the anthers. Alternatively, only the perianth may be removed and the rest of the bud, with the stamens intact, inoculated. In some cases complete inflorescences have been cultured to obtain androgenic haploids (Preil et al., 1977; Wilson et al., 1978). However, this simplified approach can be tried with only such genotypes where androgenesis occurs on simple nutrient media containing mineral salts, vitamins and sugars, because on such a medium the chances of the sporophytic tissue proliferating are very remote. However, where growth hormones are essential to induce androgenic development of pollen grains the sporophytic tissues should be removed as far as possible. The gap between bud collection and anther/pollen culture should not exceed 2 h.

The anther cultures are generally maintained in alternating periods of light (12–18 h; 5000–10 000 lx m^2) at 28°C and darkness (12–6 h) at 22°C (Vasil, 1973). However, optimal storage conditions need to be determined for individual systems. For example, the anther cultures of *Brassica* species are very sensitive to light and, therefore, should be maintained in the dark throughout.

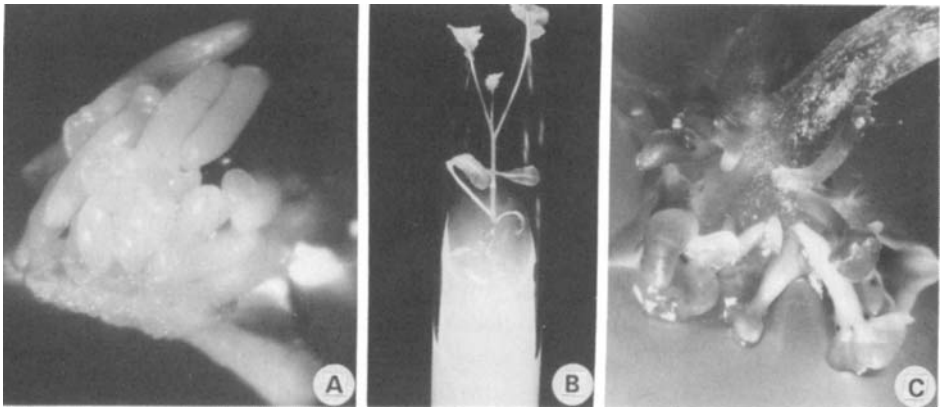


Fig. 7.1. Androgenesis in anther cultures of *Brassica juncea*. (A) Numerous pollen embryos emerging from a burst anther, 4 weeks after culture. (B) A plantlet derived by normal germination of a pollen embryo. (C) A pollen plant bearing secondary embryos close to the radicular end (after Agarwal and Bhojwani, 1993).

In responsive anthers, the wall tissues gradually turn brown and, depending on the species, after 3–8 weeks they burst open due to the pressure exerted by the growing pollen callus or pollen embryos (Fig. 7.1A). The embryos may germinate on the original medium or require transfer to another medium to form plantlets. After they have attained a height of about 3–5 cm, the individual plantlets or shoots are excised and transferred to a medium which would support good development of the root system. The rooted plants are transferred to sterilized potting-mix in small pots or seed trays. Procedures for the transplantation of plants out of cultures are discussed in Chapter 16.

The plants arising from different pollen grains in an anther would be genetically different. To achieve rapid clonal multiplication of the desired genotypes, for breeding or other experimental purposes, micropropagation through shoot multiplication may be tried (for details see Chapter 16).

7.2.2. Isolated pollen culture

There are many problems associated with raising haploids through anther culture. The pollen grains within an anther lobe being genetically heterogeneous, the plants arising from an anther would constitute a heterogeneous population. Mixing of calli of different pollen origin within an anther lobe enhances the chances of regenerating chimeric plants. Furthermore, if the proliferation of anther wall cells occurs concomitant with the callusing of pollen the tissue finally derived would not be purely of

gametophytic origin. In the cultures of anthers showing asynchronous pollen development the older grains may suppress the androgenic response of younger grains by releasing toxic substances as observed in *Brassica napus* (Kott et al., 1988). Isolated pollen culture cannot only circumvent these problems but also offers many other advantages: (a) it is a haploid, single cell system, (b) a homogeneous population of pollen grains at the developmental stage most suitable for androgenesis can be obtained by gradient centrifugation (Kyo and Harada, 1990), (c) isolated microspores can be genetically modified by exposing them to mutagenic treatments or insertion of foreign genes before culture (see Section 7.9.4) and the new genotypes selected at an early stage, and (d) according to Aslam et al. (1990) and Siebel and Pauls (1989), in rapid cycling *B. napus*, pollen culture is 60 times more efficient than anther culture in terms of embryo production.

The first report of callus formation in isolated pollen culture of an angiosperm (*Brassica oleracea* and the hybrid *B. oleracea* × *B. alboglabra*) was published in 1970 by Kameya and Hinata. Since then the technique of pollen culture has been considerably improved and androgenic plants through isolated pollen culture have been raised for many crop plants, including *Brassica carinata* (Chuong and Beversdorf, 1985), *B. campestris* (Ziemborska and Pauw, 1987; Baillie et al., 1992), *B. napus* (Chuong et al., 1988; Takahata et al., 1991), *B. nigra* (Lichter, 1989), *B. oleracea* (Takahata and Keller, 1991), *B. rapa* (Burnett et al., 1992), *Hordeum vulgare* (Datta and Wenzel, 1988), *Oryza sativa* (Chen et al., 1980a; Cho and Zapata, 1988, 1990; Datta et al., 1990), *Petunia* (Sangwan and Norreel, 1975), *Nicotiana rustica, N. tabacum* (Imamura et al., 1982), *Triticum aestivum* (Datta and Wenzel, 1987) and *Zea mays* (Pescitelli et al., 1989; Gaillard et al., 1991).

The initial success with isolated pollen culture was based on the use of some kind of a nurse tissue or its extract. Sharp et al. (1972) raised haploid tissue clones from isolated pollen grains of tomato by plating them on small filter paper pieces placed over cultured anthers of the same species. Similarly, Pelletier and Durran (1972) used anther cultures of *Nicotiana glutinosa* or *Petunia hybrida* or the petal callus of the latter as an effective nurse tissue for isolated pollen culture of *N. tabacum*. Coculture of ovaries has been used to nurse the formation of embryos from isolated pollen of wheat (Datta and Wenzel, 1987). For most of the cereals a pre-culture of anthers for 2–7 days is essential or promotory to obtain embryogenic response in isolated pollen cultures (Wilson et al., 1978; Sunderland and Xu, 1982; Cho and Zapata , 1988; Pescitelli et al., 1989). The pollen grains are released from the cultured anthers either mechanically or the cold treated anthers cultured on liquid medium dehisce after

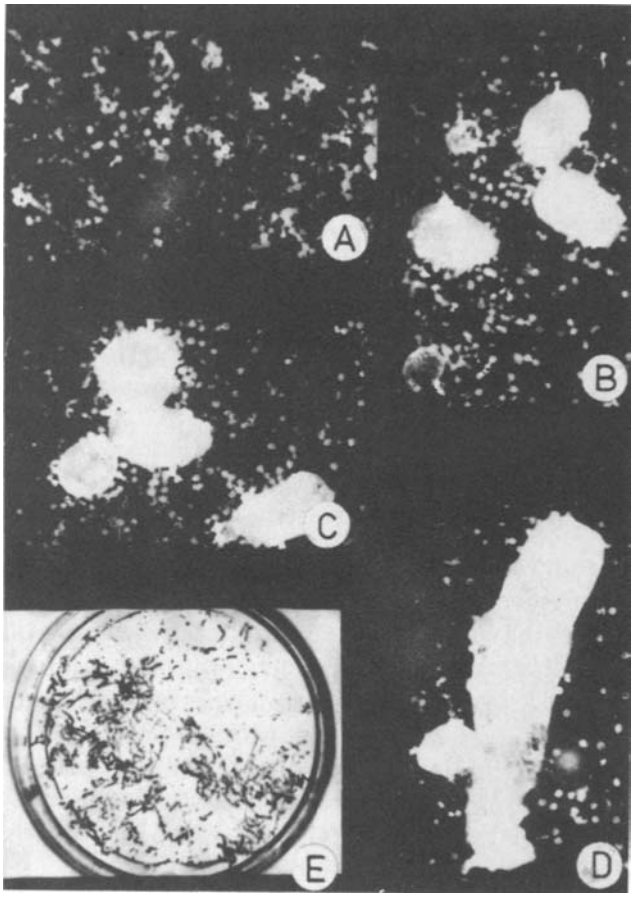


Fig. 7.2. Stages in embryo development in isolated microspore culture of *Nicotiana tabacum*. (A–E) 3, 10, 15, 18 and 20 days after culture, respectively (courtesy of C. Nitsch, France).

2–7 days liberating the pollen grains into the medium. The latter approach, called 'float culture method' (Sunderland and Roberts, 1977, 1979) has generally proved better than mechanical isolation of pollen from fresh or pre-cultured anthers (Chen et al., 1980; Pescitelli et al., 1989). These observations suggest that anther wall provides a suitable environment necessary to trigger androgenesis. Based partly on the analysis of non-embryogenic anthers (taken directly from flower buds) and embryogenic anthers (cultured for about 7 days), Nitsch (1974) highlighted the importance of glutamine, *l*-serine and myo-inositol as constituents of media for ab initio pollen culture of *Datura* and tobacco (Fig. 7.2).

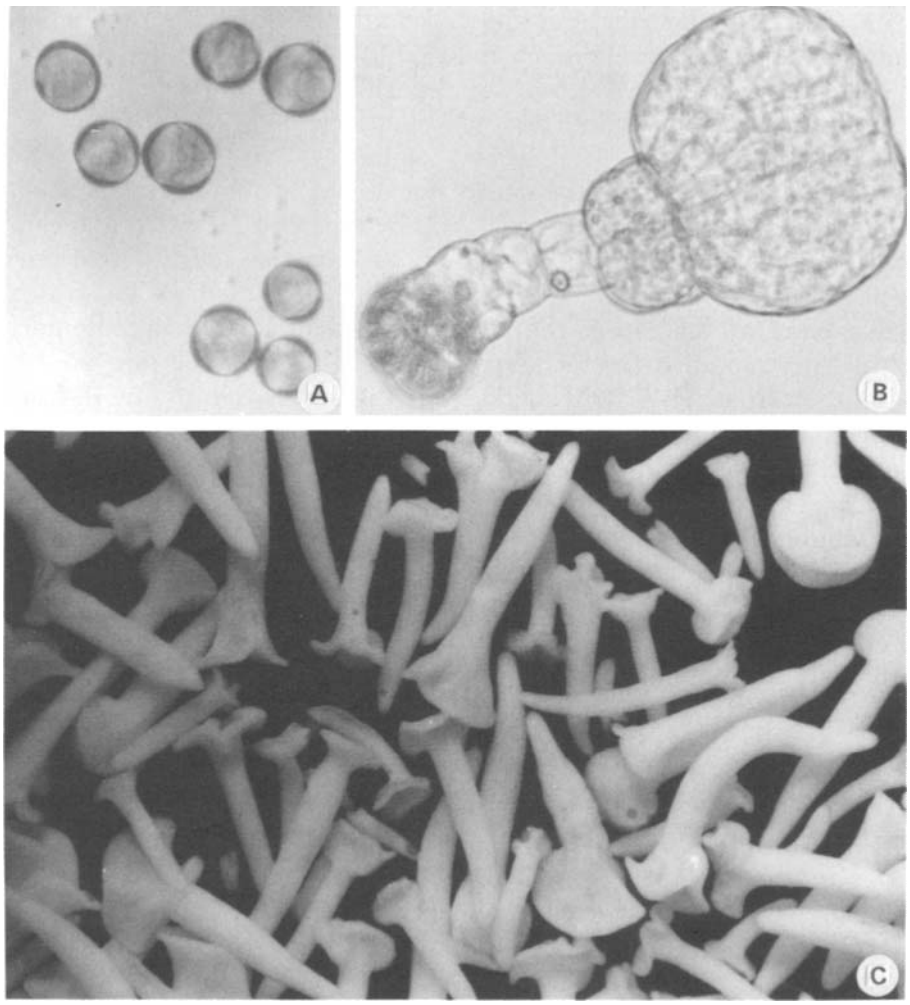


Fig. 7.3. Isolated pollen culture of *Brassica napus*. (A) Isolated pollen at the time of culture. (B) A young pollen embryo with prominent suspensor, from a 7-day-old culture. (C) Mature dicotyledonous pollen embryo, from a 21-day-old culture (courtesy of L. Kott, Canada).

Plant regeneration from isolated pollen grains, without pre-culture of anther or the use of a nurse tissue has been reported in barley (Wei et al., 1986), maize (Gaillard et al., 1991) and several species of *Brassica*. Of these *B. napus* has been investigated most thoroughly (Fig. 7.3), and the system is being employed to understand the cellular basis of induction and for classroom demonstration of androgenesis. A protocol for plant regeneration from *ab initio* pollen culture of *B. napus* is given in Appendix 7.I.

To improve the efficiency of isolated pollen culture for the production of haploids, Wenzel et al. (1975) introduced the technique of density gradient centrifugation which allows the separation of embryogenic grains from a mixture of embryogenic and non-embryogenic grains obtained after crushing the anthers. The anthers of barley were collected at the proper stage of development and gently macerated to obtain a suspension of pollen grains. After removing the debris by repeated filtration and centrifugation the suspension was layered on 30% sucrose solution and centrifuged at 1200 g for 5 min. The androgenic, vacuolated pollen grains formed a band at the top of the sucrose solution. Rashid and Reinert (1980) slightly modified the technique and used 55% Percoll and 4% sucrose solution, instead of 30% sucrose, for the separation of starch-free, embryogenic grains of tobacco. Percoll gradient centrifugation was found very useful to collect highly embryogenic grains of maize (Gaillard et al., 1991). The grains collected at the interface of 40/50% Percoll showed maximum androgenic response.

Isolated pollen culture is not only more efficient but also more convenient than anther culture. The tedious process of dissection of individual anthers is avoided. Instead, the entire buds within a suitable size range are crushed and the embryogenic grains are then separated by gradient centrifugation (Fig. 7.4).

7.3. FACTORS AFFECTING ANDROGENESIS

7.3.1. Physiological status of the donor plants

Physiology of the donor plants, which is affected by its age and the environmental conditions under which it has been grown, significantly influences the androgenic response. Generally, the buds from the first flush of flowers show better response than those borne subsequently. The anthers excised towards the end of the flowering season exhibit a delayed response, in addition to a considerably low frequency of sporophyte formation (Sunderland, 1971; Olesen et al., 1988; Sato et al., 1989). Where it is necessary to continue experiments over an extended period, the unused buds should be removed from the plant to prevent fruit formation and, thus, maintain the plants young (Dunwell, 1985; Sato et al., 1989). In *Brassica rapa*, however, pollen grains from old, sickly looking plants produced more embryos than those from young and healthy plants (Burnett et al., 1992). Similarly, the plants of *Brassica juncea* sown 2 months late in the season (in December), which reduced the vegetative growth period from 11 to 8 weeks, yielded more androgenic anthers than those sown in October (Agarwal and Bhojwani, 1993).

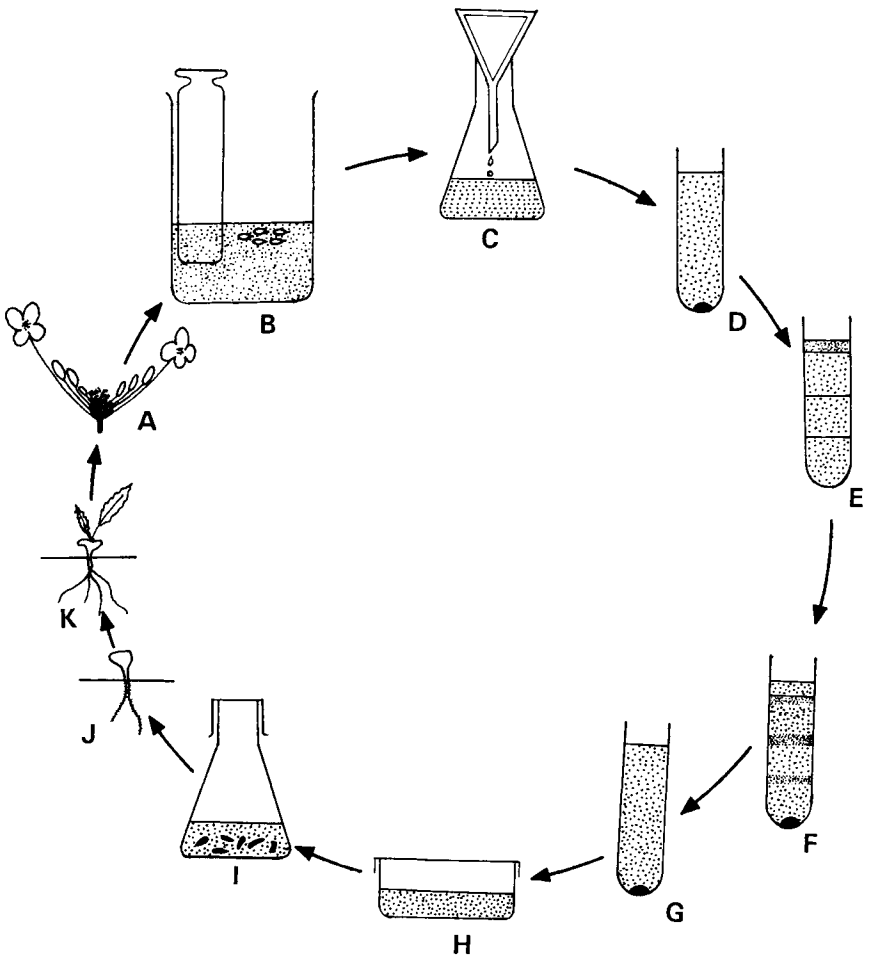


Fig. 7.4. Summary diagram of a protocol for isolated pollen culture of *Brassica napus*. The surface sterilized buds (A) of suitable size are crushed to release the pollen grains in B_5 medium containing 13% sucrose (B_5-13) in a glass homogenizer (B) and the medium is filtered through 42 μm nylon mesh to remove large debris (C). The filtrate is centrifuged at 1000 rev. min^{-1} for 3 min (D) and, after discarding the supernatant solution, the pellet is suspended in the B_5-13 medium and gently loaded on the 24%/32%/40% Percoll gradient solution (E) and centrifuged at 1000 rev. min^{-1} for 5 min. The two upper layers (F) are pipetted out and mixed with the B_5-13 medium. The suspension is again centrifuged at 1000 rev. min^{-1} for 5 min (G) and the supernatant medium is pipetted out and the pollen grains are suspended in NLN medium adjusting the plating density of the pollen grains to 2–5 $\times$ 10^4 ml^{-1} (for composition see Appendix 7.I). The suspension is plated as thin layer in petri plates (H) and incubated in the dark at 32°C for 3–5 days and then at 25°C. The regenerated tissue/embryos are transferred to 18 ml of hormone-free NLN medium in conical flasks (I) maintained on a shaking machine at 60 rev. min^{-1} at 32°C. Finally, the mature embryos are transferred to solidified B_5 medium containing 2% sucrose for germination (J,K). Fertile plants can be obtained by diploidization of pollen plants (after Ohkawa, 1988).

Exposure of the donor plants to stresses, such as nutrient stress (Sunderland, 1978) and water stress (Wilson et al., 1978) are also reported to promote androgenesis. Treating the donor plants with the substances which interfere with the normal development of pollen grains, such as the feminizing agents (etherel, auxin, antigibberellin) and gametocidal compounds, improves the yield of embryogenic grains (Heberle-Bors, 1985). Wang et al. (1974) observed that the inflorescences of *Oryza sativa* treated with etherel for 48 h at 10°C provided more responsive anthers than the untreated controls. In *B. juncea* etherel treatment of the donor plants enhanced the response from 5 to 13% (Agarwal and Bhojwani, 1993). In wheat, Picard et al. (1987) recorded 10–20% increase in the production of androgenic haploids and homozygous diploids and acceleration of embryo development by 8 days when the donor plants were treated with the gametocidal compound, Feridazone-potassium (Rohm & Hess Co.).

In *B. napus*, growing the donor plants under comparatively lower temperatures improved the yield of pollen embryos. Keller and Stringam (1978) observed that the yield of pollen embryos per 1000 cultured anthers from plants grown at day/night temperatures of 15/10, 20/15 and 25/20°C was 979, 579 and 267, respectively.

7.3.2. Stage of pollen development

Selection of appropriate age of pollen grains is very critical in the induction of androgenesis. Generally, the pollen grains around the first mitosis are most responsive. The anthers of *Datura innoxia*, *Nicotiana tabacum* and *Paeonia hybrida* gave best response when the pollen were just before, at or just after the pollen mitosis. The early bicellular stage of pollen is best for *Atropa belladonna* and *Nicotiana sylvestris* and absolutely necessary for *N. knightiana*. The pollen of rice (Raghavan, 1990) and most *Brassica* species (Leelavathi et al., 1984; Sharma and Bhojwani, 1985; Dunwell et al., 1985) are most vulnerable for embryogenic division at the late uninucleate stage.

Kott et al. (1988) have reported that in *B. napus* binucleate pollen grains are not only non-embryogenic but can also suppress embryo formation by the embryogenic, uninucleate grains by secreting hydrophilic, heat stable toxins. The toxins produced by the binucleate pollen also induced abnormalities in the developing pollen embryos. Therefore, in anther and pollen cultures the presence of some binucleate grains could be detrimental. The younger grains do not produce toxins. However, according to a recent study by Takahata et al. (1993), in *B. oleracea* a pollen population with mean nucleus number per pollen of 1.68 (50–80% bicellular grains) gave maximum yield of pollen embryos.

Generally, bud size is used as an index of the pollen stage. However, it is important to note that the size of the bud enclosing pollen at the optimum developmental stage may vary with the growing conditions and the age of the plant (Takahata et al., 1993). In this regard pollen culture has the advantage that from a heterogeneous population the pollen grains of the undesirable stage can be excluded through gradient centrifugation before culture (see Section 7.2.2).

The stage of pollen development at culture may also affect the ploidy level of the pollen plants. In anther cultures of *Datura innoxia* (Engvild et al., 1972) and *Petunia sp.* (Engvild, 1973; Raquin and Pilet, 1972) while uninucleate microspores produced mainly haploids the binucleate pollen formed plants of higher ploidy.

7.3.3. Anther wall factor(s)

There is ample evidence suggesting that the anther wall plays an important role in pollen-embryo development (Wernicke and Kohlenbach, 1977; Sunderland, 1978; Weatherhead and Henshaw, 1979). Pelletier and Ilami (1972) conducted a series of transplantation experiments and demonstrated that pollen from one cultivar of tobacco would successfully develop into an embryo even if transferred into the anthers of another cultivar. This work introduced the concept of 'wall factor'. Subsequently, nursing effects of whole anthers for androgenic development of isolated pollen of the same species (Sharp et al., 1972) as well as of different species (Pelletier and Durran, 1972) were reported. Even the extract of anthers stimulated pollen-embryo production (Debergh and Nitsch, 1973). Media conditioned by growing barley anthers or ovary for 7 days considerably increased the androgenic response in barley float anther cultures (Xu et al., 1981). The role of anther wall factor(s) in pollen embryogenesis is also suggested by the histological studies of cultured anthers (Haccius and Bhandari, 1975; Raghavan, 1978).

7.3.4. Genotype

The androgenic response is greatly influenced by the genotype of the donor plant. Generally, hybrids are more androgenic than their parents. Irikura (1975) reported that out of 118 clones and nine interspecific hybrids belonging to 46 species of *Solanum* genus only 46 species and 4 hybrids produced plants of pollen origin. Genotypic variation now seems to be of wide occurrence (Wenzel et al., 1977; Jacobsen and Sopory, 1978; Bhojwani and Sharma, 1991).

The observed intraspecific variation is often so great that while some lines of a species exhibit good androgenesis others are extremely poor performers or completely non-responsive (Jacobsen and Sopory, 1978; Schaeffer et al., 1979; Wenzel and Uhrig, 1981; Lazar et al., 1984; Datta and Wenzel, 1987; Keller and Armstrong, 1983; Orton and Browers, 1985; Swanson et al., 1987). In general, japonica rice is more responsive than indica rice (Cho and Zapata, 1990). Similarly, among the crop brassicas, *B. napus* seems to be most responsive and *B. juncea* the least.

Ockendon (1985) compared the androgenic responses of seven F_1 hybrid genotypes of Brussel's sprouts (*Brassica oleracea* cv *gemmifera*) and classified them into three categories: two were highly responsive (up to 180 and 376 embryos per 100 anthers cultured), one was moderately responsive (with up to 53 embryos) and four were virtually non-responsive. An important point made in this paper, which may be true for most *Brassica* species, is the inconsistency in the response of various genotypes, so that the genotypes expected to give good results in fact give very poor or zero response in some experiments. For example, in one experiment the cv 'Grower' yielded 28.4% embryogenic anthers with 110 embryos per 100 anthers cultured but in another experiment these values were 1.4 and 4.7, respectively. In such cases the genotypic variability should be concluded by pooling results of several experiments, each with large numbers of replicates. This intravarietal variation could be due to a complex interaction between plant genotype and the environment in which they are raised. For example, the age and growth conditions of the plants can cause a significant change in the bud length at which pollen are at the optimal developmental stage (Thurling and Chay, 1984; Takahata et al., 1993).

In nature haploidy is controlled by a single gene (hap) called the 'haploid inhibitor gene' (Kasha and Sequin-Swartz, 1983). There is sufficient evidence to suggest that in vitro androgenesis is also under genetic control, and this trait can be transferred from responsive clones to originally non-responsive clones. By intercrossing poorly responding clones of *Solanum tuberosum* (H^2236, H^2258 and H^2439) Jacobsen and Sopory (1978) could produce a line (H^3703) which showed better response than either of the parents. Following a similar approach, Foroughi-Wehr et al. (1982) improved the androgenic response of originally poorly responding lines of *Hordeum vulgare*.

In the dioecious *Melandrium*, which shows chromosomal basis of sex determination, only the pollen with X chromosome are competent to form pollen plants. Wu et al. (1990) observed that all the androgenic plants of this plant were phenotypically and cytologically female. In tetraploid *Melandrium* even a single Y chromosome is able to suppress the effect of three X chromosomes.

7.3.5. Pre-treatment of cultured anthers/pollen grains

Application of certain physical and chemical treatments to cultured anthers or pollen grains, prior to their transfer to standard culture room conditions, has proved essential or promotory for in vitro androgenesis.

(a) *Temperature shock.* In many species the incubation of anther/pollen cultures at a low temperature (4–5°C) for various periods before shifting them to 25°C enhanced the androgenic response (see Bhojwani and Sharma, 1991). For example, in *Nicotiana tabacum* up to 58% of the anthers yielded embryos if the buds were pre-treated at 5°C for 72 h as against 21% anthers from buds maintained at 22°C for the same period (Nitsch, 1974). Sunderland and Roberts (1979) reported that in *N. tabacum* var. White Burley pre-treatment of buds at 7–9°C is more effective than that at 5°C. Moreover, the optimum duration of cold treatment may change with the temperature (7 or 9°C) and the stage of pollen development. In rice the highest frequency of pollen callusing occurred when the excised panicles were treated at 13°C for 10–14 days (Genovesi and Magill, 1979). The optimum treatment for *Hyoscyamus niger* is storing the buds, at or just after pollen mitosis, at 15°C for 5 days (Sunderland and Wildon, 1979). For *Secale cereale* storage of spikes at 6°C for 6–10 days prior to anther culture has been described as optimal (Wenzel et al., 1977).

In some plants, such as *Capsicum* (Dumas de Vaulx et al., 1982), oat (Rines, 1983), and some genotypes of wheat (Hu, 1985; Li et al., 1988) an initial high temperature shock has proved beneficial. A high temperature shock (30–35°C) for the initial 1–4 days of culture is essential to induce androgenesis in most *Brassica* species (Keller and Armstrong, 1979, 1983; Klimaszweska and Keller, 1983; Sharma and Bhojwani, 1985). Hamaoka et al. (1991) observed that the efficiency of pollen embryogenesis in anther cultures of *B. campestris* treated at 35°C for 24 h was 20 times higher than the untreated controls. Pechan et al. (1991) and Fabijanski et al. (1991) have shown the appearance of some high molecular weight proteins in the pollen grains of *B. oleracea* and *B. napus* during heat treatment which may be associated with the induction of androgenesis.

(b) *Centrifugation.* In *Datura innoxia* the centrifugation of anthers at 40 g for 5 min after cold treatment of buds at 3°C for 48 h improved the percentage of androgenic anthers (Sangwan-Norreel, 1977). The promotion was especially striking (60%) in the cultures of anthers at the early binucleate stage; in the cultures at the late uninucleate stage the en-

hancement over the control was only 7%. Similarly, in the cultures of pollen grains isolated from cold treated buds centrifugation at 120 g for 15 min not only increased the number of pollen embryos but also brought about rapid and synchronous development of embryos.

One of the reasons for substantially high androgenic response in pollen culture than in anther culture of *B. napus* (Aslam et al., 1990) could be centrifugation which is required to obtain clean pollen preparation for culture. To test this hypothesis, Aslam et al. (1990) compared the androgenic response of anther cultures from untreated buds and those from buds centrifuged at 400 g for 4.8 or 12 h or at 280 g for 5 or 10 min. All centrifugation treatments markedly improved the embryo yield over the control. With the optimum treatment (400 g for 8 min) the mean number of embryos per anther was 9.5 as compared to 0.2 in the untreated control.

(c) *γ-Irradiation*. Judicious application of γ-irradiation to anthers before culture has been reported to promote pollen callusing and pollen embryogenesis in *Nicotiana* and *Datura* (Sangwan and Sangwan, 1986), wheat (Wang and Yu, 1984; Yin et al., 1988b), rice (Yin et al., 1988a) and *B. napus* (MacDonald et al., 1988). In wheat-irradiation at 1, 3 and 5 Gys quadrupled the yield of pollen embryos and enhanced the frequency of regeneration of green plants and the production of doubled haploids (Ling et al., 1991). Irradiation induced pollen embryogenesis in otherwise non-amenable genotypes of wheat.

Low doses (10 Gy) of irradiation greatly enhanced anther culture efficiency (number of responding anthers) in two cultivars of *B. napus* ssp *oleifera* (MacDonald et al., 1988). In the cv Ariana, irradiation of young buds (2.6–3 mm) doubled or tripled the frequency of responsive anthers and almost quadrupled the number of embryos per 1000 plated anthers. Even old buds (3.1–3.5 mm), which normally do not exhibit androgenesis, became responsive after γ-irradiation. For the other cultivar (Primor) γ-irradiation was detrimental when applied to young buds but proved promotory in the case of older buds. γ-Irradiation also caused a decline of pollen embryogenesis in isolated pollen cultures.

Irradiation is known to inactivate nuclei (Zelcer et al., 1978) and alter the levels of auxins and cytokinins in the tissues (Degani and Pickholtz, 1982). These actions may be involved in the promotion of androgenesis by low irradiation in some systems.

7.3.6. Culture medium

Normally, only two mitotic divisions occur in a microspore but androgenesis involves repeated divisions. A variety of treatments are known to

induce additional divisions in the pollen grains. In field-grown plants of wheat sprayed with etherel (2-chloroethylphosphonic acid), Bennett and Hughes (1972) recorded multicellular grains. Incorporation of this compound in the nutrient medium was later shown to enhance the androgenic response in anther cultures of tobacco (Bajaj et al., 1977). Multiple divisions in the pollen grains of datura and tobacco can be induced by simply excising the anthers and placing them in a humid atmosphere (Pelletier and Ilami, 1972) or planting them on agar-sucrose plates (Nitsch, 1969; Sunderland, 1974).

That sucrose is essential for androgenesis was first demonstrated by Nitsch (1969) for tobacco and later by Sunderland (1974) for *Datura innoxia*. Although Sharp et al. (1971) claimed that in tobacco pollen embryos could develop in the complete absence of sucrose, this observation could not be confirmed by Dunwell (see Sunderland, 1974). Sucrose is included in all the anther culture media and is generally used at a concentration of 2–4%. For wheat, however, Ouyang et al. (1973) found that 6% sucrose promoted pollen callusing and inhibited the proliferation of somatic tissues. Similarly, for potato 6% sucrose proved distinctly superior to 2% or 4% sucrose in terms of the number of anthers forming pollen embryos (Sopory et al., 1978). All *Brassica* species require 12–13% sucrose for androgenesis in anther and pollen cultures. According to Dunwell and Thurling (1985), high sucrose concentration favours better survival of pollen grains, thus improving the frequency of androgenesis in *B. napus*. Last and Brettell (1990) have reported that most of the cultures of wheat showed higher androgenic responses when sucrose was substituted by maltose in the medium.

Although androgenic development of pollen grains in *Nicotiana tabacum* and *Datura innoxia* can be induced on agar plates containing only sucrose, on such a simple medium the development proceeds only up to the globular stage. For further development of the embryos mineral salts are required. Possibly, the nutrients and growth factors necessary for the induction and early development of the androgenic embryos are supplied by the anther wall or pollen itself.

The available literature does not allow recommendation of an anther culture medium of general applicability. The requirements vary with the genotype and, probably, the age of the anther and the conditions under which the donor plants are grown. Unlike tobacco and datura, most species exhibit androgenesis on a complete nutrient medium (mineral salts, vitamins and sucrose), with or without growth substances. The basal media commonly used are the formulations recommended by Nitsch (1969) or Murashige and Skoog (1962). Some other media specially formulated for anther or pollen culture are listed in Table 7.2. Nitsch (1972)

TABLE 7.2

Composition of some of the media used for anther and pollen culture (unless mentioned otherwise, all concentrations are in mg l^{-1})

Constituents	Media					
	N&N[a]	N_6[b]	Potato[c]	Nitsch[d]	KA[e]	NLN[f]
KNO_3	950	2830	1000	950	2500	125
NH_4NO_3	725	–	–	725	–	–
$NaH_2PO_4 \cdot 2H_2O$	–	–	–	–	150	–
KH_2PO_4	68	400	200	68	–	125
$(NH_4)_2SO_4$	–	463	100	–	134	–
$MgSO_4 \cdot 7H_2O$	185	185	125	185	250	125
$CaCl_2 \cdot 2H_2O$	166	166	–	166	750	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	100	–	–	500
KCl	–	–	35	–	–	–
$FeSO_4 \cdot 7H_2O$	27.8	27.8	–	27.8	–	27.8
Na_2·EDTA	37.3	37.3	–	37.3	–	37.3
Fe·EDTA	–	–	37	–	–	–
Sequestrene 330Fe	–	–	–	–	40	–
KI	–	0.8	–	–	0.75	–
H_3BO_3	10	1.6	–	–	3	10
$MnSO_4 \cdot 4H_2O$	25	4.4	–	–	–	25
$MnSO_4 \cdot H_2O$	–	–	–	–	10	–
$ZnSO_4 \cdot 7H_2O$	10	1.5	–	–	2	10
$Na_2MoO_4 \cdot 2H_2O$	0.25	–	–	–	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	–	–	–	0.025	0.025
$CoCl_2 \cdot 6H_2O$	–	–	–	–	0.025	0.025
myo-Inositol	100	–	–	4502	100	100
Thiamine·HCl	0.5	1	1	–	10	0.5
Pyridoxine·HCl	0.5	0.5	–	–	1	0.5
Nicotinic acid	5	0.5	–	–	1	0.5
Glycine	2	2	–	–	–	2
L-Glutamine	–	–	–	730	800	800
Glutathione	–	–	–	–	–	30
L-Serine	–	–	–	105	100	100
Folic acid	5	–	–	–	–	0.5
Biotin	0.5	–	–	–	–	0.5
NAA	–	–	–	–	0.1	0.5
2,4–D	–	2	1.5	–	0.1	–
BAP	–	–	–	–	–	0.05
Kinetin	–	0.5	0.5	–	–	–
Potato extract %	–	–	–	10[g]	–	–
Sucrose %	2	5–12	9–10	2	10	13
Agar %	0.8	0.8	0.55–0.7	–	0.8	–

[a]Nitsch and Nitsch (1969) Science 163: 389–304 (tobacco anther culture).
[b]Chu (1978) Proc. Symp. Plant Tissue Culture, Peking, pp. 45–50 (cereal anther culture).

TABLE 7.2 (*continued*)

[c]Chuang et al. (1978) Proc. Symp. Plant Tissue Culture, Peking, pp. 51–56 (wheat anther culture).
[d]Nitsch (1977) Fundamental and Applied Aspects of Plant Cell, Tissue and Organ Culture. Springer, pp. 268–278 (isolated pollen culture of tobacco).
[e]Keller and Armstrong (1977) Can. J. Bot. 55: 1383–1388 (*Brassica* anther culture).
[f]Polsoni et al. (1988) Can. J. Bot. 66: 1681–1685 (isolated pollen culture of *Brassica*).
[g]100 g diced potato tuber boiled in distilled water for 25–30 min and then strained and filtered.

had shown that of the various minerals iron is crucial for pollen-embryo development in tobacco. In media lacking in iron (Nitsch, 1972) or containing it below the threshold concentration of 40 μM Fe·EDTA (Vagera et al., 1979), the embryo development was arrested at the globular stage. Iron proved most effective when supplied during the 2nd and 3rd weeks of anther culture, confirming its requirement for post-inductive development of the pollen embryos. A chelated form of iron, such as Fe·EDTA (Nitsch, 1969) and Fe·EDDHA (Rashid and Street, 1973) is more effective than ferric citrate.

In China considerable work has been done to develop media that would favour the formation of green haploid plants in anther cultures of cereals at a high frequency. Low inorganic nitrogen, particularly ammonium, in the medium is reported to promote androgenesis (Clapham, 1973, Chu et al., 1975) and the yield of green plants (Olesen et al., 1988a) in some cereals. Ten times reduction in NH_4NO_3 concentration in LS medium substantially enhanced the overall androgenic response in *Lolium perenne* and *L. multiflorum* (Bante et al., 1990). Even KNO_3 in the medium was inhibitory for embryogenesis. Halving the concentration of KNO_3 in MS basal medium remarkably enhanced pollen embryogenesis in *Hevea brasiliensis* (Chen, 1990a). A potato medium, with the 6 major salts at reduced concentration, iron (no minor salts), thiamine and 10% potato extracts, besides growth regulators and sucrose (see Table 7.2) gave considerably higher numbers of green plants in anther cultures of wheat compared to N_6 medium (Chuang et al., 1978). The media used for isolated pollen culture are generally very low in overall salt concentration (see Table 7.2).

In the solanaceous plants, where androgenesis occurs via direct pollen embryo formation, as in several species of *Nicotiana* and *Datura* and *Atropa belladonna*, the presence of a growth adjuvant is generally not required, and full haploid plants are formed on basal media. In some cases even vitamins are not essential. However, most *Brassica* species require an auxin and a cytokinin for direct pollen embryogenesis. For the major-

ity of non-solanaceous species known to exhibit androgenesis via a callus phase it is essential to fortify the medium with growth regulators, complex nutrient mixtures (yeast extract, casein hydrolysate, potato extract, coconut milk), either alone or in different combinations.

Sometimes it may be possible to change the mode of androgenic development by a judicious change of growth adjutants in the medium (Raghavan, 1978). For example, in *Triticum aestivum* callusing of pollen occurs if the medium contains 2,4-D and lactalbumin hydrolysate, but if the basal medium is supplemented with coconut milk pollen grains directly develop into embryos (Ouyang et al., 1973, cited in Clapham, 1977).

The addition of ascorbic acid and glutathione, and glucose in place of sucrose in the basal medium has proved stimulatory for androgenesis in rye (Wenzel et al., 1977). Incorporation of activated charcoal (AC) into the nutrient medium also stimulated androgenesis in some systems (Anagnostakis, 1974; Bajaj et al., 1977), presumably by removing the growth inhibitors from the medium (Keller and Stringam, 1978; Kohlenbach and Wernicke, 1978; Weatherhead et al., 1979). In tobacco, at the optimal concentration of 2%, it increased the frequency of cultured anthers forming plants from 41% (without AC) to 91% (Bajaj et al., 1977). For potato optimal concentration of AC turned out to be 0.5%; higher concentrations (0.75%, 1%) were not as good (Sopory et al., 1978). Addition of 100 g l^{-1} of Ficoll (type 400) has been reported to significantly enhance the formation of green pollen plants in anther cultures of wheat (Devaux, 1992).

7.3.7. Culture density

The culture density is a critical factor in isolated pollen culture, as also in single cell and protoplast culture. Huang et al. (1990) made a detailed study of the effect of culture density on embryogenesis in pollen cultures of *B. napus*. According to this report the minimum density required for embryogenesis is 3000 pollen ml^{-1} of the culture medium but highest embryo yield was obtained at 10 000–40 000 pollen ml^{-1}. This high plating density is crucial only for the initial couple of days. Dilution of the density from 30 000 to 40 000 to 1000 pollen ml^{-1} after 2 days of culture did not reduce the embryogenic frequency. The media conditioned by growing pollen at high densities (30 000–40 000 ml^{-1}) for 2 days stimulated embryogenesis in pollen cultures at low plating densities (3000–10 000 pollen ml^{-1}).

Arnison et al. (1990) reported the effect of culture density in anther cultures of *B. oleracea*. The frequency of pollen embryogenesis was enhanced if the anther culture density was increased from 3 anthers per

4 ml to 12–24 anthers per ml of the medium. However, this observation is contradictory to the findings of Cardy (1986), according to which in *B. napus* the response was better at low density (2 anthers ml^{-1}).

7.3.8. Effect of gaseous environment

Effect of gaseous environment on anther culture has been rarely investigated. Horner et al. (1977) reported ethylene production in anther cultures of *Nicotiana tabacum*. Dunwell (1979) demonstrated that the composition of the gas mixture that surrounds the anthers has profound influence on the number of embryos produced in anther cultures of *N. tabacum*. The removal of CO_2 from the culture vessel by KOH absorption resulted in a decline in anther culture response. Incubation of *Lolium perenne* and *L. multiflorum* anthers at elevated concentration of CO_2 (2%) almost doubled their androgenic response (Bante et al., 1990), possibly CO_2 inhibited the action of ethylene (Kang et al., 1967). The inhibitors of ethylene production, such as $AgNO_3$ and n-propysallate, promoted pollen embryogenesis in anther cultures of Brussel's sprouts (Biddington et al., 1988) and potato (Tiainen, 1992).

7.3.9. Effect of light

Light does not seem to be necessary for the induction of androgenesis. For pollen culture of *Datura innoxia* (Sangwan-Norreel, 1977), *Nicotiana tabacum* (Sunderland and Roberts, 1977) and *Annona squamosa* (Nair et al., 1983) an initial incubation of cultures in dark followed by diffuse light was found to be suitable. Isolated pollen cultures are more sensitive to light than anther cultures (Nitsch, 1977).

In *Brassica juncea* (Sharma and Bhojwani, 1989) and *Hordeum vulgare* (Xu, 1990) species light is detrimental even for anther cultures.

7.4. ONTOGENY OF ANDROGENIC HAPLOIDS

In the normal course of pollen development the uninucleate microspores undergo an asymmetric division, cutting a small generative cell and a large vegetative cell. The former is initially attached to the intine (inner wall of the pollen grain) but eventually it comes to lie freely in the cytoplasm of the vegetative cell. Whereas the generative cell divides further, forming sperms, the vegetative cell remains quiescent (see Fig. 7.5) (Bhojwani and Bhatnagar, 1990).

In *Datura* (Sunderland, 1974) and *Nicotiana tabacum* (Nitsch and Nitsch, 1969) simply detaching the anthers from the flower buds and

placing them on sucrose-agar plates induces an altogether different mode of development in the microspores. Numerous cell divisions occur within the original wall of the pollen grain, and a multicellular structure is formed. If suitable culture conditions are provided these tissues further develop into androgenic sporophytes.

7.4.1. Induction

For most species a suitable stage for the induction of androgenesis lies between just before to just after pollen mitosis. During this phase of development the cells are non-committal in their developmental potential because most of the sporophyte-specific gene products are eliminated from the cytoplasm before meiosis (Porter et al., 1984) and the gametophyte-specific genes are generally transcribed after pollen mitosis (Scott et al., 1991). After the first mitotic division the cytoplasm gets populated with gametophytic information and it gradually becomes irreversibly programmed to form male gametophyte. This stage is reached within 24 h after pollen mitosis (Mascarenhas, 1971).

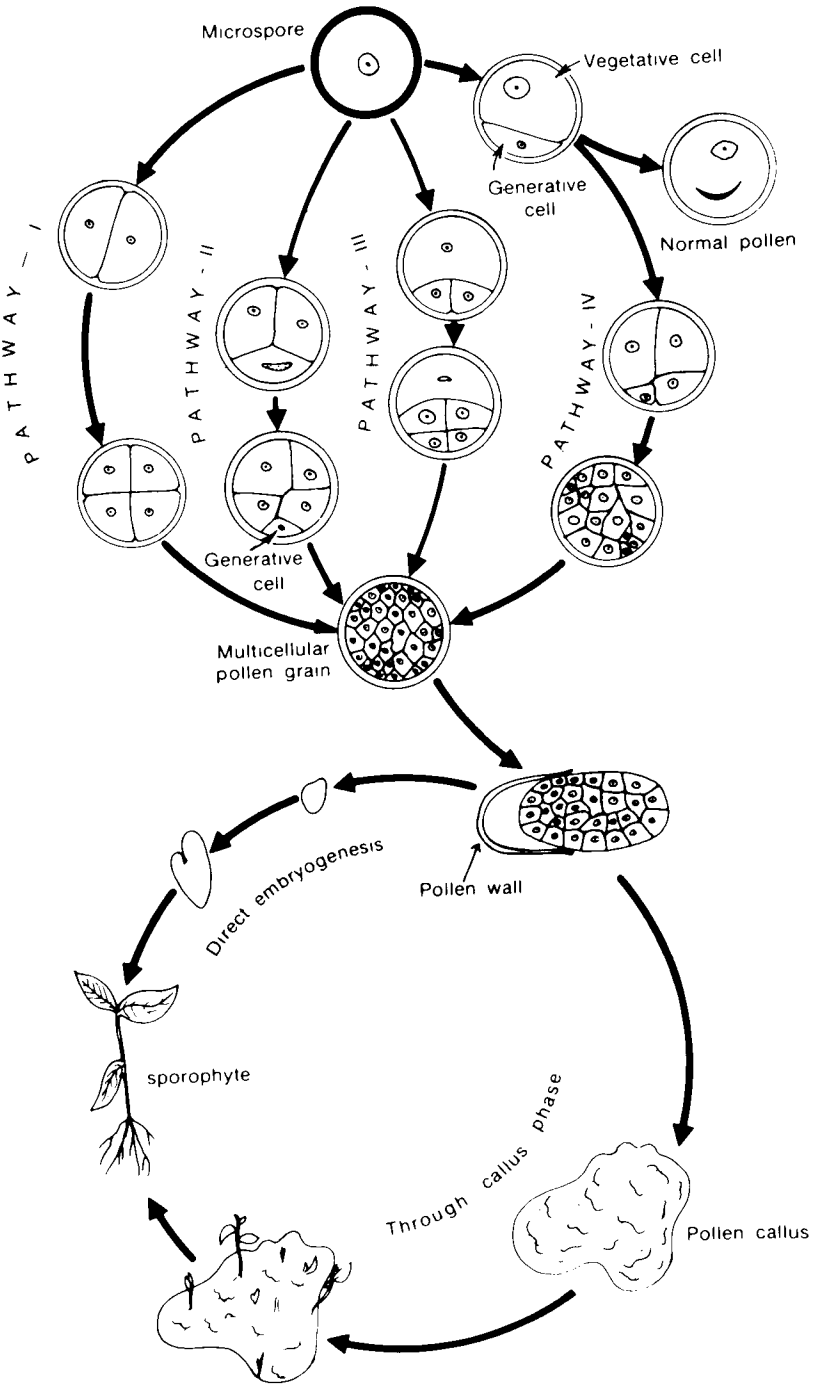
A variety of stresses applied during the labile developmental period of the pollen grain can mask the gametophytic programme and induce the expression of sporophyte-specific genes and, thus, induce the grains to switchover from gametophytic mode to sporophytic mode of development. In some cases the stress of excising the anthers and placing them under culture conditions is adequate to bring about this shift. In others, treatments such as temperature shocks (high or low), high osmolarity, and starving the grains of sugar or other nutrients are required to induce or promote the induction of androgenesis.

Aruga et al. (1985) reported that placing the anthers of *Nicotiana tabacum* in sugar-free medium for a few days immediately after culture suppressed gametogenesis and induced androgenesis. Sugar-starvation caused a loss of the ability of the generative cell nucleus to synthesize DNA even after transfer to sugar-containing medium. However, the vegetative cell nucleus exhibited the unique potentiality to synthesize DNA even in the absence of sucrose (normally the vegetative nucleus remains arrested in the G_1 phase of the cell cycle). Maintaining the anthers in sugar-free medium for 6 days gave maximum androgenic response upon transfer to complete medium. Induction of androgenesis by sugar-starvation has also been observed in microspore culture of *Hordeum vulgare* (Wei et al., 1986). Similarly, while direct culture of tobacco pollen, at the mid bi-nucleate stage, on basal medium containing glutamine favoured gametophytic development leading to the formation of mature pollen, glutamine-starvation of the pollen for the initial 24–48 h favoured

androgenic development on glutamine-containing medium (Kyo and Harada, 1985, 1986). During glutamine-starvation some new phosphoproteins appeared, and ATP level and the amount and rate of protein synthesis rapidly decreased. Zarsky et al. (1990) have suggested that the starvation of pollen grains before exposing them to full nutrient medium probably causes repression of the gametophytic cytoplasm and differentiation of vegetative cell pushing it from G_1 to S phase of cell cycle which is required for embryogenic pathway of pollen development.

The cytology of pollen embryogenesis has been studied chiefly in *Nicotiana tabacum* and *Brassica napus*. In *N. tabacum*, where androgenic embryos arise from the vegetative cell, for some time after pollen mitosis all the pollen grains develop similarly: increase in size, achieve higher stainability with acetocarmine and accumulate starch grains in the vegetative cell. Whereas most of the grains continue to gain further in these features up to maturity, a small proportion of the pollen (ca. 0.7%), called 'S-grains' or 'P-grains' or simply 'androgenic grains', do not increase much in size, their cytoplasm remains faintly staining, and their nuclei clearer. The occurrence of two structural types of grains in an anther is described as 'pollen dimorphism'. Normally the embryogenic grains would be non-functional male gametophytes but given appropriate conditions they form embryos (Horner and Street, 1978; Horner and Mott, 1979; Heberle-Bors, 1985). Pollen dimorphism has also been observed in the cereals, wheat (Zhou, 1980), rice (Cho and Zapata, 1990) and barley (Dale, 1975). The frequency of embryogenic grains can be increased above the level of its natural occurrence by dedifferentiation of the gametophytically programmed grains under inductive conditions (Bhojwani et al., 1973; Dunwell, 1978; Heberle-Bors, 1983). During dedifferentiation lysosome-like multivesicular bodies appear which may be involved in the breakdown of the cytoplasm. Towards the end of the inductive period, in terms of cell organelles, only a few mitochondria and structurally simplified plastids are left in the vegetative cell (Dunwell, 1978). The ribosomes are completely washed out. Following the first sporophytic division a fresh population of ribosomes and other organelles appears. In *Hyoscyamus niger* where the induction of pollen embryo development occurs within a couple of hours after anther culture, fresh RNA synthesis is noticeable as early as the first hour of culture (Raghavan, 1979).

In *Brassica napus*, which belongs to androgenic pathway I (Fig. 7.5), and where late uninucleate stage of pollen development is most labile to the induction of embryogenesis, the induction of androgenic grains occurs within 8 h of initiation of microspore culture and precedes the first embryogenic nuclear division (Pechan et al., 1991). The first cytological changes associated with the switch to sporophytic development is the


Microspore
Vegetative cell
Generative cell
Normal pollen
PATHWAY - I
PATHWAY - II
PATHWAY - III
PATHWAY - IV
Generative cell
Multicellular pollen grain
Pollen wall
Direct embryogenesis
sporophyte
Through callus phase
Pollen callus

loss of vacuole, the movement of the asymmetrically placed nucleus to the central position due to apparent loss of microtubule cytoskeleton investing the nucleus, and the appearance of starch containing plastids and globular domain within the cytoplasm (Zaki and Dickinson, 1990). A 12 h pulse treatment of pollen grains with 25 g l^{-1} of colchicine, an antimicrotubule drug, before the first mitosis significantly increased the number of grains undergoing symmetrical division and the production of pollen embryos in isolated pollen cultures (Zaki and Dickinson, 1991).

Before the dedifferentiated cell undergoes the first androgenic division it synthesizes a thick somatic cell wall around the plasma membrane giving it a sporophytic characteristic (Sangwan-Norreel, 1978; Rashid et al., 1982; Zaki and Dickinson, 1990). The first division is clearly a somatic type division in which a normal cell plate is formed which is traversed by plasmodesmata and contains no callose (Zaki and Dickinson, 1990).

7.4.2. Early segmentation of microspores

Based on the few initial divisions in the microspores four modes of in vitro androgenesis have been identified (see Fig. 7.5).

(i) *Pathway I.* The microspores divide by an equal division, and the two identical daughter cells contribute to the sporophyte development. In this pathway distinct vegetative and generative cells are not formed. This pathway has been commonly observed in *Brassica napus* (Zaki and Dickinson, 1992).

(ii) *Pathway II.* The uninucleate microspores divide by a normal unequal division, and the sporophytes arise through further divisions in the vegetative cell. This mode of development is commonly encountered in *Nicotiana tabacum* (Sunderland and Wicks, 1971; Horner and Street, 1978), *Hordeum vulgare* (Clapham, 1971), *Triticum aestivum* (Ouyang et al., 1973), *Triticale* (Wang et al., 1973) and *Capsicum annuum* (Kuo et al., 1973).

Fig. 7.5. Diagram showing the origin of sporophytes from pollen grains in anther cultures. A microspore may follow any of the four pathways to form multicellular pollen grains. The latter may directly form embryo or produce sporophytes through a callus phase (modified from Bhojwani and Bhatnagar, 1990).

(iii) *Pathway III.* In *Hyoscyamus niger* the pollen embryos are predominantly formed from the generative cell alone. In such cases the vegetative cell either does not divide at all or does so only to a limited extent. In either case it forms a suspensor-like structure at the radicular end of the generative cell-derived embryo proper (Raghavan, 1976a, 1978).

(iv) *Pathway IV.* As in pathway II, vegetative and generative cells are formed but in this case both the cells divide further and participate in the development of the sporophyte. This mode of androgenesis has been reported in *Datura innoxia* (Sunderland, 1974). However, Sunderland and Dunwell (1974) are of the opinion that this pathway might be operative in other plants which form high frequency non-haploids, such as *Datura metel* (Narayanaswamy and Chandy, 1971) and *Atropa belladonna* (Zenkteler, 1971).

7.4.3. Later development

Irrespective of the early pattern of divisions, the responsive pollen grains finally become multicellular and burst open to release the tissue which has an irregular outline. In several cases (e.g. *Atropa, Brassica, Datura, Hyoscyamus, Nicotiana*) this cellular mass gradually assumes the form of a globular embryo and undergoes the normal stages of post-globular embryogeny (heart-shaped, torpedo-shaped, and cotyledonary stage; see Fig. 7.5). By this method a pollen grain forms only one plant. However, in several species where androgenic sporophytes have been obtained through anther culture (*Arabidopsis, Asparagus, Triticale*) the multicellular mass liberated from the bursting pollen grain undergoes further proliferation and forms a callus which may later differentiate plants on the same medium or on a modified medium. In some plants, such as *Oryza sativa*, by varying the composition of the medium androgenic plants can be obtained via embryo formation (Guha et al., 1970) or through callusing (Niizeki and Oono, 1968). This is also possible in *Hyoscyamus niger* (Raghavan, 1978).

Generally, the pollen embryos have been described as lying freely in the anther locule but Nitsch (1969) observed that in tobacco at least some young embryos are attached to the locular wall by a small suspensor-like structure. Using an improved technique of paraffin sectioning (see Bhandari, 1976), in which the pollen and pollen embryos are retained in their original position during processing of the material, Haccius and Bhandari (1975) made detailed ontogenetic studies on pollen embryos of tobacco. They observed that about 82% of the well developed pollen embryos had their basal end adhering to a supporting tissue (see Fig. 7.6A–

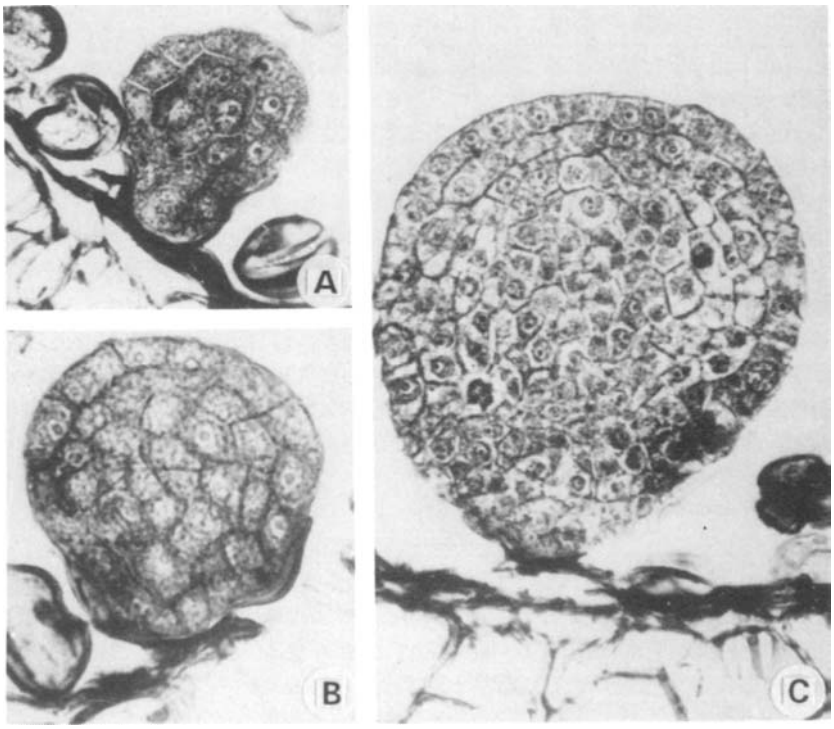


Fig. 7.6. Pollen embryos of *Nicotiana tabacum* at different stages of development. Note that the basal end of the embryos are attached to the anther wall (after Bhandari, 1976).

C), which could be the thecal wall, other embryos or clusters of dead pollen. This suggests that for establishment of initial polarity and for normal development of pollen embryos a temporary support may be essential. These investigators also observed that the site of the exine rupturing is so regulated as to expose the plumular end of the proembryonal mass. Further development of the globular embryos and differentiation of protoderm occurs only if the exine ruptures in such a way that the open end of the exine is not in direct contact with the supporting tissue (see Fig. 7.7).

7.5. PLANT REGENERATION FROM POLLEN EMBRYOS

Regeneration of plants from pollen callus or pollen embryos may occur on the original medium or it may require transfer to a different medium. The pollen embryos exhibit considerable similarity with zygotic embryos in their morphology and certain biochemical features (Holbrook et al., 1990). However, often the pollen embryos do not germinate normally. In

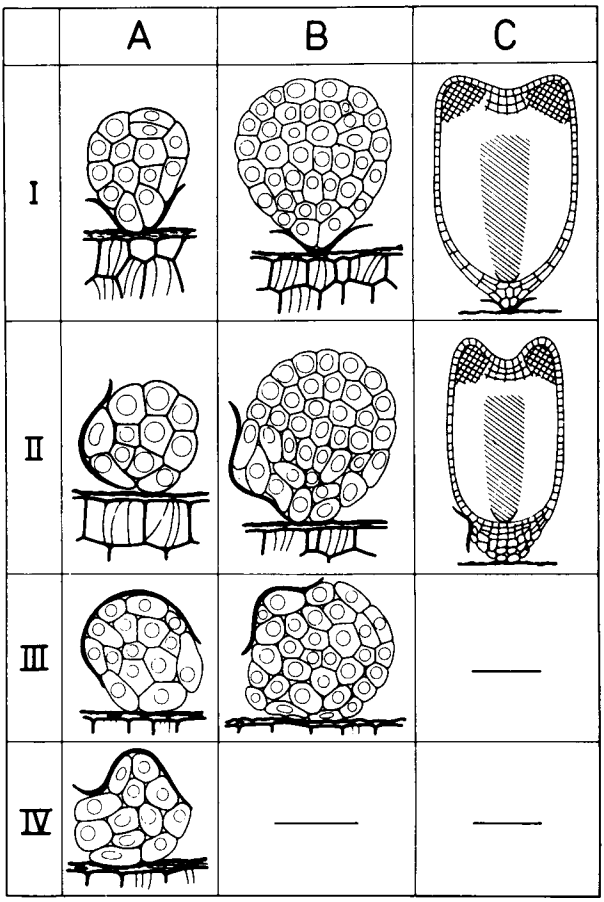


Fig. 7.7. Diagram to show the relationship between the place of exine bursting (A) and the fate of the pollen embryos (B,C) in *Nicotiana tabacum*. Mature embryos develop only if the exposed part of the proembryonal mass is away from the place of attachment to the supporting tissues (I,II) not otherwise (III,IV) (after Haccius and Bhandari, 1975).

Datura innoxia pollen embryos frequently produce secondary embryos on stem surface, and all such embryos which produce secondary embryos are haploid and the others non-haploids (Lenee et al., 1987). To raise full plants from pollen embryos it is necessary to excise a cluster of the secondary embryos along with a part of the parent embryo and plant them on fresh medium. They do not germinate if left on the pollen embryo or removed individually (Sangwan-Norreel, 1983).

In most of the *Brassica* species pollen embryos exhibit very poor germination (10–30%; Fig. 7.1B). On the germination medium the hypocotyl elongates, the cotyledons turn green, a primary root develops but the

plumule rarely produces a shoot. The recalcitrant embryos regenerate plants through adventitious shoot bud differentiation or secondary embryogenesis (Fig. 7.1C) from the epidermal cells of the hypocotyl (Loh and Ingram, 1982; Chuong and Beversdorf, 1985; Sharma and Bhojwani, 1989). ABA and cold treatment (at 4°C for 6–12 h) and partial desiccation of the embryos of *B. napus* promoted their germination (Kott and Beversdorf, 1990). The best medium for the germination of *Brassica* pollen embryos is to transfer the dicot embryos to B_5 + 0.1 mg l^{-1} GA_3 and expose them to 5°C for 10 days before transferring them to 25°C in light. With this protocol up to 90% germination of pollen embryos has been achieved in *B. napus* (Kott and Beversdorff, 1990). Swanson et al. (1987) reported that shaking of globular and heart-shaped embryos obtained from isolated pollen grains of *B. napus* dramatically improves the speed and synchrony of embryo development as well as the quality of the embryos produced. ABA and cold treatment also enhanced normal germination of pollen embryos of *B. juncea* (Agarwal and Bhojwani, 1993).

7.6. GYNOGENESIS

Development of plants from unfertilized cells of the female gametophyte (embryo sac), in floret, ovary or ovule culture is becoming an attractive alternative to anther culture for haploid production (Yang and Zhou, 1990). It was first reported in barley by San Noeum (1976). Since then gynogenetic haploids have been raised for 19 species belonging to 10 families (Table 7.3). Gynogenetic haploids mostly arise from unfertilized egg cell (parthenogenesis), as observed in barley (Huang et al., 1982), sunflower (Yang et al., 1986) and sugarbeet (Olesen et al., 1988b). However, in rice the gynogenic haploids arise through synergid apogamy (Zhou et al., 1986; He and Yang, 1988). The gynogenic plants may arise through direct embryogenesis, or the gametic cells may form a callus followed by plant regeneration on another medium. Although the available data on in vitro gynogenesis is not enough to make generalisations (Yang and Zhou, 1990) the key factors affecting it are summarised below:

7.6.1. Explant

Young flowers, ovaries or ovules have been used as the explant to produce gynogenic haploids. Generally, ovules attached to the placenta respond better than isolated ovules. In *Gerbera jamesonii*, so far, only isolated ovule culture has been successful. In *Helianthus annuus* ovule culture proved superior to ovary or floret culture (Yan et al., 1988). In sugerbeet ovule culture has been mostly used although Van Geyt et al.

TABLE 7.3

List of species for which gynogenetic haploids have been raised through ovary/ovule culture

Family	Species	Explant	Reference
Chenopodiaceae	*Beta vulgaris*	Ovule	Hosemans and Bossoutrot (1983); Bornman (1985); Lux et al. (1990)
Asteraceae	*Gerbera jamesonii*	Ovule	Sitban (1981); Cappadocia et al. (1988); Tosca et al. (1990)
	Helianthus annuus	Ovary, Ovule	Cai and Zhou (1984)
Brassicaceae	*Brassica oleracea*	Ovule	Dore (1989)
Cucurbitaceae	*Cucumis melo*	Ovule	Katoh et al. (1993)
Euphorbiaceae	*Hevea brasiliensis*	Ovule	Chen et al. (1990)
Poaceae	*Coix lacryma-jobi*	Ovary	Li and Zhang (1984)
	Hordeum vulgare	Ovary	San Noeum (1976); Gu and Zheng (1984)
	Oryza sativa	Ovary	Asselin de Beauville (1980)
	Triticum aestivum	Ovary	Zhu and Wu (1979)
	Zea mays	Ovary	Ao et al. (1982)
Liliaceae	*Allium cepa*	Ovule	Campion and Alloni (1990); Keller (1990)
	A. tuberosum	Ovary	Tian and Yang (1989)
	Lilium davidii	Ovary	Gu and Zheng (1983); Prakash and Giles (1986)
Moraceae	*Morus alba*	Ovary	Lakshmi Sita and Ravindran (1991)
Salicaceae	*Populus simonigra*	Ovary	Wu and Xu (1984)
Scrophulariaceae	*Mimulus luteus*	Ovary	Hess and Wagner (1974)
Solanaceae	*Nicotiana tabacum*	Ovary	Zhu and Wu (1979); Wu and Chen (1982)
	Petunia axillaris	Ovule	DeVerna and Collins (1984)
	Solanum tuberosum	Ovule	Tao et al. (1985)

(1987) have reported that ovary culture was better. In rice the best results of gynogenesis were obtained when unhusked flowers with pistil and stamens attached to the receptacle were inoculated on liquid medium; the response was less effective when stamens were removed and was worst when single pistils were used (see Yang and Zhou, 1982). Similarly, in barley whole florets (with or without stamen) inoculated vertically on solid medium performed better than randomly placed single pistils (Huang et al., 1982).

The stage of the female gametophyte at culture is crucial. Mostly, the explants cultured at nearly mature embryo sac stage gave best results

(Yang and Zhou, 1990, Yang et al., 1986). Rice is an exception, where inoculation of ovaries at 1–4 nucleate stage of female gametophyte development proved most responsive (Zhou et al., 1986).

7.6.2. Pre-treatment

A beneficial role of cold treatment on gynogenesis has been reported. Pretreating the capitula of sunflower at 4°C for 24–48 h before culture significantly increased the induction frequency (Yan et al., 1987). Cai et al. (1988) observed a promotory effect of cold treatment of the young panicles of rice, at 7°C for 1 day before ovary culture.

In *Cucumis melo* pollination of pistils with irradiated pollen was essential to obtain ovules capable of forming gynogenetic haploids (Katoh et al., 1993).

7.6.3. Culture medium

The culture media used for the production of gynogenetic haploids vary considerably. The most widely used basal medium in these studies happens to be N_6 (Yang and Zhou, 1982). However, N_6 and MS were equally good for the production of maternal haploids of maize (Genovesi, 1990). Generally, sucrose is used at higher levels: 3–l0% for barley, 8–l4% for wheat, 5–12% for maize and 3–6% for rice and gerbera.

For the graminaceous species the induction medium is generally supplemented with 2,4-D (2 mg l^{-1}) or MCPA (0.l25–0.5 mg l^{-1}). The best combination of growth regulators for parthenogenetic haploid production in ovary/ovule culture of *Beta vulgaris* was found to be 0.3 mg l^{-1} BAP, 0.l mg l^{-1} NAA and 0.05 mg l^{-1} 2,4-D (Van Geyt et al., 1987). In sunflower, on the other hand, a hormone-free medium gave higher induction frequency than with MCPA (Yan et al., 1987).

Mostly solid media have been used to induce gynogenesis. However, in rice at the induction stage liquid medium proved superior to solid medium (Zhou and Yang, 198l). On the other hand, for maize solid medium was more productive than liquid medium (Genovesi, 1990).

The cultures are initially stored in the dark.

In vitro gynogenesis is a multistage process. The special requirements of each stage ought to be studied carefully. For example, rice ovary culture comprises at least two stages. The first stage of induction is characterized by float culture on liquid medium supplemented with exogenous auxin and cultured in dark while the second stage of regeneration requires transfer of the callus to solid medium, reduction in auxin concentration and keeping the cultures in light (Zhou et al., 1986). Rarely, a

gametophytic cell may directly form an embryo capable of germination on the original medium (De Verna and Collins, 1984).

7.6.4. Practical importance

The production of haploids via gynogenesis is more tedious, less efficient and so far restricted to a very few species compared to androgenesis. However, it may prove valuable as an alternative to anther culture under special situations: (1) In plants, such as sugar beet, onion, and melon, where anther culture has not been successful, gynogenesis has yielded haploids (Van Geyt et al., 1987; Katoh et al., 1993). (2) In male sterile plants the value of haploid production via gynogenesis is obvious. Cai et al. (1988) produced haploids of a special photosensitive male sterile rice line by culturing unfertilized ovaries. (3) Albinism is a limiting factor in anther culture of some cereals (Section 7.10.3). In these crops ovary/ovule culture may provide relatively higher proportion of green haploids. For example, San Noeum and Ahmadi (1982) reported that in barley cv. Bernice 100% gynogenic plants were green as against 1% androgenic plants. Similarly, in rice cv. Zao Geng No. 19 the percentage of green plants in ovary culture was 89.3% but in anther culture it was only 36.4% (Zhou et al., 1986). (4) In rice the percentage of haploids was also higher in gynogenic plants (77.5%) than in androgenic plants (63.9%). A common problem with anther culture of *Petunia* has been high percentage of non-haploids. In contrast to this, 93% gynogenic plants of *P. axillaris* were haploid (De Verna and Collins, 1984).

7.7. HAPLOID PRODUCTION THROUGH DISTANT HYBRIDIZATION

Selective elimination of chromosomes following distant hybridization is another technique to obtain haploids of some cereals (see Fig. 7.8). Kasha and Kao (1970) observed that in the interspecific crosses between the tetraploids of *Hordeum vulgare* and *H. bulbosum* nearly all the plants were dihaploids. Similarly, in the crosses of the dihaploids of these species the progeny comprised haploids. Morphologically as well as cytologically the progeny represented *H. vulgare*. Treatment of fertilized florets with low concentrations of GA_3 (25–150 $\mu g\ l^{-1}$) for a couple of days considerably enhanced the frequency of seed-set and the recovery of haploid plants (Kasha et al., 1978). Furusho et al. (1993) have shown that the efficiency with which haploids are produced by this method varies considerably with the genotype of *H. bulbosum*. With the best clone of *H. bulbosum* (Hb 254–18) 35.7% of the pollinated florets produced haploid plants and with the worst clone only 1.5%.

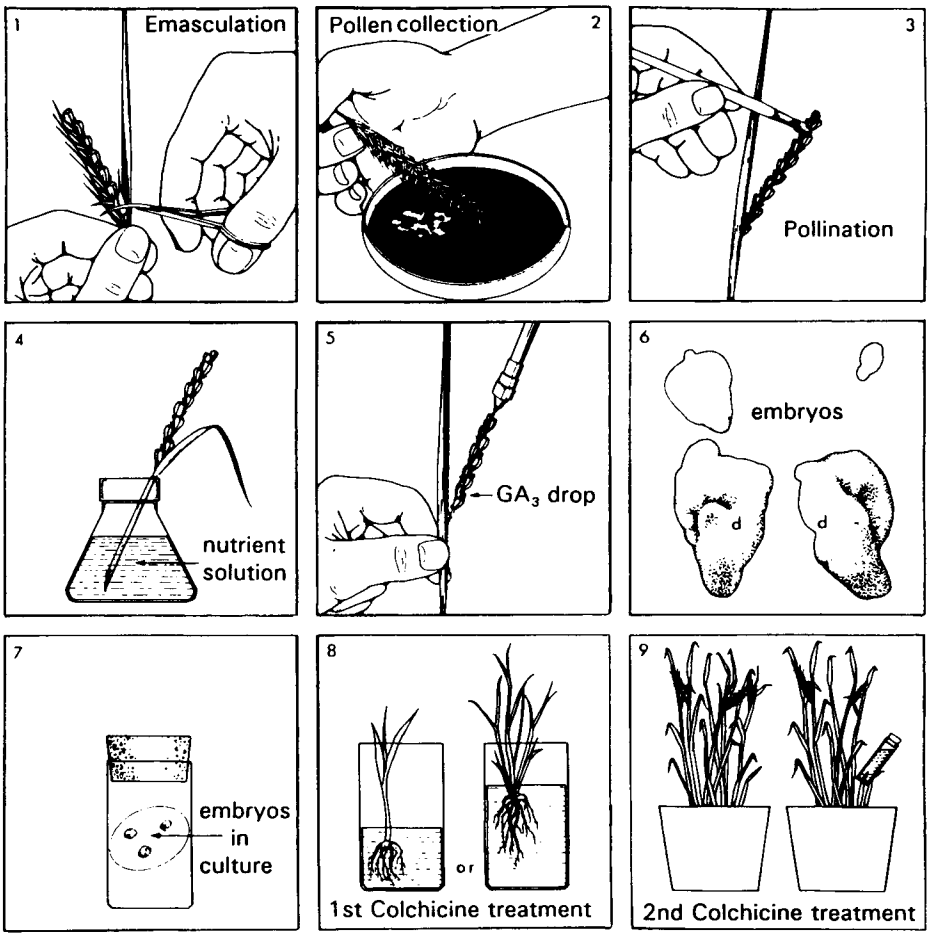


Fig. 7.8. General procedure for haploid production in barley following interspecific hybridization (courtesy of N.C. Subrahmanyam, India).

Detailed cytological studies have revealed that in these crosses double fertilization occurs normally but elimination of *Bulbosum* chromosomes during the early stages of development of the hybrid embryo leads to the formation of embryos with only *vulgare* chromosomes (Subrahmanyam and Kasha, 1973). The chromosome elimination is controlled by genes present on chromosomes 2 and 3 of *H. vulgare*. In these crosses the embryo normally aborts 10 days after pollination. For raising full plants it is essential to excise the immature embryos and rear them on an artificial nutrient medium.

High frequency gynogenic haploids of *Triticum aestivum* have been raised by crossing it with *H. bulbosum*, followed by embryo culture

(Barclay, 1975; Zenkteler and Straub, 1979; Inagaki, 1990). The immature embryos excised 2 weeks after the cross show maximum potential to form plants (Inagaki, 1990). However, wheat crosses with maize are proving more productive than with *H. bulbosum* for raising haploids (Laurie and Reymondie, 1991).

7.8. DIPLOIDIZATION TO RAISE HOMOZYGOUS DIPLOIDS

Haploids may grow normally up to the flowering stage but in the absence of homologous chromosomes meiosis is abnormal and, consequently, viable gametes are not formed. To obtain fertile, homozygous diploids for analysing the progenies and the breeding behaviour of the pollen plants the chromosome complement of the haploids must be duplicated. Spontaneous duplication of chromosomes in pollen-derived plants has been observed but its frequency is fairly low. In wheat and barley 20–50% (Henry and de Buyser, 1990) and 25–70% (Luckett and Smithard, 1991) of the pollen plants respectively, exhibited spontaneous diploidization. However, generally, the rate of spontaneous duplication of chromosomes may be very low. This can be significantly enhanced by using artificial means.

For *Nicotiana tabacum* a 0.4% solution of colchicine is recommended to diploidize the pollen plants. In practice, the young pollen-derived plants are immersed in a filter-sterilized solution of colchicine for about 96 h and then transferred to a culture medium to allow their further growth. Alternatively, the treatment is given in the form of a lanolin paste. It is applied to the axils of the upper leaves and the main axis is decapitated to stimulate the axillary buds to grow into diploid and fertile branches. Lichter et al. (1988) induced chromosome doubling in *B. napus* by injecting 2% solution of colchicine into the secondary buds. Polsoni et al. (1988) and Swanson et al. (1989) diploidized the pollen plants of this species by immersing the roots into 0.1–0.2% solution of colchicine (see also Mathias and Robbelen, 1991).

Besides bringing about chromosome duplication, colchicine treatment may also result in chromosome and gene instabilities (Burk, 1970). Therefore, the frequent occurrence of spontaneous duplication of chromosomes in differentiated plant cells (cortex, pith) and callus cells in long-term cultures (see Chapter 9) has also been exploited to raise homozygous, fertile diploids from haploid plants (see Fig. 7.9). In this method pieces of vegetative parts such as stem, root or petiole segments are cultured in a suitable medium to induce callusing. The initial callus may have some diploid cells but their frequency would increase in repeated subcultures. Such calli are transferred to the plant regeneration medium.

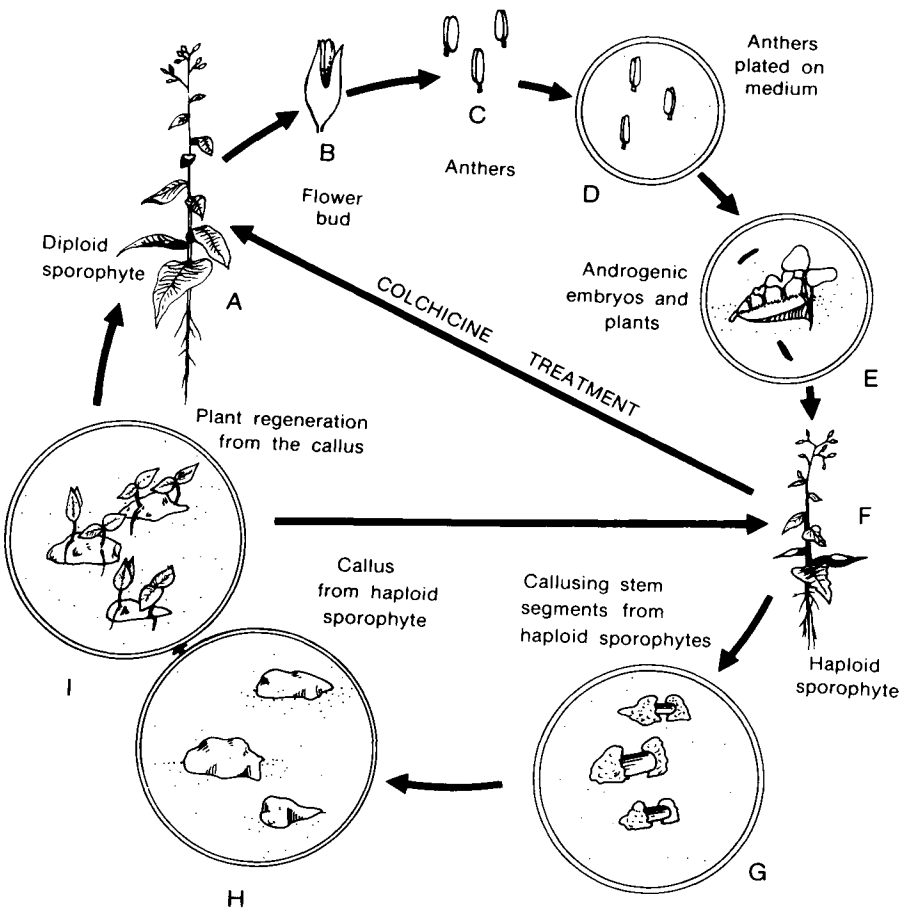


Fig. 7.9. Methods of obtaining homozygous diploids from androgenic haploids.

Many of the plants so derived are diploid. However, the ploidy of individual plants must be confirmed before incorporating them in further experiments.

7.9. APPLICATIONS

Homozygous lines of the cross pollinating species and hybrids are highly desirable to increase the efficiency of selection and production of homozygous plants. The conventional method to produce homozygous plants is lengthy and laborious, requiring 7–8 recurrent cycles of inbreeding. Moreover, this approach is impractical for self-incompatible and

male sterile plants and tree species. On the other hand, homozygous plants can be obtained in a single generation by diploidization of the haploids. Haploids are also extremely useful for detecting recessive mutants which may not express themselves in the heterozygous diploid background and, therefore, can be easily lost.

Since the discovery of haploid plants in *Datura stramonium*, by Bergner in 1921 (see Hu, 1985), several in vivo and in vitro techniques have been tried to produce haploids. However, none of the in vivo methods proved dependable due to extremely low incidence of haploid production. Of the three in vitro methods of haploid production described in this chapter, anther/pollen culture is the simplest and hitherto most efficient. Some of the applications of this technique are discussed below:

7.9.1. Shortening of breeding cycle

The most important application of androgenic haploids is in the production of stable, homozygous dihaploids (DH) in a single generation equivalent to the F_α generation of pedigree breeding and, thus, considerably shortening the breeding cycle (Henry and de Buyser, 1990; Luckett and Darvey, 1992). Normally, in a hybridization programme evaluation of lines is possible only after 4–5 years of backcrossing (F_5–F_6 generations) and it takes another 4–5 years to release a new variety. By anther culture of F_1 hybrids the various genotypes of gametes can be fixed and evaluated in the first generation. Anther culture can itself generate new recombinations and fix them simultaneously.

Nakamura et al. (1974) raised three promising doubled haploid lines through anther culture of the hybrids between MC-1610 and Coker-139. The new lines exhibited higher resistance to bacterial wilt, black shank and black root rot without losing the agronomic and chemical traits of the MC-1610. Similarly, Wark (1977) raised doubled haploids of tobacco showing high yield, good quality of cured leaves and disease resistance in a much shorter period than the normal breeding methods.

Haploid breeding, involving anther culture, has been most successfully used in China to produce several agronomically superior varieties of wheat, rice, maize and pepper, many of which are under large scale cultivation (Hu and Huang, 1987). According to Huang (1987) 81 new varieties or strains of rice and 20 new varieties of wheat have been developed through anther culture. For example, the release of the winter wheat variety 'Jinghua No. 1', in which anther culture was done in F_1 generation and evaluation of line in H_1 generation, took 6 years from the time the cross was made (see Table 7.4). By the conventional method it would have taken another 4–5 years. The desirable characters of this cultivar,

TABLE 7.4

Steps involved in the development of the wheat cultivar 'Jinghua No. 1' (after Hu and Huang, 1987)

Year	Description	Generation	Years consumed
1976	Hybridization:(Lovrin 18x 5238–036) × Hongliang No. 4	F_1	
1977–1978	Anther culture and chromosome doubling	H_1	
1978–1979	Evaluation of lines	H_2	
1979–1980	Trial test, multiplication of seeds	H_3	6
1980–1981	Trial test, regional test, performance test, and multiplication of seeds	H_4	
1981–1982	Trial test, regional test, performance test, multiplication of seeds and nomination of the cultivar	H_5	

which covered over 170 000 ha in 1984, are high yields (4.5–6 tons per ha), resistance to rust, short stature, and wide adaptation. Some of the rice cultivars produced in China through anther culture are 'Hua Yu No. 1', 'Xin Xiu', 'Hua Han Zao', 'Zhongua Nos 8', 9 and 10', and 'Shan Hua 369'. The latter cultivar showed 4.4–11.4% increase in yield over the best parent, early maturity, high protein content (10–12%), cold tolerance of seedlings and a uniformity in the heading and maturing stages. 'Hai-Hua No. 3', a sweet pepper cultivar selected directly from pollen derived plants, yield 10–59% over the control. The other attributes of this cultivar are early maturity, desirable fruit colour and size, good quality, resistance to diseases and tolerance to heat.

Outside China, a doubled haploid wheat variety 'Florin' (Ets Desprez), obtained by anther culture of the F_1 cross Wizard × Iena, was produced in France in 1985 (de Buyser et al., 1987). Only 64 green pollen plants were required to obtain this cultivar.

While anther culture of F_1 plants (H_1-DH) has yielded promising results, Snape and Simpson (1981) have suggested that F_2-DH lines should permit better selection than F_1-DH since linkages may be involved in determination of a character. Another strategy adopted to improve some polygenic traits involve one cycle of anther culture followed by sexual hybridization between different genotypes of anther derived plants and then another cycle of anther culture of the sexual hybrids (see Hu and Huang, 1987).

Anther culture is being routinely used as an adjunct to a broccoli breeding programme (Springer and Bailey, 1990).

7.9.2. Gametoclonal variations

Besides yielding haploids, in vitro androgenesis provides a unique opportunity to screen the gametophytic variation, caused by recombination and segregation during meiosis, at the sporophytic level. The gametoclonal variants being hemizygous express even the recessive traits in the R_0 plants unlike somaclonal variants which require selfing and progeny analysis. A gametoclone of tomato, which bears fruits with higher solid content than the parent cultivar, has been selected (Morrison and Evans, 1988). Similarly, Schaeffer et al. (1984) obtained dwarf rice plants with longer grains, higher levels of seed storage proteins, shorter stature and more highly tillered in selfed progeny of pollen plants. Physiological and phenotypic variability occurred in pollen plants of *Datura* (Grange et al., 1984; Herouart et al., 1988). For example, the leaf alkaloid content of *D. innoxia* plants obtained from in situ cuttings and seed-derived plants was nearly uniform while that of pollen plants was quite variable. In certain pollen plants alkaloid content was five times higher than in the control. Some of the variations for alkaloid content persisted even after selfing.

Of the 40 000 plants derived through selfing of a temperature sensitive strain of *Nicotiana tabacum* none survived exposure to the low temperature regime of 13°C day/8°C night, but 2 out of 366 dihaploids produced through anther culture survived cold treatment (Matzinger and Burk, 1984). Breeding experiments revealed that these variants appeared due to changes in the genetic factor of the cytoplasmic organelles.

In oil yielding brassica, cultivars with low erucic acid content are required for the production of edible oil. High erucic acid oil is also important commercially as it is an excellent lubricant and is used to produce water repellents, plasticizers, waxes and surface active agents. Siebel and Pauls (1989) observed that some of the pollen derived spontaneous diploids from crosses between low and high erucic acid lines of *Brassica napus* produced higher erucic acid oil than either of the parents; some erucic acid-free lines also occurred in these androgenic plants.

7.9.3. Mutagenesis

Detection and isolation of recessive mutants in the haploid state and rapid obtainment of the mutated gene in a homozygous diploid state is a special merit of haploidy in higher plants. Application of mutagenic treatment at the microspore stage, which is a single celled structure, has the added advantage of obtaining solid mutants.

Ethyl nitrosourea (20 mM) and γ-irradiation (0.5 Krad) have been successfully used to create herbicide resistant mutants of *Brassica napus*.

Since microspore embryogenesis in this plant is sensitive to physical disturbances during the first day of culture it has been recommended that mutagenic treatment should be applied to the microspores 16–24 h after culture at which time most of the microspores are ready to enter the first cell division (Huang, 1992). Swanson et al. (1988, 1989) have selected *B. napus* plants resistant to the sulfonylurea herbicides (e.g. chlorsulfuron and imidazolinones). This resistance is created due to point mutation in the gene responsible for the synthesis of the enzyme acetohydroxy acid synthase which is the first enzyme common to the synthesis of leucine, valine and isoleucine. The wild form of this gene is sensitive to the herbicide. The mutant gene has been cloned and introduced in *B. napus* and tobacco to produce transgenics with elevated levels of resistance to the herbicides (see Huang, 1992). Kott and Beversdorf (1990) mutagenized large scale cultures of *B. napus* followed by in vitro selection for resistance to several herbicides to isolate array of mutants currently under field trials.

Through microspore mutagenesis, Turner and Facciotti (1990) have obtained a mutant of *B. napus* with high oleic (75.3%) and low lanoleic acid (8.7%) content. Similarly, Huang et al. (1991) have isolated *B. napus* mutants with lower saturated fatty acid or higher oleic acid than the parent cultivar.

7.9.4. Genetic transformation

Agrobacterium is a superior vehicle for transformation of dicots but its use with monocots is very limited. Therefore, alternative methods, such as microinjection, electroporation etc., are being tried. Another problem with somatic protoplasts/cell transformation in most monocots and some dicots is the poor regenerability of plants after DNA insertion. In such cases immature pollen embryos, which exhibit high regenerative capacity may offer excellent recipient cells. The pollen embryos being haploid the proof of a successful transformation is facilitated because it is expressed homozygously.

Neuhaus et al. (1987) produced transgenic plants of *B. napus* by microinjection of DNA into individual cells of immature embryos. The 12-cell stage of the embryo was optimum for this purpose, considering the optical control during microinjection, high survival rates and overall efficiency until plant regeneration. As determined by DNA dot blot analysis, 27–50% of the primary regenerants showed transformation. The transformants were chimeric but solid transformants were obtained from them via secondary embryogenesis. Transformants of *B. napus* have also been produced through co-cultivation of *Agrobacterium* with microspores

(Pechan, 1989) and microspore-derived embryos (Swanson and Erickson, 1989).

The ability of isolated microspores of *Hordeum vulgare* to undergo androgenesis after PEG-induced uptake of foreign DNA with frequencies comparable to the untreated controls suggests that it may be possible to obtain solid transformants directly by transforming single microspores (Kuhlmann et al., 1991).

7.9.5. Production of super-males of *Asparagus officinalis*

In *Asparagus officinalis*, a dioecious crop species, an inbred population is produced through sib crosses between pistillate (XX) and staminate (XY) plants which yield 50% males and 50% females. However, the commercially desirable features of this crop are uniform male population with spears having low fibre content. Thevenin (1974) and Tsay et al. (1982) reported the production of haploids of this species by anther culture which could be diploidized to raise homozygous males (YY), also called super-males. The advantage of having super-males is that when such plants are crossed with females they yield a homogeneous male population (see Fig 7.10). Corriolis et al. (1990) have announced the release of the first homogeneous, all male F_1 hybrid of *A. officinalis*, called 'Andreas', using homozygous supermales derived from pollen embryos and a homozygous female parent obtained by diploidizing a spontaneous parthenogenetic haploid. 'Andrea' is a very regular, high yielding variety with large spear diameter and very tight head.

7.10. LIMITATIONS

The practical applications of androgenic haploids are still limited as the desired success with anther and pollen culture has been largely restricted to only a few families, such as Brassicaceae, Poaceae and Solanaceae. Other problems associated with the realization of the full potential of anther and pollen culture in crop improvement are discussed below.

7.10.1. Low yields

Generally only 5–8% of the total pollen grains in a responding anther undergo androgenic development (Raghavan, 1976b, Sangwan-Norreel, 1983; Sharma and Bhojwani, 1985), and a very small proportion of these androgenic grains develop into full sporophytes. This turns out to be a serious limitation in obtaining full range of gametic segregants of interest to plant breeders (Petolino, 1992).

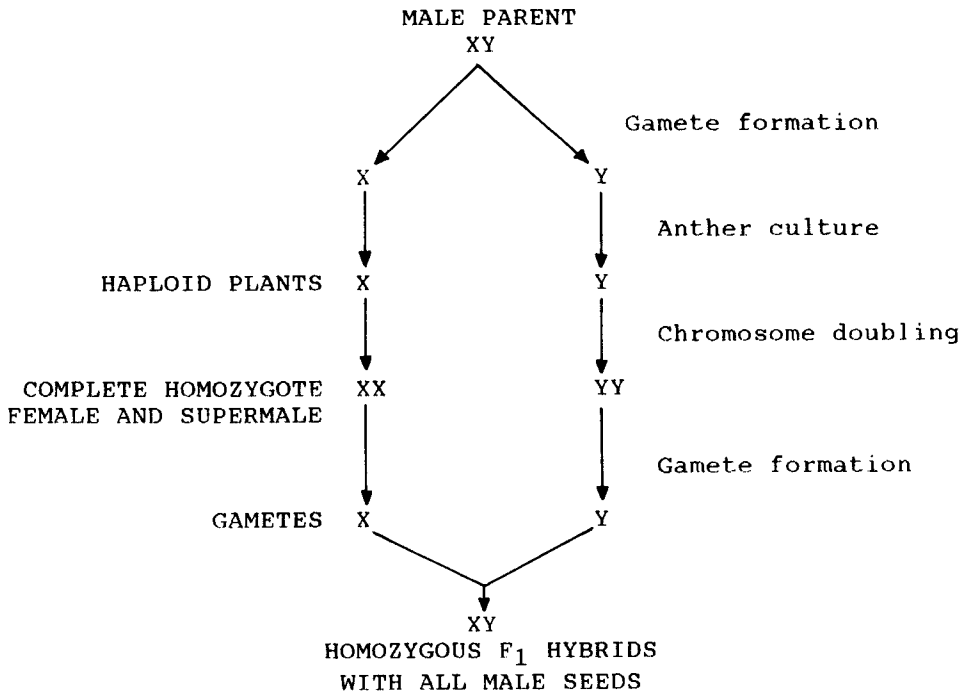


Fig. 7.10. Flow chart of the technique used to produce all male seeds of *Asparagus officinalis* through anther culture (after Tsay, 1983).

7.10.2. Conversion of pollen embryos into plants

One of the major problems in anther/pollen culture of *Brassica* species is the structural, physiological and biochemical abnormalities of pollen embryos so that 70–80% of the embryos are incapable of normal germination (Swanson et al. 1987; Sharma and Bhojwani, 1989; Taylor et al. 1990). Plant regeneration from such embryos occurs through secondary embryogenesis (Fig. 7.1C) or adventitious shoot bud differentiation from the hypocotyl and cotyledons.

Taylor et al. (1990) have shown that the pollen embryos of *B. napus* do not accumulate napin or cruciferin which are seed-specific proteins of this species. ABA application induced the transcription of the genes responsible for the synthesis of these proteins but the gene expression remained blocked at the transcription level. However, ABA application was effective in improving the germinability of the pollen embryos. Besides ABA, cold treatment (4°C) of the pollen embryos for 6–12 days has been found to enhance the incidence of normal germination of pollen embryos

of *B. napus* (Kott and Beversdorf, 1990) and *B. juncea* (Agarwal and Bhojwani, 1993).

7.10.3. Albinism in cereals

A serious problem associated with the application of anther/pollen culture to cereal improvement is the occurrence of albinos with high frequencies (Bhojwani, 1988). The degree of albino formation may vary with the variety. For example, rice varieties 'Hung Chin No 16', 'Ching Hung No 2' and 'Lien Chiang No 2' formed albinos at a rate of 100, 50 and 70%, respectively (Wang et al., 1978). Similar genotypic variation has been reported for wheat (Schaeffer et al., 1979). Since the albino plants cannot survive in nature they are of no agronomic value.

The albino plants are morphologically normal except for the absence of chlorophyll. In the androgenic albinos of rice the plastids showed poor differentiation of grana, and the Fraction-1-Protein and 23S and 16S subunits of rRNA were totally lacking (Wang et al., 1978; Sun et al., 1979). This may be due to deletion of ptDNA. Bernard (1980) has suggested that high temperature increases the difference between the speed of replication of the nuclear material and the cell organelles, resulting in retarded or incomplete development of the plastids leading to albino phenotypes. According to him the development of functional photosynthetic system is promoted by low temperature treatment. However, in *Lolium perenne* the donor plants grown in greenhouse at 16–35°C yielded higher proportion of green plants than those grown in growth chamber at 15°C (Olesen et al. , 1988). Recently, Kao et al. (1991) have reported that in *Hordeum vulgare* direct embryogenesis from pollen, using well buffered, high Ficoll/high sucrose medium and proper aeration yielded higher numbers of green plants than the callus pathway. In the latter case the callus cells are under anaerobic conditions, causing the accumulation of lactate and alcohol dehydrogenase which may be damaging the plastids.

7.10.4. Genetic changes

Instability of genetic material during androgenesis could be an hindrance in the use of haploids. In tobacco the androgenic plants exhibited growth depression, reduced agronomic performance and abnormalities such as reduced leaf and flower size and tumerization of leaf cells, which could be correlated to amplification of DNA (De Paepe et al., 1981, 1982, 1983; Dhillon et al., 1983; Reed and Wernsman, 1989). Large scale deletion of plastid DNA has been detected in albino plants derived from pollen grains of wheat (Day and Ellis, 1984, 1985) and rice (Harada et al.,

1991). Absence of such deletions in callus derived from rice seeds suggests that this phenomenon may be specific to anther culture. According to Harada et al. (1991) the deletion of ptDNA during androgenesis may be due to intramolecular recombination(s) between repeated DNA sequences which are known to occur in plants.

7.11. CONCLUDING REMARKS

Of the three in vitro methods of haploid production described in this chapter, the bulbosum method is applicable to only a couple of cereals. Gynogenesis, which has been achieved in over a dozen species, is valuable only where anther culture is not applicable or is beset with some problem. Androgenesis continues to be the most efficient technique to produce haploids because of the occurrence of thousands of haploid cells per anther as compared to, at the most, seven cells per cultured ovule.

During the last 10 years the list of species for which androgenic haploids have been produced has enlarged considerably. The exploitation of pollen plants in crop improvement by the Chinese is outstanding. They have produced several improved cultivars of the major cereals through anther culture of F_1 or F_2 hybrids. However, outside China such examples are very few. This could be because anther culture is labour intensive and the frequencies with which androgenesis occurs is generally very low, so that the number of anthers to be cultured to obtain a reasonable number of pollen plants is very large. In this regard, isolated pollen culture holds great potential. Pollen culture is not only less laborious but also useful for crop improvement through biotechnological methods. The area of pollen culture is expected to receive more research input in future.

Success in anther/pollen culture is highly genotype-specific, limiting its application to only some cultivars of crop plants. For example, the success with maize anther culture, outside China, is largely restricted to the germplasm which is not of commercial importance (Petolino, 1992). It is desirable that the protocol of anther/pollen culture should be genotype non-specific so that it is applicable to a broad germplasm base, and does not require refinement each time a new cultivar is produced by the breeders. In this regard it is encouraging to note that RFLP probes linked to the genes conferring high androgenic response have been identified (Cowen et al., 1992, cited in Petolino, 1992).

APPENDIX 7.I

Protocol for isolated pollen culture of *Brassica napus* (after Coventry et al. 1988).[3]

(a) Grow the donor plants under controlled conditions; of 16 h photoperiod (approx. 200 μmol m^{-2} s^{-1}) and at 24 ± 2°C/21 ± 12°C day/night temperature. Water and fertilize the plants daily.

(b) Collect healthy buds at the late uninucleate stage (approx. 4.5 mm long) and surface sterilize them in 5.6% sodium hypochlorite solution for 10–15 min (the buds should be processed for pollen culture immediately after plucking).

(c) Rinse the buds three times in cold, sterile distilled water for 5 min each time.

(d) Transfer the buds to the blender cup under aseptic conditions, add cold B_5 liquid medium containing 13% sucrose (ca. 30 ml medium in a 50 ml blender cup) and blend them for a total of 20–30 s alternating between high and low speeds.

(e) Filter the contents of the blender through two layers of nested sterile filters (Nytex 63 μm top and 44 μm bottom).

(f) Centrifuge the filtrate at 900–1000 rev. min^{-1} for 5–8 min. Discard the supernatant, resuspend the sediment in cold B_5 medium and spin as above. Repeat this process four times.

(g) Finally, suspend the microspores in 50 ml of NLN[4] liquid medium and adjust the plating density to 7.5×10^3–10^4 ml^{-1} using a haemocytometer.

(h) Dispense 10 ml of pollen suspension into petri plates (100 × 15 ml) using a sterilized pipet.

(i) Seal the petri plates with a double layer of parafilm.

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[3] Dr Y. Ohkawa (Chugoku Natn. Agric. Exp. Stn. Fukuyama, Japan) has slightly modified the above protocol by: (i) reducing the concentration of major salts of NLN to half- strength, and (ii) introducing gradient centrifugation after step 5 to enrich the population of embryogenic grains. The suspension from step 5 is centrifuged at 1000 rev. min^{-1} for 3 min. The supernatant is discarded and the pellet suspended in 1 ml of B_5 medium containing 13% sucrose. The suspension is gently loaded on a Percoll gradient 24, 32 and 42% in screw cap centrifuge tubes and centrifuged at 1200 rev. min^{-1} for 5 min. The fraction at the interphase of 24 and 32% Percoll, containing the predominantly late uninucleate pollen and cultured largely as described above.

[4] Composition of NLN medium (mg l^{-1}): KNO_3 (125), $MgSO_4 \cdot 7H_2O$ (125), $Ca(NO_3) \cdot 4H_2O$ (500), KH_2PO_4 (125), Fe-330 (40), $MnSO_4 \cdot 4H_2O$ (22.3), H_3BO_3 (6.2), $ZnSO_4 \cdot 7H_2O$ (8.6), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.025), $CoCl_2 \cdot 6H_2O$ (0.025), myo-inositol (100), nicotinic acid (5), glycine (2), pyridoxine·HCl (0.5), thiamine·HCl (0.5), folic acid (0.5), biotin (0.05), glutathione (30), L-glutamine (800), L-serine (100), sucrose (130 000), NAA (0.5) and BAP (0.05), pH 6.

(j) Place the petri plates in a light-proof box and place in an incubator at 30°C for 14 days.

(k) After 2 weeks shift the box to a slow shaker (60 rev. min^{-1}), for 7 days to ensure proper development of the embryos.

(l) Transfer individual embryos to B_5 solid medium (ca. 10 embryos/plate), seal the plate with parafilm and incubate at 4°C with 8 h photoperiod for 10 days.

(m) Transfer the plates to a 27°C incubator with 12 h photoperiod. Under these conditions the embryos should develop into plantlets which can be transferred directly to soil.

(n) For mutant selection, after step 10 expose the pollen to mutagenic treatments and after step 11 transfer the embryos to a 250 ml flask containing 50–75 ml of B_5 liquid medium (1% sucrose) containing 0.1 mg l^{-1} GA_3 (B_5G_1) and incubate them on a shaker (80 rev. min^{-1}) in light. In 3–5 days the embryos turn green. Replace the medium with the B_5G_1 medium containing the filter-sterilized selection agent (e.g. herbicide). The embryos which remain green and survive are selected and passed through steps 12 and 13 for plant regeneration.

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Chapter 8

Triploid Production

8.1. INTRODUCTION

Endosperm is a unique tissue. In the majority of flowering plants (over 81% of families) it originates from the fusion product of three haploid nuclei (one from the male gametophyte and two from the female gametophyte) and is, therefore, triploid. The endosperm is the main nutritive tissue for the embryo and, also, a dynamic centre of developmental influences on the embryo (Raghavan, 1976a). Dysfunction of endosperm, or its complete absence, usually causes abortion of the embryo. The endosperm may be consumed wholly by the developing embryo, so that the mature seed is non-endospermous (legumes, cucurbits), or it may persist and store abundant reserve food in the mature seed (cereals, castorbean, coconut, coffee). During seed germination these substances are digested and utilized for the initial growth of the seedling.

The endosperm tissue offers an excellent system for experimental morphogenic studies. Besides being triploid, it is a homogeneous mass of parenchymatous tissue, lacking the differentiation of vascular elements. Whereas all other ovular tissues have been reported to form embryos in nature, there is no confirmed instance of the endosperm doing so (Bhojwani and Bhatnagar, 1990).

During the last 30 years the potential of endosperm cells for unlimited growth and organogenic differentiation in vitro has been well established. The endosperm tissue being triploid, the plantlets formed from it are also triploid. This technique of endosperm culture may be profitably exploited as an alternative to crossing tetraploids and diploids for raising triploids in plant improvement programmes (Johri and Bhojwani, 1977; Bhojwani, 1984).

In nature the endosperm cells store large quantities of reserve food material in the form of starch, proteins, lipids and fats, which are mobilized during embryo development and seed germination. It is, therefore, an ideal system to study the metabolism of these natural products (Sobolev et al., 1971; Keller et al., 1972; Chu and Shannon, 1975).

8.2. CALLUSING

The earliest attempt to grow endosperm tissue in culture was made by Lampe and Mills (1933, cited by LaRue, 1936). They grew young corn endosperm on a nutrient medium enriched with the extract of potato or young corn and obtained slight proliferation of cells adjacent to the embryo. However, the first extensive work on the growth and differentiation of endosperm tissue in cultures was undertaken by LaRue and his associates at the University of Michigan, Ann Arbor, MI, USA. After several years of sustained work, in 1949, LaRue reported the establishment of a continuously growing tissue from the immature maize endosperm. Subsequently, many other workers examined tissue cultures of maize endosperm from diverse angles.

8.2.1. Explant

The cereal endosperm proliferates only if excised during a proper period of development: 9–10 days after pollination (DAP) in *Lolium perenne* (Norstog, 1956a), 8–11 DAP in *Zea mays* (Straus and LaRue, 1954; Tamaoki and Ullstrup, 1958), 8 DAP in *Triticum aestivum* and *Hordeum vulgare* (Sehgal, 1974) and 4–7 DAP in *Oryza sativa* (Nakano et al., 1975). The mature endosperm in these plants has not been amenable to culture, except for the solitary report by callusing and plant regeneration in the cultures of mature endosperm of two cultivars of rice, by Bajaj et al. (1980). Tamaoki and Ullstrup (1958) suggested that certain physiological changes occur in corn endosperm 12 DAP that render it incapable of responding to the treatments in culture. Plant physiologists regard the mature cereal endosperm, except for a few outer layers which constitute the aleurone tissue, as dead (Varner, 1971). Incidentally, a dicot (*Cucumis sativus*) has also been reported to exhibit similar behaviour; the endosperm proliferates only when it is excised 7–10 DAP (Nakajima, 1962). The best stage for endosperm culture of tomato was after 37–50 days from fruit-set by which time the fruits had attained 75–90% development (Kagan-Zur et al., 1990).

In 1963 Mohan Ram and Satsangi demonstrated the potentiality of mature endosperm cells to divide. They observed proliferation of endosperm in germinating seeds of *Ricinus communis* previously soaked in 2,4-D solution. These authors subsequently established tissue cultures of mature endosperm of this euphorbiaceous plant (Satsangi and Mohan Ram, 1965). To date continuously growing tissues have been raised from the mature endosperm of several dicots. Most of the plants which have responded favourably belong to the families Euphorbiaceae, Santalaceae

and Loranthaceae. With respect to the first two families, it was observed that the initial association of the embryo was essential for inducing proliferation of the endosperm from fully mature and dried seeds. Shortly after the endosperm has started callusing, the embryo can be removed without affecting the growth of the former. When mature endosperm with the embryo intact is planted on a suitable nutrient medium, the embryo shows initial stages of germination, such as elongation of the hypocotyl and the expansion of the cotyledons (Bhojwani and Johri, 1971) (see Fig. 8.1A). If not removed at this stage, the embryo may also proliferate, resulting in a mixed callus. It is, therefore, advisable to remove the embryo soon after the endosperm shows proliferation.

The role of embryo in the proliferation of mature endosperm remains a matter of speculation. It is evident from the work of Brown et al. (1970) and Kagan-Zur et al. (1990) that some factor(s) contributed by the germinating embryo is(are) essential for the activation of mature and dried endosperm of castor bean (Fig. 8.2) and tomato, respectively. Brown et al. (1970) observed that endosperm pieces from dried seeds of castor bean did not grow in cultures. However, if endosperm was excised from seeds soaked in 3.5% $Ca(OCl)_2$ solution for 22 h, some explants did proliferate.

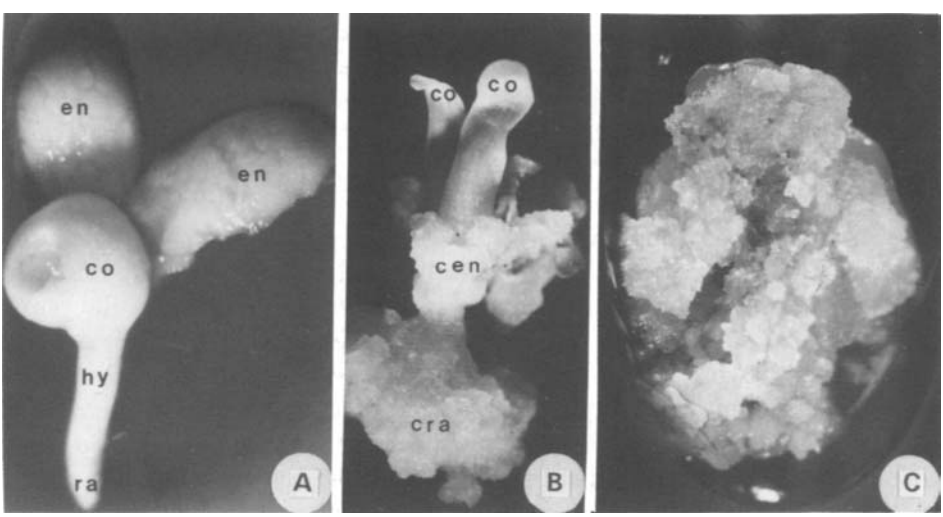

Fig. 8.1. In vitro proliferation of mature endosperm in the cultures of de-coated seeds of *Croton bonplandianum*. (cen, callused endosperm; co, cotyledon; cra, callused radicle; en, endosperm; hy, hypocotyl; ra, radicle). (A) Ten-day-old culture of de-coated seed, showing germinated embryo with enlarged and coiled cotyledons, and elongated hypocotyl; also note proliferation on the inner side of the two halves of endosperm. (B) Four-week-old culture, showing enlarged cotyledons. The radicle as well as endosperm has proliferated. (C) Profusely growing endosperm callus (after Bhojwani and Johri, 1971).

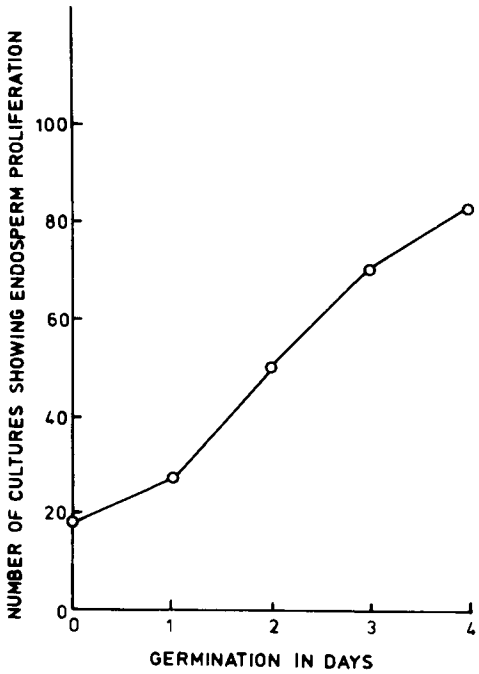

Fig. 8.2. *Ricinus communis*. Effect of germination period on the proliferation of excised endosperm; 0 day refers to seeds soaked for 22 h (drawn from the data of Brown et al., 1970).

The number of proliferating endosperm pieces further increased if the soaked seeds were allowed to germinate, and there was a direct relationship between the number of days after seed germination and the number of cultures showing endosperm proliferation (Fig. 8.2). For tomato endosperm culture, soaking the seeds in water for 3 days proved optimum in terms of the number of responding explants (Kagan-Zur et al., 1990). It is known that during germination, embryo releases gibberellin-like substances (Ogawa, 1964; Ingle and Hageman, 1965). In cereals gibberellin helps in the activation and de novo synthesis of certain enzymes. Bhojwani (1968) noted that GA_3 was able to replace the 'embryo factor' for inducing proliferation of mature endosperm of *Croton bonplandianum*. These observations were subsequently supported by the work of Srivastava (1973) on *Putranjiva roxburghii*.

8.2.2. Culture medium

LaRue (1949) cultured corn endosperm on media containing various supplements such as tomato juice (TJ), grape juice, green-corn juice, yeast extract (YE), or cow's milk. Of all these, 20% TJ supported maximal

growth. Sternheimer (1954) confirmed the superiority of TJ over other tested substances for corn endosperm callus growth. However, Straus and LaRue (1954) noted that the callus growth on TJ-supplemented medium was erratic and unpredictable. These investigators used canned TJ and, therefore, it is unlikely that for every experiment the juice was derived from tomatoes of the same age and variety. Cytokinin-like activity in TJ is known to decrease correspondingly with the age of the fruit (Bottomley et al., 1963). This may be one of the reasons for the erratic and unpredictable growth of corn endosperm on media supplemented with TJ.

Straus and LaRue (1954) and Tamaoki and Ullstrup (1958) demonstrated that YE could substitute for TJ to a considerable extent. Yeast extract also supported good growth of *Lolium* endosperm callus (Norstog, 1956a). In the process of evolving a purely synthetic medium, Straus (1960) found asparagin (1.5×10^{-2} M) to be superior to TJ or YE. Nakajima (1962) carried out a series of experiments and reported that for satisfactory growth of endosperm callus of *Cucumis* a combination of an auxin, a cytokinin, and a source of organic nitrogen such as YE or casein hydrolysate (CH) was necessary. Later it was shown that mature endosperm tissue of *Santalum* (Rangaswamy and Rao, 1963), *Ricinus* (Satsangi and Mohan Ram, 1965), *Croton* (Bhojwani and Johri, 1971), and *Jatropha* (Srivastava, 1971) grew best on a nutrient medium supplemented with 2,4-D, kinetin and YE. In *Putranjiva roxburghii*, a tree member of the Euphorbiaceae, best growth of the endosperm callus was noted on a medium containing IAA, kinetin and CH (Srivastava, 1973).

From amongst the various sugars tested, sucrose at a concentration of 2–4% supported best growth of the endosperm callus of maize (Straus and LaRue, 1954) and castor bean (Johri and Srivastava, 1972). Arabinose, cellobiose, galactose, lactose, mannose and sorbose were inhibitory. Interestingly enough, starch as an exclusive source of carbon in the medium supported the growth of the endosperm callus of *Asimina triloba* (Lampton, 1952).

8.2.3. Physical factors

Unfortunately, not much attention has been paid to the influence of physical factors, such as light and pH, on the growth of endosperm tissue. The information on this aspect is available mainly with reference to maize and castor bean.

(i) *Light.* The corn endosperm grows better when maintained in the dark than in light (Straus and LaRue, 1954), whereas *Ricinus* endosperm

grows better under continuous light (Srivastava, 1971). Light did not have a significant effect on the endosperm cultures of *Lolium* (Norstog, 1956a). The endosperm callus of coffee thrived best under 12 h light/dark conditions (Keller et al., 1972).

(ii) *Temperature*. The optimum temperature for the growth of endosperm callus is reported to be around 25°C (Straus and LaRue, 1954; Johri and Srivastava, 1973).

(iii) *pH*. The optimum pH of the culture medium varies from 4.0 for *Asimina* (Lampton, 1952) to 5.0 for *Ricinus* (Johri and Srivastava, 1973), 5.6 for *Jatropha* and *Putranjiva* (Srivastava, 1971, 1973) and 6.1 for *Zea mays* (Straus and LaRue, 1954).

8.3. HISTOLOGY AND CYTOLOGY OF CALLUS

In *Zea mays* the endosperm becomes cellular 3 DAP and the divisions continue throughout the tissue mass up to 7–8 DAP. The meristematic activity then becomes restricted to the outermost cell layer. At the time of excision, 12 DAP, the endosperm comprises a comparatively homogenous tissue with a peripheral layer of meristematic cells. In cultures, cells of this layer undergo anticlinal and periclinal divisions to add to the girth of the outermost layer as well as to the mass of general endosperm tissue. By the third day the peripheral layer becomes four cells thick and during the next 24 h it adds up many more layers. This is followed by differential growth at localized areas leading to the formation of nodules. These meristematic nodules may arise directly from the peripheral layer or cells located immediately below the outermost layer (Straus, 1954; Sehgal, 1969).

On a medium fortified with 2,4-D, kinetin, and YE the proliferation of mature endosperm begins 10–12 days after inoculation. In *Croton*, callusing starts on the inner side of the endosperm which becomes visible when the endosperm bursts open into two halves (Fig. 8.1A,B). The embryo is removed at this stage, and the proliferation extends all over the endosperm tissue (Fig. 8.1C). In the cultures of *Santalum* endosperm callusing starts with the differentiation of concentric layers of meristematic tissue about five to six layers below the epidermis (Rangaswamy and Rao, 1963). In the presence of kinetin alone, or in combination with IAA, the callusing is initiated with the differentiation of localized, peripheral meristems leading to the formation of nodular outgrowths on the surface of endosperm as observed in *Osyris wightiana* (Johri and Bhojwani, 1965) and *Putranjiva roxburghii* (Srivastava, 1971).

Irrespective of the composition of culture medium, the endosperm callus of cereals remains purely parenchymatous. This is also true for *Cucumis* (Nakajima, 1962). However, tissues derived from the endosperm of Euphorbiaceae, Loranthaceae and Santalaceae members readily differentiate tracheidal elements (Johri and Srivastava, 1973; Johri and Bhojwani, 1977). The tracheidal elements may appear scattered or in clusters. The slow growing and compact callus shows a higher degree of tracheidal differentiation. Organogenic differentiation from endosperm generally occurs only in those plants which exhibit tracheidal differentiation in cultures.

The endosperm tissue is well known for a high degree of polyploidization of its cells during in vivo development. It also exhibits various kinds of mitotic irregularities such as chromosome bridges and laggards (Bhojwani and Bhatnagar, 1992). These cytological features are also common in long-term tissue cultures of various organs, including endosperm (see Chapter 9). Straus (1954) reported that in established cultures of corn endosperm, the polyploid, hypoploid and aneuploid cells were as common as those showing normal chromosome number. Thirty percent of the total cells observed in anaphase exhibited chromosome bridges and laggards. Cells of ploidy higher than $3n$ have also been reported in endosperm cultures of *Croton* (Bhojwani and Johri, 1971), *Jatropha* (Srivastava, 1971), and *Lolium* (Norstog, 1956a; Norstog et al., 1969). It is, however, interesting that in over 10-year-old calli of rye-grass endosperm the majority of cells remained triploid (Norstog et al., 1969). A remarkable stability in chromosome number of endosperm cells in vivo as well as in vitro is shown by mistletoes, viz., *Dendrophthoe falcata*, *Taxillus vestitus* and *T. cuneatus* (Johri and Nag, 1974). Neither the composition of the medium (Matthysse and Torrey, 1967) and the length of the culture period, known to influence polyploid mitosis in pea (see Chapter 9), nor any other treatment altered the chromosomal constitution of endosperm cells in *Dendrophthoe*. Probably, in this taxon the natural mechanism leading to polyploidy is either lacking or inoperative.

8.4. ORGANOGENESIS

As early as 1944, LaRue reported that excised endosperm pieces of castor bean regenerated roots. Three years later, LaRue (1947) published that in maize endosperm cultures '...less than one in thousand developed roots and a single one formed root-shoot axis with miniature leaves'. However, neither of these communications described the nutrient medium or other culture conditions which favoured differentiation. None of the later workers achieved organogenic differentiation from endosperm

cultures of castorbean (Satsangi and Mohan Ram, 1965; Johri and Srivastava, 1973). Regarding the maize endosperm, Straus (1954), one of LaRue's students remarked: 'Since then, the tissue has passed through approximately 95 transfers and has produced an estimated 15 kg of tissue. Not a single example of complex differentiation was observed during this period.' Sehgal (1969) also failed to obtain differentiation of organs in endosperm cultures of maize.

Organ formation from endosperm tissue, supported with histological details, was first demonstrated in *Exocarpus cupressiformis* (Santalaceae) by Johri and Bhojwani (1965). They observed that in 'seed' cultures of *Exocarpus* on a medium supplemented with IAA, Kinetin and casein hydrolysate (CH), 10% cultures formed shoot buds all over the endosperm (Fig. 8.3A). In a single explant as many as eight buds developed. The origin of these buds from endosperm was confirmed through histological studies (Fig. 8.3B,C). In situ the buds did not grow beyond 1.25 cm. On the other hand, if the buds were excised and planted on a fresh medium of the same composition they formed slow growing callus which later differentiated shoot buds that showed limited growth. The buds were, as expected, triploid.

Stimulated by the report of Johri and Bhojwani, extensive studies were undertaken to raise full triploid plants through endosperm culture, and to date organogenic differentiation in endosperm cultures has been achieved in several parasitic and autotrophic species, including some important tree species (Table 8.1). The success in raising triploid plantlets of *Actinidia* (Mu et al., 1990), *Citrus* (Wang and Chang, 1978; Gmitter et

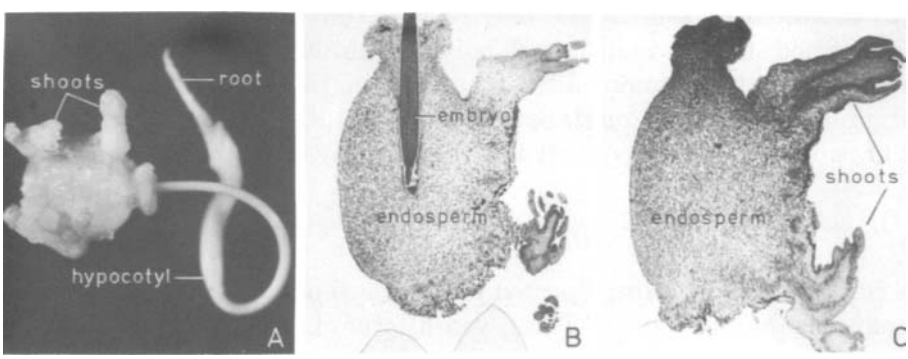

Fig. 8.3. *Exocarpus cupressiformis*. (A) A 5-week-old 'seed' (endosperm + embryo) culture showing shoot buds on the endosperm and germination of embryo. (B,C) Sections of the 'seed' shown in (A); note the origin of the shoots from the periphery of endosperm (after Johri and Bhojwani, 1965).

TABLE 8.1

Species which have been reported to form shoots or whole plants from endosperm tissue

Species	Reference
Actinidiaceae	
Actinidia chinensis	Gui et al. (1982)
Actinidia hybrids	Mu et al. (1990)
Annonaceae	
Annona squamosa	Nair et al. (1986)
Apiaceae	
Petroselinum hortense	Masuda et al. (1977)
Euphorbiaceae	
Codiaeum variegatum	Chikkannaiah and Gayatri (1974)
Jatropha panduraefolia	Srivastava (1971)
Putranjiva roxburghii	Srivastava (1973)
Loranthaceae	
Dendrophthoe falcata	Nag and Johri (1971)
Scurrula pulverulenta	Bhojwani and Johri (1970)
Taxillus vestitus	Nag and Johri (1971)
Poaceae	
Oryza sativa	Nakano et al. (1975), Bajaj et al. (1980)
Rosaceae	
Prunus persica	Liu and Liu (1980)
Pyrus malus	Mu et al. (1977)
Rutaceae	
Citrus grandis	Wang and Chang (1978)
C. sinensis	Gmitter et al. (1990)
Santalaceae	
Exocarpus cupressiformis	Johri and Bhojwani (1965)
Santalum album	Lakshmi Sita et al. (1980)
Solanaceae	
Lycium barbarum	Li and Zhang (1990)

al., 1990), apple (Mu et al., 1977), pear (Zhao, 1983) and sandalwood (Lakshmi Sita et al., 1980) are especially encouraging.

8.4.1. Factors affecting shoot-bud differentiation

The endosperm may form buds either directly, or it may first proliferate into a callus mass followed by organogenesis. For shoot-bud differentiation from endosperm tissue an exogenous cytokinin is always necessary. In 'seed' cultures of *Scurrula pulverulenta* on a medium supplemented with IAA, kinetin and CH, 15% of the explants showed surface

callusing of the endosperm. In another 13% cultures green buds developed from the unproliferated regions of endosperm (Fig. 8.4A). Omission of CH from the medium, promoted shoot bud differentiation to 26%. Further studies revealed that kinetin alone was sufficient to induce shoot bud differentiation; CH and IAA counteracted the effect of kinetin. Increase in the concentration of kinetin from 1 to 10 mg l^{-1} brought about corresponding increase in the number of cultures forming buds from endosperm. *Taxillus vestitus* endosperm showed similar response (Nag and

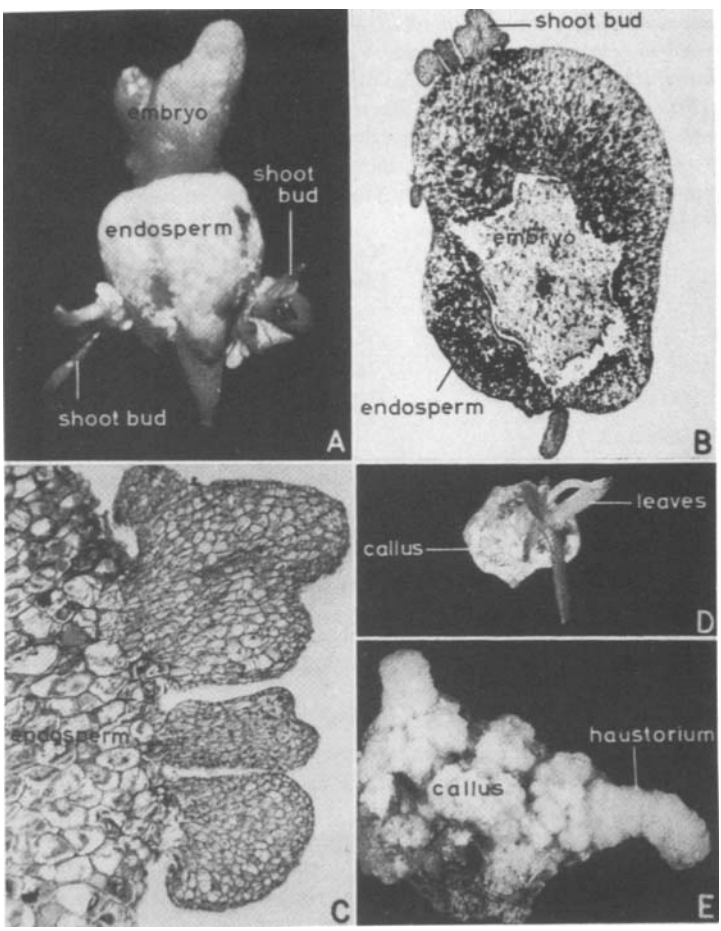

Fig. 8.4. *Scurrula pulverulenta.* (A) A 16-week-old culture of 'seed' on White's medium supplemented with zeatin (10^{-5} M); note the differentiation of shoot buds from endosperm. (B) Transection of a cultured 'seed' showing buds arising from the periphery of endosperm. (C) Portion from (B) to demonstrate the peripheral origin of buds. (D,E) Endosperm buds subcultured on a medium enriched with IAA, kinetin and CH. The callused buds have formed a shoot (D) and haustoria (E) (after Bhojwani and Johri, 1970).

Johri, 1971). However, in *Dendrophthoe* and *Leptomeria* kinetin-induced bud differentiation occurred only in the presence of a low concentration of an auxin, such as IAA or IBA. Here, an increase in the concentration of kinetin also enhanced the response, whereas increase in the concentration of auxin suppressed it (Nag and Johri, 1971). Endosperm pieces of *T. vestitus* pre-soaked in 0.025% kinetin solution for 24 h differentiated buds even on a cytokinin-free medium. Of the various cytokinins tested for bud formation from endosperm, 2ip has proved most effective, and triacanthine the least (Fig. 8.5) (Bhojwani and Johri, 1970; Johri and Nag, 1970). Unlike *Scurrula*, in *Dendrophthoe* and *Leptomeria acida* CH had a promotive effect on shoot bud differentiation (Nag and Johri, 1971). In *D. falcata*, with 2000 mg l^{-1} of CH bud formation occurred in 46% cultures compared to 32% in its absence (Nag and Johri, 1971).

In systems other than endosperm where cytokinins have been reported to favour bud formation it is a case of promotion rather than induction. In this connection Miller (1961) remarked that the fact to be kept in mind is

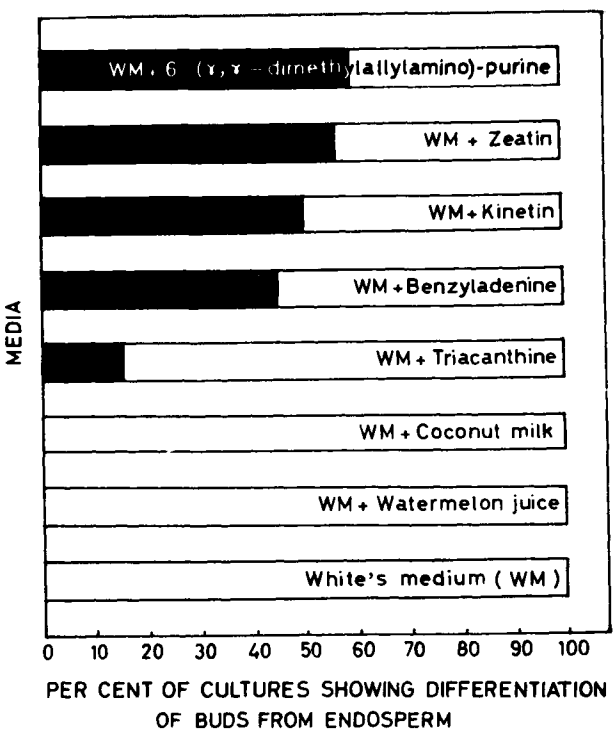

Fig. 8.5. *Scurrula pulverulenta*. The influence of various cytokinins, coconut milk, and watermelon juice on shoot-bud formation from endosperm. Solid portion of bars represent percentage cultures forming buds (after Bhojwani and Johri, 1970).

that all the plants reported to respond to kinetin in terms of increased budding show some formation of shoot structures even in the absence of kinetin. Thus, a cytokinin is known to cause bud formation only in those cases which possess an inherent tendency for such a morphogenesis. The endosperm tissue neither forms buds in vivo nor in cultures in the absence of a cytokinin. It is, therefore, a distinct case of bud induction by cytokinins.

Young endosperm (12–14 weeks post anthesis) of *Citrus grandis*, *C. sinensis* and *C.* × *paradisi* produced amorphous callus on Murashige and Tucker (1969) medium (MT) containing 2 mg l^{-1} 2,4-D, 5 mg l^{-1} BAP and 1000 mg l^{-1} CH. Doubling the salt concentration in MT (2MT) and supplementing it with GA_3 induced the differentiation of globular embryos from the calli of *C. grandis* and *C. sinensis*. However, the optimum medium for embryogenesis was 2MT containing 2 mg l^{-1} GA_3, 0.25 mg l^{-1} BAP, 2 mg l^{-1} adenine and 500 mg l^{-1} CH (Gmitter et al., 1990). On this medium dicotyledonous embryos developed only in the case of *C. sinensis*. These embryos germinated on 2MT + 5 mg l^{-1} GA_3 but root development did not occur. Plantlets were recovered by micrografting the shoots onto the diploid rootstock seedlings. Eventually triploid plants of endosperm origin were established in soil (Gmitter et al., 1990).

In *Taxillus*, the embryo had an adverse effect on bud differentiation from endosperm. The number of buds per culture and number of cultures forming buds (Fig. 8.6) was comparatively higher when endosperm halves were cultured compared to that when endosperm with the embryo

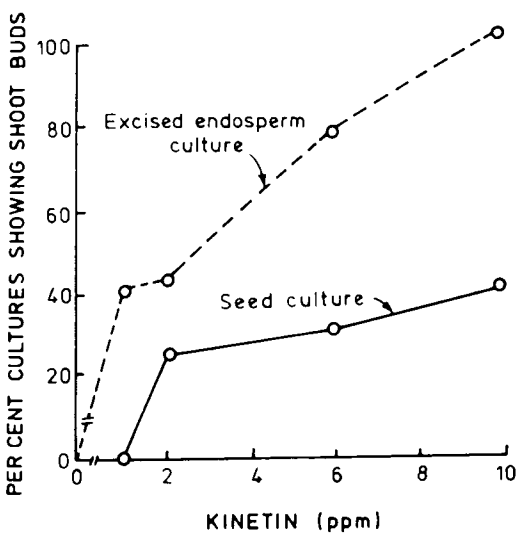

Fig. 8.6. *Taxillus vestitus*. Influence of kinetin and the presence of embryo on shoot bud differentiation from endosperm (after Johri and Nag, 1970).

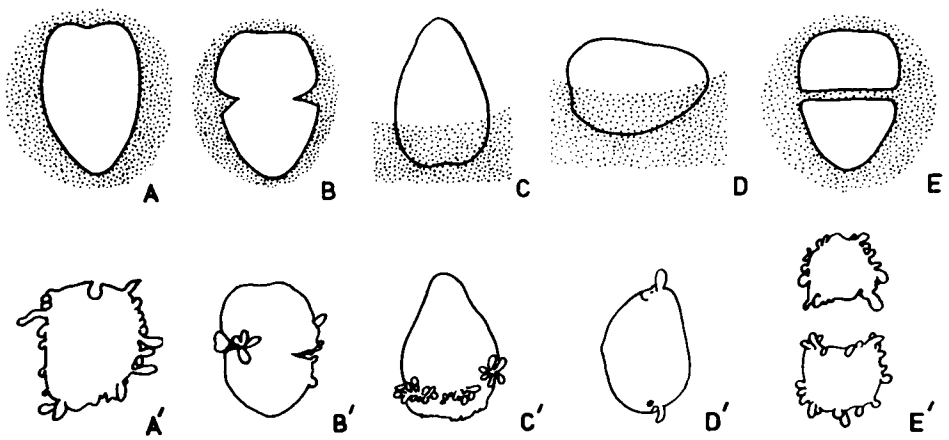

Fig. 8.7. *Taxillus vestitus*. Effects of injury and the position of explant on semi-solid medium on bud differentiation from endosperm. Stippled portion represents the medium. (A–E) are the treatments and (A′–E′) their responses. (A,A′) Half-split (longitudinally) endosperm with cut surface in contact with medium. (B,B′) Two V-shaped pieces removed from endosperm, and planted as in (A). (C,C′) Longitudinal half of endosperm, planted vertically with half-portion embedded in the medium. (D,D′) Same as (C) but planted horizontally. (E,E′) Half-split endosperm cut into two transverse pieces, and planted as in (A). Invariably, buds arise close to the injury, just above the level of the medium (after Nag and Johri, 1971).

intact was cultured. However, subsequent development of buds was better in the presence of embryo. Injury to endosperm enhanced the response (Fig. 8.7). In addition, the position of the endosperm on the culture medium had a significant effect on the differentiation and distribution of buds (Fig. 8.7). Irrespective of the position of endosperm on the medium, the buds invariably first appear along the injury (Fig. 8.7A–E, A′–E′). When the half-split endosperm of *T. vestitus* was planted with the cut surface in contact with the medium (White's medium with 5 mg l^{-1} kinetin) 100% cultures formed 12–18 buds (Fig. 8.7AA′), whereas if the cut surface was kept away from the medium only one to three buds developed in 30% cultures. If the half-split endosperm was planted vertically or horizontally the buds first appeared from the epidermal cells near the cut end situated immediately above the level of the medium (Fig. 8.7CC′, DD′). Thus, the site of bud formation in these segments was almost predictable. Such a system is ideal for studying cellular changes underlying shoot-bud differentiation.

8.4.2. Ontogeny of shoot buds

The differentiation of shoot buds from intact endosperm in *Scurrula pulverulenta* occurs as a result of meristematic activity of the epidermal

as well as hypodermal cells (Fig. 8.4B,C) which are, compared to the cells of the deeper region of endosperm, smaller and richly cytoplasmic (Bhojwani and Johri, 1970). In *Taxillus* the buds originated exclusively from the epidermal cells (Johri and Nag, 1970). The initiation of buds had no correlation with the differentiation of vascular elements in the parent tissue (Fig. 8.4B,C). The young buds appeared as mere parenchymatous outgrowths and, subsequently, vascular traces differentiated in them. The endosperm tissue per se, however, did not show any vascular differentiation.

In *Scurrula* the buds formed directly from the endosperm (without callusing) showed limited growth in situ. If excised and planted on a fresh medium of the same composition, the buds instead of growing into larger shoots, proliferated into a compact callus. These calli as well as those derived by direct proliferation of endosperm tissue, differentiated shoot buds and/or haustoria (Fig. 8.4D,E). In *Dendrophthoe*, *Nuytsia* and *Taxillus* a tissue of unlimited growth was obtained on White's medium fortified with 5 mg l^{-1} IBA, 5 mg l^{-1} kinetin and 2000 mg l^{-1} casein hydrolysate. If IBA was replaced by 2.5 mg l^{-1} IAA the endosperm calli in *Dendrophthoe* and *Taxillus* differentiated buds and haustoria. An increased concentration of kinetin enhanced shoot formation and suppressed haustorium formation, whereas an increase in the concentration of IAA promoted haustorium formation and adversely affected shoot differentiation (Nag and Johri, 1971). Being stem parasites, *Dendrophthoe*, *Scurrula* and *Taxillus* lack a true root system; instead, haustorial structures are formed.

In autotrophic taxa organogenesis from endosperm tissue is usually preceded by callusing of the endosperm. The calli of *Croton* and *Jatropha* grow best on a medium supplemented with 2,4-D, kinetin, and yeast extract but on this medium the tissue remains unorganized (Bhojwani, 1966). Upon transfer to the basal medium the endosperm callus of *Croton* forms numerous roots which grow to various lengths (Bhojwani and Johri, 1971). Shoot formation did not occur in this tissue. The endosperm calli of *Jatropha* (Srivastava, 1971) and *Putranjiva* (Srivastava, 1973) form roots as well as shoots. In the latter, root and shoot may differentiate as a bipolar axis (Fig. 8.8). On a medium supplemented with IAA, kinetin, and CH, which supports best growth of *Putranjiva* endosperm tissue, the callus assumed a nodulated appearance followed by organogenesis. After 8 weeks about 80% of the cultures formed buds (Srivastava, 1973). Within the next 8 weeks the shoots attained a height of about 4 cm and bore three or four pairs of leaves. In about 20% cultures the root-shoot axis was quite distinct. The plantlets of endosperm origin were not transplanted to the soil.

Fig. 8.8. *Putranjiva roxburghii.* Plantlet formation from endosperm callus (after Srivastava, 1973).

In endosperm cultures of *Citrus* (Wang and Chang, 1978) and *Santalum* (Lakshmi Sita et al., 1980) triploid plants developed through somatic embryogenesis.

It is important that most of the organs and plantlets differentiated from endosperm tissue are triploid, and morphologically and anatomically comparable to their counterparts formed by the zygotic embryo. Masuda et al. (1977) reported that plants originating from the endosperm callus of parsley were predominantly diploid. From their work it is not very clear whether the callus actually developed from endosperm or any other tissue of the seed.

8.5. APPLICATIONS OF ENDOSPERM CULTURE

Triploid plants are usually seed-sterile and, consequently, undesirable for plants where seeds are of commercial importance. However, there are instances where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. It is with reference to these plants that

triploidy can be exploited for plant improvement. Some of the economically important plants whose triploids are already in commercial use include several varieties of apple, banana, mulberry, sugar beet, tea, and watermelon (Elliott, 1958). Triploid plants of *Petunia axillaris* raised from microspores, were more vigorous and much more ornamental than their parental anther-donor diploids (Gupta, 1982). Natural triploids of tomato produced larger and tastier fruits than the diploid counterpart (Kagan-Zur, 1990).

Triploid quaking aspen (*Populus tremuloides*) has more desirable pulpwood characteristics than its diploid.

Triploid production by the conventional techniques involves chromosome doubling followed by crossing the tetraploids with their diploids. This approach is not only laborious but in many cases may not be possible due to high sterility of tetraploids (Gupta, 1982; Esen and Soost, 1973). The demonstration of the cellular totipotency of triploid cells of endosperm has created a new approach to the production of a large number of triploids in one step.

8.6. CONCLUDING REMARKS

It has been established that triploid cells of endosperm are totipotent. Plants have been regenerated from endosperm tissue of some economically important taxa such as, apple, pear, kiwifruit, citrus and sandalwood. Although the chromosome number of most of these plants showed variation and mixoploidy (Mu and Liu, 1979; Gu et al., 1987; Mu et al., 1990) all the plants derived from the endosperm of *Citrus sinensis* were uniformly triploid with $2n = 3x = 27$ (Gmitter et al., 1990).

Growth promoting factors of the endosperm are not specific to the embryo of the same species (Williams and De Lautour, 1980) (also see Section 11.2.4). Even cultured endosperm tissue has been shown to retain the ability to support the growth of young embryos (Pieczure, 1952; Norstog, 1956a). This feature of the cultured endosperm tissue may allow rearing of full plants from isolated very young hybrid embryos which normally abort prematurely.

Endosperm is a reservoir of stored food which is mobilized during embryo development and germination. It, therefore, provides an excellent experimental system to study biosynthesis and metabolism of these natural products. Chu and Shannon (1975) have described maize endosperm callus as a useful model system to study in vivo starch biosynthesis. Endosperm callus of coffee is shown to synthesize caffeine (Keller et al., 1972). The levels of the alkaloid in the callus increases by a factor of three after 2 weeks and by a factor of six after 4–5 weeks of culture.

Chapter 9

Variant Selection

9.1. INTRODUCTION

For a considerable time, the frequently observed variability in plant populations regenerated from tissue cultures was ignored, and organogenic and embryogenic differentiation in callus and suspension cultures was regarded as a potential method for rapid clonal multiplication of plant species. This being the fastest in vitro method for asexual multiplication of several plant species (Chapter 16), efforts are continuing to develop methods for large scale production of somatic embryos, in bioreactors, which can be converted into synthetic seeds for mechanized planting in the field (Chapter 6). However, a major obstacle in realization of this goal is the inherent genetic instability of cultured cells, endangering the clonal nature of the plants produced by this method, and so far there is no effective method known to control this phenomenon. The extent of variation in tissue culture raised plants is often so great that tissue culture is proving to be a rich source of genetic variability, suitable for crop improvement (Sections 9.3, 9.7).

The variations observed in tissue cultures may be due to physiological changes induced by the culture conditions. Such variations are temporary and disappear when the culture conditions are removed. However, sometimes the altered phenotype may persist over a longer period, well after the inductive conditions are withdrawn and may be passed from one cell generation to another. Such variations, which are also ultimately reversible, and are not sexually transmitted, are caused by epigenetic changes. The cultured cells also exhibit genetic variation (Table 9.1). Thus, the plants regenerated from tissue cultures show a range of variability, ranging from temporary changes in the phenotype to sexually heritable mutations. Although tissue culture induces the most obvious changes in the nuclear genome of plants, the genetic make-up of the cytoplasmic organelles can also be altered (Section 9.5).

The first formal report of morphological variation, observed in plants regenerated from sugarcane tissue cultures, was published in 1971, by Heinz and Mee. Since then several useful variants of sugarcane resistant to fungal and viral diseases have been isolated in Fiji, Hawaii and Tai-

TABLE 9.1

Criteria for the classification of tissue culture variation as genetic or epigenetic

Feature	Genetic	Epigenetic
1. Frequency of occurrence	Low; 10^{-5}–10^{-7} per cell generation	High; 10^{-3} per cell generation
2. Nature of change	Random	Directed
3. Stability of change in somatic lineages	Usually stable	Stable; but reversal can occur at high rates
4. Sexual transmission of the change	Yes	No

Based on Stafford (1991).

wan. This was followed by similar observations with other crops. Larkin and Scowcroft (1981) reviewed the scattered literature on the occurrence of variability in tissue cultures and suggested that this variation among regenerated plants could be useful for the development of new cultivars. This paper proved to be a major thrust in the exploitation of tissue culture-generated variability in crop improvement. To date, at least ten new varieties of crop plants, based on the utilization of tissue culture derived variability, have been released (Table 9.2).

9.2. TERMINOLOGY

Genetic variants selected through tissue culture have been variously referred to as calliclones (from callus cultures; Skirvin, 1978), protoclones (from protoplast cultures; Shepard et al., 1980) and phenoclones (Sibi, 1976). Larkin and Scowcroft (1981) proposed a general term 'Somaclonal variation' to describe the genetic variation in plants regenerated from any form of cell culture. Accordingly, the plants derived from cell and tissue cultures are termed 'somaclones', and the plants displaying variation as 'somaclonal variants'. Evans et al. (1984) suggested that the plants regenerated from cell cultures of gametic origin be termed 'gametoclones' to distinguish them from somatic cell derived regenerants.. Some examples of gametoclonal variation are described in Section 7.9.2. In this chapter some prominent examples of somaclonal variants showing agronomic promise are described, and the possible causes and origin of these variations are discussed.

The plants regenerated from tissue cultures are designated as R_0 generation and their successive sexual generations as R_1, R_2, and so on.

TABLE 9.2

Crop species in which new varieties have been developed from somaclones

Crop	Country and institution	New trait	Reference
Geranium	Dept. Horticulture, Pardue Univ., USA	Vigour and attractive flowers	Skirvin and Janick (1976b)
Sweet potato	North Carolina Research Service, USA	Colour, shape and baking quality of roots	Moyer and Collins (1983)
Sugarcane	Sugarcane Research Centre, Fiji	Yield and disease resistance	Cited in Daub (1986)
Maize	Molecular Genetics, USA	Tryptophan content	Anonymous (1987)
Tomato	DNA Plant Technology of New Jersey, USA	Dry matter content	Evans (1989)
		Disease resistance	Evans (1989)
Rice	Plantech Research Institute, Japan	Yield	Anonymous (1989)
	Univ. Agricultural Sciences, Godollo, Hungary	Disease resistance	Heszky and Simon-Kiss (1992)
Celery	DNA Plant Technology of New Jersey, USA	Processing, yield efficiency	Orton and Romig (1990)
Brown mustard	ICAR, New Delhi India	Yield	Katiyar and Chopra (1995)

Based on Semal and Lepoivre (1990).

9.3. SELECTED EXAMPLES OF SOMACLONAL VARIANTS

To be of agronomic use, a somaclonal variant must fulfil certain basic requirements (Gunn and Day, 1986): (i) it must involve useful character(s); (ii) it must be superior to the parents in the character(s) in which improvement is sought; (iii) the improved character(s) must be combined with all other desirable characters of the parent; and (iv) the variation must be inherited stably through successive generations by the chosen means of propagation.

Two approaches have been followed to recover somaclonal variation from cultured cells: (1) plants are regenerated from tissue or cells cultured for various periods and screened for the desired traits, and (2) for a

number of characters, such as resistance to a fungal toxin, a herbicide, a pollutant, high salt concentrations and extremes of temperature, the selection might also be made at the cell level. An advantage of the latter method is that it permits screening millions of cells in a very small space with a comparatively small input of effort and resources. However, it cannot be taken for granted that the phenotypes expressed by selected cells would also be expressed by plants regenerated from them. Similarly, certain genes may not be active at the single cell level, and specific tissue functions may occur only when the cell is an integral part of the intact plant. Therefore, the character for which cells have been selected must be rechecked at the whole plant level.

In vitro selection has been mostly made at the callus level. However, selection of single cells is expected to give better results because of a better contact of cells with the selection agent and regeneration of solid mutants.

9.3.1. Selection at plant level

(i) *Sugarcane*. The potential use of somaclonal variation in crop improvement was first demonstrated in sugarcane (Heinz et al., 1977). Plants regenerated from calli of sugarcane exhibit marked phenotypic and genotypic variations, such as cane yield, tillering, fibre content and numerous fine morphological characters. Nickell's group in Hawaii and Krishnamurthi and his co-workers in Fiji isolated, through tissue culture, subclones of established cultivars of sugarcane which showed resistance to eyespot disease (caused by *Helminthosporium sacchari*), Fiji disease (caused by an aphid transmitted virus) and downy mildew (caused by *Sclerospora sacchari*).

Krishnamurthi (1974) and Krishnamurthi and Tlaskal (1974) were able to isolate variants which showed higher resistance to Fiji disease and downy mildew than their parent clones. 'Pindar' is an important sugarcane cultivar of Fiji; it is suited to poor soils but is highly susceptible to Fiji disease. Of the 38 subclones of this cultivar obtained through tissue culture and screened for Fiji disease, four proved resistant. The resistance was maintained through several cane generations in the field. In field trials, three of these lines gave poor yields but one ('Pindar 70-31') performed as well or even better than the original cultivar. Pindar 70-31 also carried high resistance to downy mildew. This work led to the release of a new cultivar 'Ono' with higher yield and greater disease resistance (see Daub, 1986). Heinz et al. (1977) observed that mutagenic treatments did not increase the frequency of variants resistant to eyespot disease over the frequency with which they appeared spontaneously.

Q101 is an agronomically valuable sugarcane cultivar of Australia but for its susceptibility to eyespot disease. Larkin and Scowcroft (1983) isolated somaclones of this cultivar which were highly resistant or nearly immune to the fungal toxin but morphologically identical to the parent variety. Indeed, such small modifications in otherwise outstanding varieties would be especially useful.

(ii) *Potato.* Somaclonal variations have been studied extensively in potato. Most of the popular cultivars of potato are highly heterozygous and, therefore, their improvement by conventional breeding practices is difficult. In this regard tissue culture techniques have proved useful. Shepard et al. (1980) screened about 2500 regenerants from protoplast cultures of the American cultivar 'Russet Burbank' and selected about 60 protoclones which displayed stable improvement of agronomic traits, including resistance to late blight (caused by *Phytophthora infestans*) and early blight (caused by *Alternaria solani*). Similarly, two variants regenerated from a single callus of the cultivar 'Bintje' showed field resistance to late blight (Fourage et al., 1987). Several other potato somaclones expressing higher resistance to common scab than the parental cultivar have also been identified (Thompson et al., 1986a; Fourage et al., 1987). Gunn and Day (1986) isolated protoclones of potato which had tuber yields equal to or better than the parents and were disease resistant.

Thompson et al. (1986a) found potato protoclones which were field-resistant to potato virus Y (PVY) and potato leafroll virus (PLRV). Selection of somaclones allowing low aphid colonization may indirectly contribute to the control of a number of aphid transmitted virus diseases, such as PRLV and PVY. A somaclone variant of the potato cultivar 'Roxane', selected on the basis of its low colonization by *Myzus persicae* (Fig. 9.1), in the green house, showed field resistance to PVY, with a lower percentage of virus-infected tubers than the parental cultivar (see Semal and Lepoivre, 1990).

At the Rothamstead Plant Breeding Station (UK) plants were regenerated from 14 cultivars of potato which varied in such characters as yield, tuber shape and tuber colour (Bright et al., 1985; Wheeler et al., 1985). A white skinned tuber trait, obtained as a variant from the red skinned 'Desiree' variety, has been stably expressed through tuber generations. Chromosomally normal plants with properties differing from the parental line have been obtained and evaluated for field trails. Similarly, a somaclone of the cultivar 'Bintje' showed improved field performance during four successive years, with a yield of marketable tubers (Fig. 9.2) significantly higher (15–20%) than the parent cultivar (Meulemans et al., 1987).

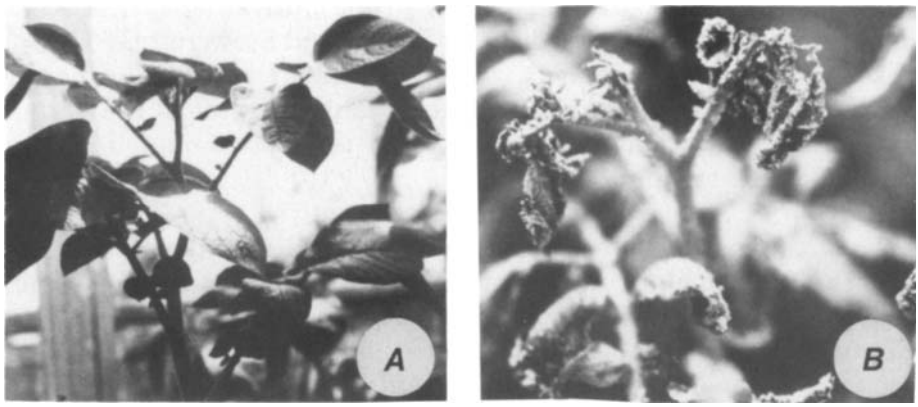

Fig. 9.1. Colonization by *Myzus persicae* of a somaclonal variant (A) compared to the parental potato cv. Roxane (B) (after Semal and Lepoivre, 1990).

Cassells et al. (1983) demonstrated that plants regenerated directly from stem pieces, without a callus phase, also carry considerable variation. Direct regeneration of plants may be a better source of useful variants than protoplast or callus cultures which induce gross abnormalities.

Due to difficulties in performing sexual crosses (limited flowering or sterility of many cultivars) and because of the tetraploid status of most potato genome, no genetic analysis of potato somaclones has been presented so far (Semal and Lepoivre, 1990).

(iii) *Banana*. Tissue culture raised banana plants exhibit a range of morphological variations, including dwarfism, abnormal leaf, pseudostem pigmentation, persistence of flowers and split fingers (Israeli et al., 1991). Drew and Smith (1990) and Smith and Drew (1990a) reported that 22% of the total population of plants regenerated from callus cultures were dwarf, and this trait was retained through five vegetative cycles.

All commercial banana cultivars of Taiwan are susceptible to Race 4 of *Fusarium oxysporum* f. sp. *cubense*. The traditional breeding methods to select *Fusarium* wilt resistant lines of Cavendish banana were unsuccessful (Su et al., 1986). However, several somaclones of this cultivar showed very high resistance to the pathogen (Hwang, 1991). Cultures were initiated from shoot-tips of suckers of the susceptible cultivar and the adventitious shoot buds produced by them were put through 6–7 monthly cycles of multiplication. Of about 20 000 of these plants screened in the field, 6 showed very high resistance to the pathogen (0–10% incidence of disease compared to 60% in the parent cultivar). However, all the 6 variants were agronomically inferior. Through a repeat course of in

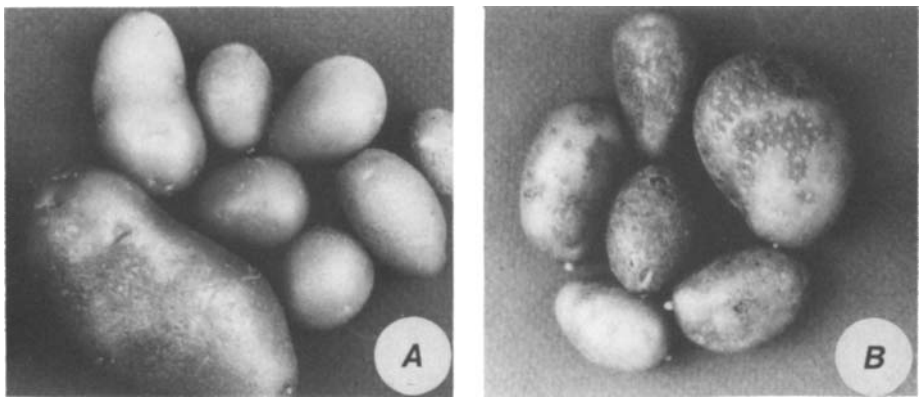

Fig. 9.2. Symptoms of common scab expressed in a somaclonal variant (A) compared to the parental potato cv. Desiree (B) (after Semal and Lepoivre, 1990).

vitro shoot multiplication, as before, from shoot-tips of the resistant variants, Hwang (1991) was able to select at least 3 lines with improved yield. These plants also retained the improved resistance to the disease. One of the three variants (GCTCV-215–1) combined the high resistance trait (5.2–17.2% incidence of disease) with the agronomic traits comparable to that of the parent cultivar.

(iv) *Strawberry*. Toyoda et al. (1991) have reported the selection of strawberry plants with stable resistance to *F. oxysporum* f. sp. *fragriae* by directly transferring the plants regenerated from leaf callus of a susceptible variety to soil infected with the pathogen. In the soil, where almost all the plants of the parent cultivar (control) died after showing the symptoms of the disease, 2 out of the 1225 regenerants grew well and produced 3 daughter generations. Direct application of the pathogen to the roots of the third runner generation plants of the putative somaclone followed by their planting in pathogen infested soil did not induce disease symptoms, and the plants continued to reproduce normally. Under identical conditions all the control plants died within 3 weeks.

(v) *Geranium*. This ornamental plant is traditionally propagated from leaf cuttings. Skirvin and Janick (1976a) compared the plant populations raised from root, stem and petiole cuttings in vivo and those differentiated from their callus cultures. Whereas the plants from in vivo stem cuttings were uniform, those from in vivo root and petiole cuttings and from callus were quite variable. They have released a new scented variety 'Velvet Rose' from a calliclone obtained from the cultivar 'Rober's

Lemon Rose' (Skirvin and Janick, 1976b). The new cultivar has double the chromosome number of the parent and is selected for its general attractiveness and vigour.

(vi) *Paspalum dilatatum*. It is a C_4 forage grass, showing poor seed-set and is susceptible to ergot under New Zealand conditions. Since the productive biotype is an obligate apomict and lacks sexual cross compatibility, the traditional breeding method for its improvement is of little use (Burton, 1962). Attempts to induce variation using irradiation have not been successful (Bashaw and Hoff, 1962). Therefore, its genetic improvement through somaclonal variation would be of particular importance. Davies et al. (1986a) recovered somaclones in batches of plants regenerated from long-term embryogenic callus of this grass. The regenerants showed extensive phenotypic variation in field trials but none displayed resistance to ergot, and seed-set was low. These findings were recently confirmed by Burson and Tischler (1993). While most of the somaclones screened by them displayed low seed germination, one of the variants had significantly higher germination than the common dallisgrass.

(vii) *Tomato*. Somaclonal variants of tomato for several characters, such as fruit colour, plant architecture and characters for mechanical harvesting, have been isolated by Evans and Sharp (1983; see also Evans et al., 1984b; Evans and Bravo, 1986). Detailed genetic analysis of these variants has revealed stable genetic changes caused by single gene mutations, fruit colour being recessive and *Fusarium* resistance as dominant trait. One of the somaclones of tomato, with very high (20%) dry matter content, an enhanced taste and a better texture and colour has been registered as a new variety by DNA Plant Technology Corporation, USA (Evans, 1989).

(viii) *Celery*. Wright and Lacy (1988) observed that the plants regenerated from tissue cultures of celery ranged from highly resistant to highly susceptible to three fungal and one bacterial pathogens. A celery somaclone (UC-T3) exhibiting significantly higher resistance to *F. oxysporum* f.sp. *apii* Race 2 than the parent cultivar (Tall-Utah 527OR) was isolated by Heath-Pagliuso et al. (1988, 1989). Through detailed breeding experiments, Heath-Pagliuso and Rappaport (1990) established that the improved resistance of the UC-T3 was heritable and probably controlled by two genes.

(ix) *Rice*. Several reports of the selection of somaclonal variants from somatic and gametic tissue cultures of rice have been published (Oono,

1978; Chaleff, 1980; Chen et al., 1980; Fukui, 1983). Oono (1988) reported that in 72% of the 762 regenerated lines that were screened, variation were noted for such characters as seed viability, plant height, tillering and chlorophyll deficiency, and somaclonal variants were stable through at least one seed generation. From a single callus formed in seed cultures, Fukui (1983) raised 12 plants with 90% fertility. The progeny of these plants showed variation for leaf colour, early heading, albino and short culm. The author has concluded that the mutations for the four traits occurred independently and successively.

Anther and pollen cultures are a good source of variation (Section 7.9.2). The pollen plants being haploid, stable variants can be obtained in one generation.

Schaeffer et al. (1984) obtained useful variants by selfing anther-derived plants of rice. Chinese workers have produced new varieties of many cereals, including rice, through anther culture. Through a combination of anther culture and somatic tissue culture, Heszky et al. (1992) have evolved an improved variety of rice, 'DAMA'. The 'pollen haploid somaclone' method (PHS method) is summarized in Fig. 9.3.

9.3.2. Selection at cell level

There are two basic strategies for in vitro mutant selection (Gonzales, 1994): (i) Single-step selection: the selection agent is used at a concentration two or three times the MIC (minimum concentration resulting in 100% inhibition). This is the simplest method with clear results and the least likelihood of escapes. (ii) Multiple-step selection: this strategy may be applied where single-step selection does not work. In this approach the concentration of the selective agent is considerably less than the MIC, usually near I_{50} (concentration resulting in 50% inhibition). The concentration is gradually increased in frequent successive subcultures to allow the fast growing resistant cells to outgrow the sensitive wild-type cells. By this process it should be possible to eventually obtain vigorously growing cultures at the inhibitor level well above the MIC established for the original unselected cultures.

(i) *Disease resistance*. Resistance to most plant diseases is not mediated by single genes and may not be detectable in cultures. However, sometimes the toxic effect of a phytotoxin on tissue, cell or protoplast culture is equivalent to its effect on the whole plant (Earle, 1978). Furthermore, if a phytotoxin or toxins is/are the sole determinant of pathogenicity, direct selection of a large population of disease resistant totipotent cells is possible by incorporating the toxin into the medium. This hy-

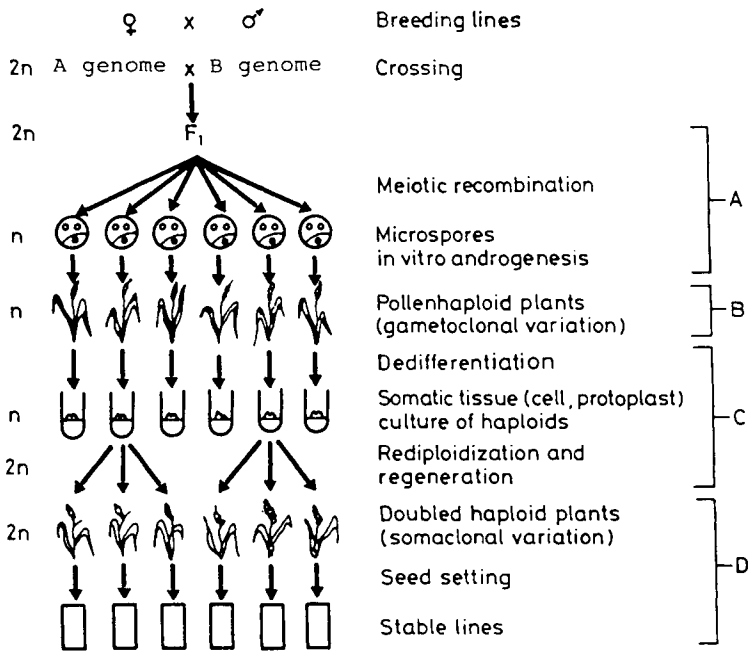

Fig. 9.3. Diagrammatized summary of the pollen haploid somaclonal method (PHS method) for increasing genetic variability in breeding material (after Heszky et al., 1989).

pothesis received experimental support when Carlson (1973) reported that plants regenerated from tobacco protoplasts (treated with the mutagen ethyl methane sulphonate; EMS) selected for resistance to methionine sulfoximine (MSO) showed enhanced resistance to *Pseudomonas tabaci*. While MSO is no longer considered to be an analogue of the *P. tabaci* phytotoxin (Thanutong et al., 1983), this observation raised the possibility of selecting phytotoxin-resistant cells/plants in vitro. Excellent reviews on in vitro selection of disease resistance are written by Daub (1986) and Van den Bulk (1991). Some examples of stable disease resistant somaclones obtained by selection at plant and cell levels are listed in Table 9.3.

Maize plants with Texas male sterile cytoplasm (cms-T) are sensitive to the toxin of *Drechslera maydis* Race T, which causes southern corn leaf blight. With the objective of raising resistant male sterile maize, Gengenbach and his associates made recurrent selection of non-mutagenized embryo callus with progressively higher concentrations of T-toxin and isolated toxin-resistant calli (Gengenbach and Green, 1975) and later whole plants (Gengenbach et al., 1977). These findings were later confirmed by Brettell and Ingram (1979) and Brettell et al. (1980). However,

TABLE 9.3

Some examples of somaclones showing heritable disease resistance obtained by selection at plant level or at cell level

Crop	Pathogen	Selective agent	Culture	Resistance system	References observed
Selection at cell level					
Alfalfa	*Fusarium oxysporum* f.sp. *medicaginis*	Culture filtrate	Callus	Increased resistance	Hartman et al. (1984), McCoy (1988)
Barley	*Helminthosporium sativum*	Crude toxin	Callus	Resistance	Chawla and Wenzel (1987a)
Eggplant	Little leaf disease (mycoplasma-like organism)	Pathogen	Callus from infected tissue	No symptoms	Mitra and Gupta (1989)
Maize	*H. maydis*	Hm toxin	Callus	Resistance	Gengenbach et al. (1977)
Oats	*H. victoriae*	Victorin	Callus	Resistance to victorin	Rines and Luke (1985)
Rape	*Phoma lingam*	Culture filtrate	Suspension cells, embryo cultures	Increased resistance	Sacristan (1982, 1985)
Rice	*H. oryzae*	Crude toxin	Callus	Increased resistance	Ling et al. (1985), Vidyasekaran et al. (1990)
Sugarcane	*H. sacchari*	Toxin	Callus	Increased resistance	Heinz et al. (1977), Larkin and Scowcroft (1983)
Tobacco	*Pseudomonas syringae* pv. *tabaci*	Methionine Sulfoximine	Cells, Protoplasts	No chlorotic haloes	Carlson (1973)
		Crude toxin	Callus	Resistance	Thanutong et al. (1983)
Tobacco	Tobacco mosaic virus	Virus	Callus from infected tissue	Reduced virus multiplication and restricted translocation	Murakishi and Carlson (1982), Toyoda et al. (1985, 1989)

TABLE 9.3 (*continued*)

Crop	Pathogen	Selective agent	Culture	Resistance system	References observed
Tomato	*F. oxysporum* f.sp. *lycopersici*	Culture filtrate	Callus culture	Tolerance to filtrate	Scala et al. (1984)
		Fusaric acid	Protoplasts	Resistance	Shahin and Spivey (1986)
Tomato	Tobacco mosaic virus	Virus	Infected explants	Tolerance	Cassells et al. (1986)
Wheat	*H. sativum*	Crude toxin	Callus	Resistance	Chawla and Wenzel (1987b) Chawla and Kole (1990)
Selection at plant level					
Banana[a]	*F. oxysporum* f.sp. *cubense*	–	Meristem	Increased resistance	Hwang and Ko (1988), Hwang (1991)
Celery	*F. oxysporum* f.sp. *apii*	–	Embryogenic suspension culture, callus	Increased resistance	Ireland and Lacy (1987), Wright and Lacy (1988), Heath-Pagliuso et al. (1988), Heath-Pagliuso and Rappaport (1990)
Maize	*H. maydis*	–	Callus	Resistance to Hm toxin	Brettell et al. (1980)
Peach[a]	*Xanthomonas campestris* pv. *pruni*	–	Callus	Increased resistance	Hammerschlag and Ognjanov (1990)
Potato[a]	*Alternaria solani*	–	Protoplasts	Smaller lesions	Matern et al. (1978)
Potato[a]	*Phytophthora infestans*	–	Protoplasts	Increased resistance	Shepard et al. (1980)
			Protoplasts, callus, explants	Increased resistance	Meulemans and Fourage (1986), Meulemans et al. (1986)
Potato[a]	Potato virus X, Potato virus Y	–	Callus, explants	Field resistance	Cassells et al. (1986, 1987)

Potato[a]	*Streptomyces scabies*	–	Explants	Increased resistance	Evans et al. (1986)
Rape*	*Phoma lingam*	–	Callus, embryogenic suspension	Increased tolerance	Sacristan (1982, 1985)
Rice*	*H. oryzae*	–	Callus	Resistance	Ling et al. (1985)
Strawberry[a]	*F. oxysporum* f.sp. *fragariae*	–	Callus	Resistance	Toyoda et al. (1991)
Sugarcane[a]	*Fiji virus*	–	Callus	Increased resistance	Krishnamurthi and Tlaskal (1974), Heinz et al. (1977)
Sugarcane*	*H. sacchari*	–	Suspension culture, Explants	Increased resistance	Larkin and Scowcroft (1983)
Tomato	*Tobacco mosaic virus*	–	Explants	Resistance	Barden et al. (1986)
Tomato	*F. oxysporum* f.sp. *lycopersici* race 2	–	Callus	Resistance	Smith and Murakishi (1987), Miller et al. (1985), Evans (1987)
Tomato*	*F. oxysporum* f.sp. *lycopersici* race 2	–	Protoplasts	Resistance	Shahin and Spivey (1986)

[a]Transmitted after vegetative propagation.
*In the studies marked with an asterisk, disease resistant plants were obtained from non-selected control plants of in vitro selection experiments.

in both cases toxin-resistance was accompanied by restoration of male fertility. In a later experiment, Umbeck and Gengenbach (1983) found that of the 169 plants regenerated from calli of T-toxin sensitive cms maize plants 2 were unexpectedly male fertile and toxin-sensitive. However, R_1 progeny of these plants reverted to male fertile-toxin resistant and male sterile-toxin sensitive type plants, suggesting that the two traits are closely linked. Restriction endonuclease pattern of mitochondrial DNA revealed significant changes in the mitochondrial DNA of the regenerated variant plants (Gengenbach and Connelly, 1981; Kemble et al., 1982; Kemble and Pring, 1982).

Fusarium wilt resistant somaclones of tomato could be isolated after challenging the cotyledon protoplasts with fusaric acid, a non-specific toxin, as well as from non-selected calli of a susceptible variety (Shahin and Spivey, 1986). The resistance, conferred by a single dominant gene, was transmitted through several sexual generations.

Although it is very attractive to enrich the population of cells with desirable genotypes through in vitro selection, often the somaclones from cells selected for resistance to toxin or fungus culture filtrate do not exhibit stable resistance to the pathogen (Koike et al., 1991; Toyoda et al., 1989). For example, celery cells resistant to fusaric acid did not regenerate plants with higher resistance to the *F. oxysporum* f. sp. *apii* race 2 (FOA_2) than the control, suggesting that the principal mode of infection by FOA_2 does not involve a host specific toxin (Heath-Pagliuso et al., 1988). In contrast, selection of regenerated plants in FOA_2-infested soil yielded two plants showing significantly higher resistance to the fungus than the parent cultivar. The new variation was heritable, controlled by two dominant genes (Heath-Pagliuso and Rappaport, 1990).

(ii) *Herbicide resistance.* Herbicide resistance is important not only for plant breeding purposes but also as a selective marker in genetic engineering. Herbicide resistant plants of some species have been produced by genetic engineering (Chapter 14). Somaclonal variation is a practical alternative approach for selection of herbicide resistant mutants in cases where transgenic plants are difficult to produce.

Selection of herbicide resistant phenotypes in the field is complicated by difficulties in applying high and uniform selection pressure. Comparisons of herbicide action at cell, tissue and plant levels have indicated the possibility of selecting for herbicide resistance in tissue cultures.

The feasibility of developing herbicide-tolerant plant varieties through in vitro selection was first demonstrated by Chaleff and Parsons (1978). They selected picloram-resistant tobacco cell lines by exposing cell suspension to 500 μM of the herbicide. In some of the plants regenerated

from selected cell lines herbicide tolerance was inherited as dominant or semi-dominant alleles of single nuclear genes.

Chlorsulfuron (CS) and sulfometuron methyl (SM), sulfonylurea type of herbicides, inhibit the biosynthesis of acetolactate synthase (ALS). Tobacco plants resistant to CS and SM were isolated by callus selection (Chaleff and Ray, 1984). One of the somaclones resistant to CS was shown to possess an altered form of ALS which was far less sensitive to inhibition by the two sulfonylurea herbicides than that from normal cells (Chaleff and Mauvais, 1984). The CS resistance was inherited as a single dominant or semi-dominant trait. Plants homozygous for CS resistance showed 100-fold more resistance than normal plants. Recently, barley variants resistant to CS under greenhouse conditions have been isolated (Baillie et al., 1993). *Lotus corniculatus* lines showing genetically stable resistance to the sulfonylurea herbicide, harmony, have been isolated through sequential selection at the callus, shoot and whole plant levels (Pofelis et al., 1992).

Bensulfuron methyl (BSM), another member of the sulfonylurea family of herbicides, is highly toxic to annual and perennial dicotyledonous weeds. Its toxicity to rice is not severe but it effects the initial growth of rice plants by inhibiting root elongation. Terakawa and Wakasa (1992) selected BSM-resistant calli of rice by exposing the seed-derived callus to 0.1 mM BSM. Of the 19 plants regenerated from 6 resistant calli, 2 showed inheritance of the BSM-resistance trait up to the R_2 generation. The resistance was controlled by a dominant nuclear gene.

(iii) *Salt tolerance*. Salt tolerance in crop plants is an increasingly desirable characteristic not only because of limited water supply in the world but also because of salinization of irrigated soils. Since traditional breeding methods have been slow to yield substantial improvement in salt tolerance, the alternative approach of utilizing plant cell culture and regeneration of plants from the selected cell lines has received increased attention.

Initial efforts to produce salt tolerant plants through in vitro selection was not successful because of the inability to regenerate vigorous and fertile plants from selected cell lines (Raghava Ram and Nabors, 1985; Winicov, 1994). Although we still do not understand the mechanism by which plants can acquire incremental improvement in their salt tolerance, recently it has been possible to regenerate fertile plants from cells after selection on culture media containing additional salt and to demonstrate that the salt tolerant trait can be sexually transmitted. Thus, the generation of salt tolerant plants through tissue culture appears to be a viable alternative to classical breeding.

Nabors et al. (1975, 1980) isolated tobacco cell lines tolerant to high NaCl levels (0.88%). Regenerated plants retained tolerance through two successive sexual generations.

Winicov (1991) regenerated salt tolerant alfalfa plants which were morphologically indistinguishable from their salt sensitive parents, and the growth characteristics of the F_2 seedlings were comparable to the parent seedling. Winicov (1994) has recommended that to avoid the appearance of undesirable variants along with the desired salt tolerance the alfalfa callus should be exposed to MIC_{100} salt concentration within 3 months of initiation. The selected salt tolerant lines are expected to grow better on salt containing callus proliferation medium than on salt-free callus proliferation medium. Cell lines that are stably salt tolerant will not lose their ability to grow at a high salt level even after 2 months growth on control medium. Increased vigour has been observed in several plants selected through survival on salt medium in vitro followed by immediate regeneration (McHughen, 1987). High salt medium has helped achieve plant regeneration in long-term cultures of rice and to restore the lost regeneration potential of old cultures (Binh and Heszky, 1990). Interestingly, all the plants regenerated on this medium were salt tolerant.

9.4. ORIGIN OF SOMACLONAL VARIATION

The variations observed in the plants regenerated from cultured cells are derived from two sources (Fig. 9.4): (i) some of the variations could be revelation of the inherent cellular heterogeneity of the explant, and (ii) culture conditions may bring about new genetic changes.

9.4.1. Pre-existing variability

Normal plant development (in about 90% angiosperms) is accompanied by a range of direct changes in nuclear DNA (D'Amato, 1990). Consequently, in the majority of plants, mature and differentiated tissues, such as cortex and pith, exhibit considerable variations in the chromosomal constitution of their cells (Bennici and D'Amato, 1978). In apical meristems (root tips and shoot tips), where DNA synthesis is immediately followed by karyokinesis and cytokinesis (normal cell cycle), cells are maintained at a uniform diploid level (Fig. 9.5). However, the derivatives of these meristematic cells, during their subsequent differentiation do not divide by normal mitosis but may undergo DNA duplication and endoreduplication (see Figs. 9.5, 9.6). The varying degrees of endoreduplication results in somatic cells with 4C, 8C or even higher levels of DNA (see Fig.

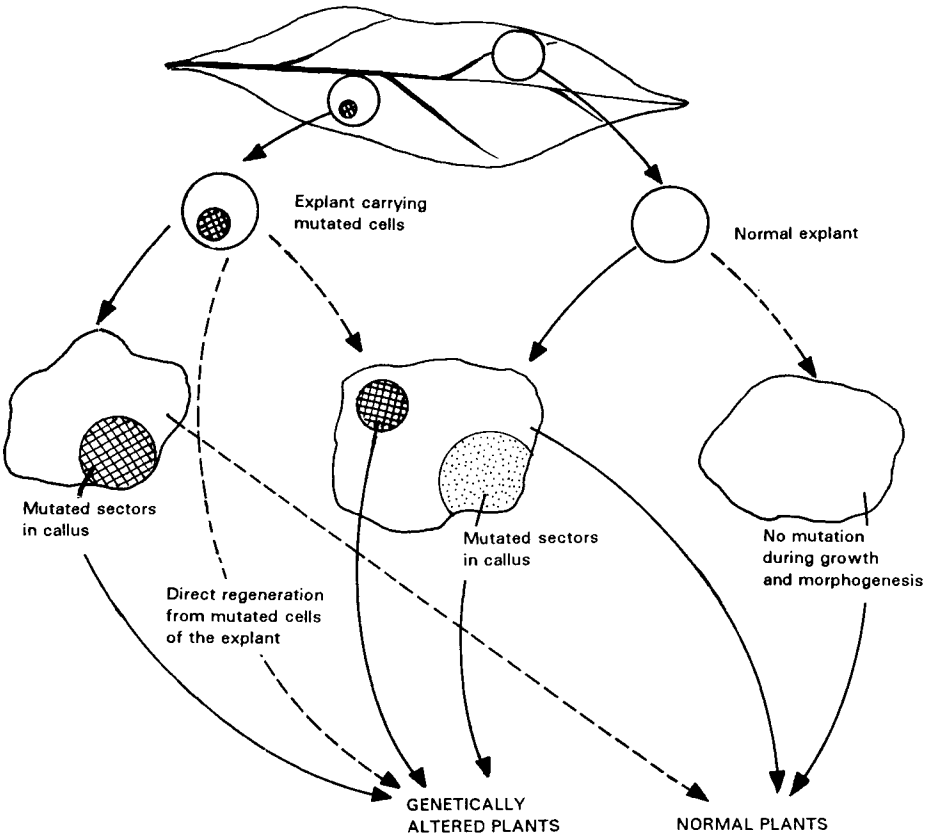

Fig. 9.4. Origin of genetic variation in plants regenerated from explant or callus cultures.

9.5). This phenomenon of polyplodization of body cells is termed polysomaty.

The widespread occurrence of polyploidy in differentiated cells may suggest that endopolyploidy of somatic cells is a general prerequisite for histological differentiation. However, this is not true because in some non-polysomatic species, such as *Helianthus tuberosus* (Partanen, 1959), *H. annuus* (Kupila, 1958; Butcher et al., 1975), *Crepis capillaris* (Fenzl and Tschermak-Woess, 1954), *Dendrophthoe falcata* (Johri and Nag, 1974) and *Lilium longiflorum* (Sheridan, 1975), normal tissue differentiation occurs in the complete absence of endopolyploidy. Polyploidy may, therefore, be an outcome of the process of differentiation. One possible significance of polysomaty could be the blockage of further division of the differentiated cells under normal conditions.

Under a normal situation, the genetic variability induced in somatic cells by polysomaty or any other kind of genetic changes remains unno-

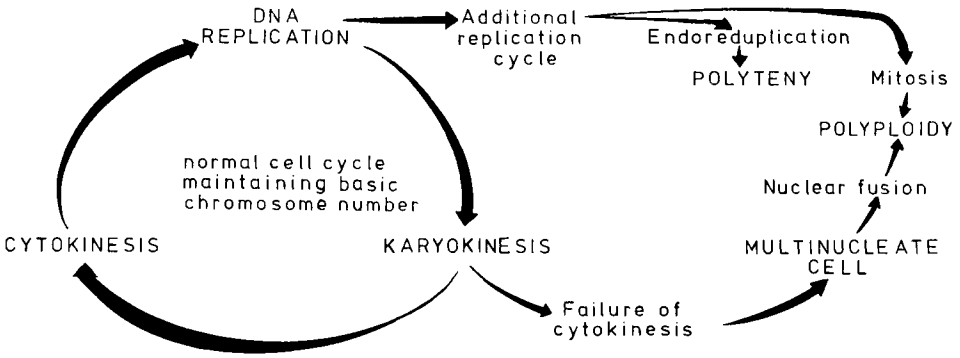

Fig. 9.5. Normal cell cycle and deviations from it, leading to polyploidization of cells in nature (after Partanen, 1965).

ticed as these cells do not divide. However, under culture conditions these cells may be induced to divide and undergo re-differentiation and express the inherent variability at whole plant level.

Torrey (1965) presented autoradiographic evidence to suggest that the first set of tetraploid cells observed in root cultures of pea, a polysomatic species, are those already present in the explant. In the first mitoses in cultures, diploid cells divided with 2n chromosomes (2 chromatid chromosomes) whereas reduplicated cells divided with 2n diplochromosomes (4 chromatid chromosomes), 2n quadruplochromsomes (8 chromatid chromosomes) or 2n polychromosomes (more than 8 chromatids, polyteny). Diplo- and quadruplochromosome mitoses have been commonly observed in the first phase of in vitro growth of tissues, such as stem pith of *Nicotiana* (Naylor et al., 1954; Patau and Das, 1961), stem internodes of haploid *Pelargonium* (Bennici et al., 1968) and the seedling callus of *Haplopappus gracilis* (Bennici et al., 1971).

Since some parts of the plants are more liable to undergo genetic changes in nature than the others, the extent of variation contributed by the donor plant is expected to vary with the explant. Accordingly, meristematic or embryonic tissues are likely to yield most stable calli and regenerated plants. The occurrence of somaclonal variation in the cultures of apical and lateral meristems of *Ipomoea batatas* is a rare exception (Moyer and Collins, 1983). All the plants of pineapple obtained from syncarp (compound ovary) or slip (small shoot which appears just below the fruit) callus were variants while only 7% of the plants obtained from crown (shoot at the top of the fruit) callus were variable (Wakasa, 1979). Similarly, many of the plants regenerated from seed callus of *Cymbopogon* sp. were atypical but those obtained from inflorescence callus closely resembled the parents, with only a negligible variation (Jagdish Chandra

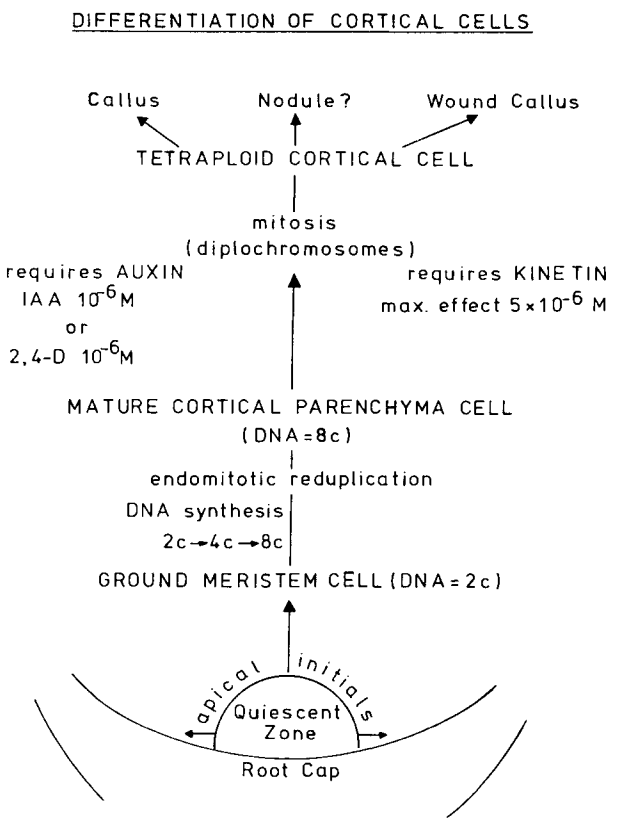

Fig. 9.6. Diagrammatic representation of a longitudinal section of a root to trace the derivation of polyploid-cortical cells from diploid cells in the root apex through endomitosis. Auxin and cytokinin induce divisions in these cells giving rise to a tetraploid cell population (after Torrey, 1965).

and Sreenath, 1982). The plants obtained indirectly from spadix explants of *Anthurium scherzerianum* were less variant than those produced from leaf segments (Geier, 1987). In potato, cotyledon-derived protoplasts produced more tetraploids than the leaf-derived protoplasts (Osifo et al., 1989).

Another type of pre-existing chromosomal variability, viz. aneusomaty, occurs only rarely in plants of hybrid origin (*Saccharum officinarum*; Heinz et al., 1969) and polyploids of recent origin (D'Amato, 1985). Every individual of sugarcane clone H50-7209 shows chromosome number mosaicism ($2n$ = 108–128; see D'Amato, 1977). In these plants the apical meristems and, consequently, the mature tissues comprise a mosaic of cells with varying proportion of different aneuploid chromosome numbers. The aneusomatic condition is transmitted to, and generally en-

hanced in, callus cultures derived from such tissues (Heinz et al., 1969; Cavallini and Lupi, 1987). Breakdown of genetic chimeras during callus or direct differentiation of adventive organs may be another source of somaclonal variation, particularly in vegetatively propagated species (Preil, 1986).

The extent of variation originating from the explant is dependent on, among other things, the age (Brossard, 1975; Castorena Sanchez et al., 1988) and type of tissue and organs (Sree Ramulu and Dijkhuis, 1986), genotype of the donor plant (Krikorian et al., 1993) and even the mode of cultivation of the donor plant (Pijnacker et al., 1989).

9.4.2. In vitro induced variability

The plant genome is under continuous flux which helps the plant adjust to changes in environmental conditions (Walbot and Cullis, 1985; Cullis, 1981). The tissue culture system is a stressful environment for the plant cells (De Klerk, 1990; Phillips et al., 1994) that could result in adaptive changes of genetic or epigenetic nature. Direct evidence for tissue culture-induced variation is the occurrence of chromosomal changes in the cultured tissues of non-polysomatic species, such as *Crepis capillaris* (Sacristan, 1971; Hahne and Hoffmann, 1986), strains of sunflower (Butcher et al., 1975), and in the cultures derived from single cells (Cooper et al., 1964; Mahfouz et al., 1983) or protoplasts (Karp et al., 1982). Generally, less variations are found in the plants than in the callus from which they are derived because in mixed population of cells, with different ploidies, euploid cells tend to be more regenerative than aneuploid cells. Several factors have been shown to influence the induction of variability in tissue cultures.

(i) *Culture medium.* It has been assumed that certain constituents of the culture medium, particularly certain growth regulators are mutagenic (Vajrabhaya, 1977; George and Sherrington, 1984). Torrey (1965) observed that in the cultures of pea root segments on a medium with 2,4-D as the sole hormone only diploid cells divided but when the medium contained kinetin and yeast extract, in addition to 2,4-D, the tetraploid cells were selectively induced to divide.

Direct as well as inverse correlations have been reported between polyploidy in tissue cultures and the presence of 2,4-D in the medium. Whereas some authors (Mitra and Steward, 1961; Melchers, 1965; Sunderland, 1977) regard this auxin as a direct inducer of polyploidy, others consider it as a factor selectively favouring the division of polyploid cells. Sunderland (1977) observed that in a medium containing 2,4-D

the suspension cultures of *Haplopappus* changed from a wholly diploid state to a wholly tetraploid state within a period of 6 months. This change was much slower if the medium contained NAA in place of 2,4-D. Similarly, chromosomal abnormalities in *Nigella sativa* tissues occurred at maximum frequency when 2,4-D, rather than IAA or NAA, was present in the medium together with kinetin (Chand and Roy, 1980). Increase in the number of sister chromatid exchanges (SCEs) per chromosome was noted in garlic roots treated with 5–15 μM 2,4-D (Dolezel et al., 1987). In wheat, 2,4-D did not induce such changes but 2,4,5-T at concentrations higher than 2 mg l^{-1} caused dramatic increases in SCEs which was partially suppressed by simultaneous addition of kinetin (Murata, 1989).

Kallak and Yarvekylg (1971) noted an inverse relationship between the concentration of 2,4-D and the degree of polyploidization of cultured cells of pea. According to these authors, 2,4-D at an auxetic (hormonal) concentration (0.25 mg l^{-1}) increased polyploid mitoses and decreased diploid mitoses but when applied at a herbicidal concentration (20 mg l^{-1}) it favoured division in diploid cells. Interestingly, the mitotic index in the callus was comparable at both the concentrations. An increase in the population of diploid cells, by selective elimination of higher ploidy cells, with an increasing concentration of 2,4-D was also observed in suspension cultures of *Haplopappus gracilus* and *Vicia hajastana* (Singh and Harvey, 1975b). In some cases cytokinin increased the extent of polyploidy in callus cultures (Torrey, 1958; Ghosh and Gadgil, 1979; Wright and Northcote, 1973) but in others either it had no effect (Butcher et al., 1975) or even reduced polyploidy and other karyotypic variability (Bennici et al., 1971; Vanzulli et al., 1980; Dolezel and Novak, 1985; Ashmore and Shapcott, 1989). Some of the potato plants (4.3%) regenerated from internode calli on medium containing zeatin showed increased resistance to *Phytophthora infestans* but none of the plants regenerated in the presence of 2ip showed increased resistance (Meulemans et al., 1986).

Besides the growth substance, some basic constituents of culture media also influence the cytological behaviour of cultured cells. In a mixed culture of a predominantly diploid and a hypertetraploid carrot cell lines the selective advantage of the latter line to proliferate on standard medium was substantially reduced in nitrogen and phosphate limiting medium (Bayliss, 1977, 1980). In suspension cultures of *Datura innoxia* organic nitrogen favoured the proliferation of diploid and tetraploid cells whereas inorganic nitrogen favoured the multiplication of haploid cells (Furner et al., 1978). Similarly, whereas B_5 medium selected for diploids, MS medium selected for tetraploid cells in *Haplopappus gracilis* (Singh,

1975b). Whites medium, which is a low salt medium, prevented the loss of embryogenic potential of carrot cells that occurred on MS medium (Smith and Street, 1974). An overdose of $CaCl_2$ in the medium increased the frequency of gross aberrations among regenerants of the potato cv 'Bintje' (Semal and Lepoivre, 1990).

(ii) *Growth pattern and mode of regeneration.* The nature of the callus may affect the variability observed in the regenerated plants. A true callus is a mass of dedifferentiated cells proliferating in an unorganized manner, and is likely to introduce considerable variability. However, in several monocots the callus often represents a mass of suppressed organs or proembryos rather than completely unorganized tissue (Humault, 1979; Wernicke et al., 1982; Wernicke and Gorst, 1987). Such calli are likely to retain a high degree of karyotypic constancy. This may explain the relative genetic stability of tissue cultures and regenerants reported in anthuriums (Geier, 1987), asparagus (Becker and Reuther, 1986), barley (Karp et al., 1987), daylily (Krikorian et al., 1981), guinea grass (Hanna et al., 1984), lily (Sheridan, 1975), maize (Edallo et al., 1981; McCoy and Phillips, 1982) and wheat (Chu et al., 1987). The same explanation is probably true for the stability observed in the cultures derived from zygotic embryos of conifers, which represent masses of proliferating embryos rather than disorganized calli (Papes et al., 1983; Schuller et al., 1989). In *Anthurium scherzerianum*, selective transfer, on to the same medium, of callus portion with or without shoot initials led to karyotypically stable and highly morphogenic sublines and highly variable, non-morphogenic sublines, respectively (Geier, 1988).

It has been suggested that regeneration via embryogenesis has better chances of obtaining genetically uniform plants than through organogenic differentiation (Vasil, 1987). However, this is not always true. Plants raised through somatic embryogenesis in petiole cultures of fennel exhibited considerable variability in various morphological parameters (Humault and Desmaret, 1990). High chromosome variability was also observed in embryogenic cultures of celery (Orton, 1985, 1987) and maize (Armstrong and Phillips, 1988). All the somatic embryos of *Bellevalia romana* were uniformly diploid up to the globular stage but during their further development chromosome mosaicism occurred (Cavallini et al., 1987).

Growth of unorganized callus was thought to be necessary for induction of variability but recent studies suggest that genetic variability is even present in population of plants directly regenerated from explants adventitiously (Cassells et al., 1983; Evans and Bravo, 1986; Bhojwani and Arora, 1992).

(iii) *Length of culture period and frequency of subculture.* Variant karyotypes commonly accumulate with increasing age of callus and, consequently, the proportion of variant plants produced during successive passages generally also increases (Geier, 1991). An increase in the frequency of regenerants displaying aneuploidy and/or chromosome structural changes with the length of culture period has been observed in garlic (Novak, 1980), maize (Lee and Phillips, 1987; Benzion and Phillips, 1988), oat (McCoy et al., 1982) and triploid ryegrass (Ahloowalia, 1983). In most of these cases the proportion of karyotypically normal plants was nearly 100% during the first passage.

There is evidence to suggest that frequent transfers compared to extended subculture intervals, yield more stable cultures. A higher degree of diploidy could be maintained in suspension cultures of alfalfa (Binarova and Dolezel, 1988), carrot (Bayliss, 1980; Bayliss and Gould, 1974), *Nigella sativa* (Ghosh and Gadgil, 1980) and tobacco (Evans and Gamborg, 1982) through frequent transfers . In *Begonia rex*, callus ageing resulted in increased aneuploidy and polyploidy (Cassells and Morrish, 1987). In suspension cultures of *Haplopappus* maintained by regular subcultures every 2 days, Singh and Harvey (1975a) observed equilibrium in the cell population of 93% diploid and 7% tetraploid cells over 300 days. Longer passages enhanced the range of nuclear abnormalities.

(iv) *Ploidy and genotype.* Genotype of the parent plant is a strong determinant of variability in cultures. Skirvin and Janick (1976a) noted that the frequency of variation observed in the regenerants of *Pelargonium* was dependent on the variety. Cummings et al. (1976) found differences in the extent of variation induced in different varieties of flax.

Similarly, ploidy of the donor plant also determines the susceptibility of cells to in vitro changes. Generally, ploidy levels lower than the usual ploidy level of the respective species prove to be more or less unstable. Sacristan (1971) compared the cytological changes in long-term cultures of haploid and diploid strains of *Crepis capillaris* and noted that diploidization in haploid tissues was more common than the occurrence of tetraploids in diploid lines.

Where comparison has been made between polyploid and diploid genotypes of the same species generally the former tended to show higher variation in culture. For example, 12 out of 47 regenerants from two 4X ryegrass genotypes lost up to three chromosomes while all the regenerants of the 2X genotypes retained the diploid chromosome number (Jackson and Dale, 1988). Similarly, cultures of a diploid line of potato did not give rise to any morphological variation whatsoever (Wenzel et al., 1979), whereas somaclones of the tetraploid variety 'Russet Burbank'

varied widely with respect to a large number of traits, many of them being of agronomic importance (Thomas et al., 1982). In contrast, autotetraploids of *Phlox drummondi* were found to be karyologically more stable than the diploids (Raja et al., 1992).

(v) *Physical factors.* Besides its chemical composition, the physical state of the medium also influences the cytological behaviour of the cultured cells. In *Hevea*, polyploidy increased when cells were cultured in suspensions but decreased when they were recultured as callus on a solid medium (Wilson et al., 1976).

Culture temperature can influence the rate of mutation. Tobacco callus incubated at 35°C remained predominantly diploid, while the same tissue cultured at 25°C showed marked karyological instability and became mainly tetraploid (Binns and Meins, 1980). In *Lilium longiflorum* the occurrence of albino seedlings from somatic embryos increased as the incubation temperature was raised above 10–15°C (Jackson and Dale, 1988).

9.5. MECHANISMS UNDERLYING GENETIC VARIATION

9.5.1. Changes in ploidy level

Ploidy changes is the most frequently observed chromosomal abnormality in cultured cells and the plants regenerated from them (D'Amato, 1985; Geier, 1991). With respect to the number of chromosomes two main types of abnormalities occur in cultured cells: (a) euploidy: increase in chromosome number in simple multiples of the basic chromosome number ($2n$, $3n$, $4n$, $5n$, etc.), and (2) aneuploidy: cells with chromosome numbers which are not simple multiples of the basic chromosome number. Geier (1991) scored 306 papers describing chromosomal variability in tissue cultures, of which 295 papers reported numerical changes (euploidy 239; aneuploidy 205).

Formation of a restitution nucleus due to the failure of spindle formation and chromosome lagging at anaphase (Bayliss, 1973; Knosche and Gunther, 1980; Dobel, 1983; Dolezel and Novak, 1985; Zhang et al., 1987) and the fusion of spindles during synchronous divisions in multinucleate cells (Mitra et al., 1960; Mitra and Steward, 1961) are common sources of the occurrence of euploid cells of the 4,8,16... series in tissue cultures. Fusion of nuclei may even occur during interphase (Constabel et al., 1975). Euploid cells of odd series ($3n$, $5n$, $7n$, etc.), which are also commonly encountered in tissue cultures (Mitra and Steward, 1961; Fox, 1963; Shamina, 1966; Sacristan and Melchers, 1969) may arise through

nuclear fusion (Sunderland, 1977) or genome segregation during polyploid mitosis (D'Amato, 1977).

Haploid cells, sometimes found in calli of diploid origin (Mitra and Steward, 1961; Gupta, 1971; Singh et al., 1972; Bennici et al. 1976, Roy, 1980; Singh, 1986; Zhang et al., 1987), probably arise through somatic pairing and reduction, a process that is well documented in intact root meristems (Mehra, 1986), and has been shown to occur at least in some callus cultures (Mitra and Steward, 1961). Nuti Ronchi et al. (cited in Nuti Ronchi, 1990) have observed all stages of meiosis in cell cultures of carrot.

With the exception of plants of hybrid origin (*Saccharum officinarum*; Heinz et al., 1969) and polyploids of recent origin, aneuploidy normally does not occur in nature. However, aneuploidy in cultured cells is not a rare feature. In the pith tissue cultures of *Nicotiana glauca*, on a 2,4-D containing medium, extensive nuclear fragmentation (amitosis) was noted during the first 2–6 days of culture (Nuti Ronchi et al., 1973). This resulted in multinucleate cells having nuclei of varying sizes. Normal mitoses in such cells is expected to produce cells with a wide range of chromosome numbers. This has been actually observed in suspensor cultures of *Phaseolus coccineus* (Bennici et al., 1976) (Fig. 9.7) and cotyledon cultures of *Vicia faba* (Cionini et al., 1978), as early as the first wave of regular mitosis in the explant. However, many of the aneuploid cells may be eliminated at later stages through mitotic selection in favour of diploid cells (Cionini et al., 1978).

The selection in favour of certain type of cells in cultures plays a significant role in establishing dominant karyotype or modal chromosome number. Singh (1975b) isolated 4-, 6-, 16-chromosome lines from tissue cultures of *Haplopappus gracilis*. In prolonged cultures, on B_5 medium supplemented with 2,4-D, the 4- and 6-chromosome lines were stable whereas the 16-chromosome line gradually lost chromosomes, and a stable karyotype with 13 chromosomes evolved. In mixed cultures of diploid and tetraploid cell lines of carrot (both lines show identical growth rates in monocultures) the frequency of tetraploid cells gradually increased and, finally, the cultures attained a tetraploid mode (Smith and Street, 1974).

The regeneration process itself acts as a screen to eliminate a portion of varying karyotypes. Generally, a strong selection acts in favour of diploids (Hahne and Hoffmann, 1986; Sengupta et al., 1988; Geier et al., 1992) or at least euploids (Taniguchi and Tanaka, 1989) during plant regeneration from callus and suspension cultures. Selection may occur at different stages in the regeneration process. Some of the cells with chromosomal variation may be totally impaired in their regeneration process

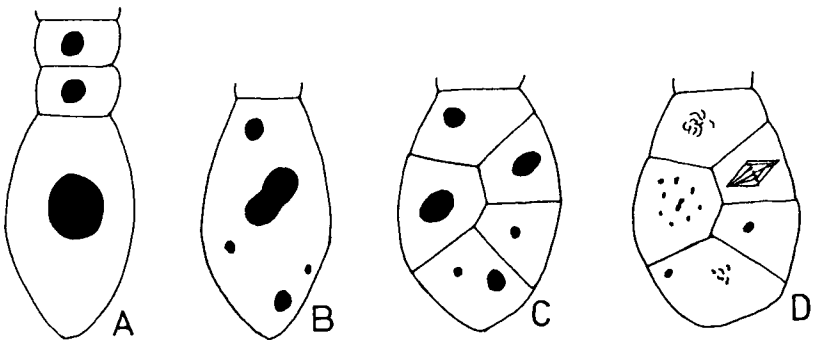

Fig. 9.7. Diagrammatic representation of nuclear irregularities reported to occur in suspensor cells of *Phaseolus coccineus*. (A) Part of the suspensor at the time of culture. (B) Fragmentation of the nucleus in the basal cell. (C) Cellularization of the basal cell; the resulting cells include nuclei of different sizes. (D) Some of the aneuploid cells are dividing (after Bennici et al., 1976).

(Murashige and Nakano, 1965; Torrey, 1967). The plants regenerated from embryogenic cell suspensions of *Euphorbia pulcherrima*, which showed DNA content ranging from 2C to 32C, were highly uniform with regard to their phenotype and ploidy level (Geier et al., 1992). The shoots regenerated from potato protoplasts showed a significant difference in their rootability depending on whether they were euploid (well rooting) or aneuploid (poorly rooting) (Fish and Karp, 1986).

In some cases, where explants or calli are aneusomatic and the regenerants are of multicellular origin, the resulting plants may be a mosaic of cells with different euploid and aneuploid chromosome number (Bennici, 1979; Lupi et al., 1981; Natali and Cavallini, 1987). The wheat plants regenerated from mesocotyl segments of Durum wheat, within 10–15 days of culture, showed chromosome number mosaicism in root-tips and shoot-tips. The constituent cells of the mosaic were hypohaploid, haploid and hypodiploid besides being diploid (Bennici and D'Amato, 1978). The aneusomaty persisted until an advanced stage of spike development but was completely eliminated before meiosis in micro- and megaspore mother cells (Lupi et al., 1981).

9.5.2. Changes in chromosome structure

The stability of chromosome number in cultured cells does not rule out their karyotypic instability. Structural rearrangements involving gain or loss of chromosome segments can lead to entirely altered karyotype while maintaining the original number of chromosomes (Lee and Phillips, 1988). *Crepis capillaris* has three pairs of chromosomes which can be

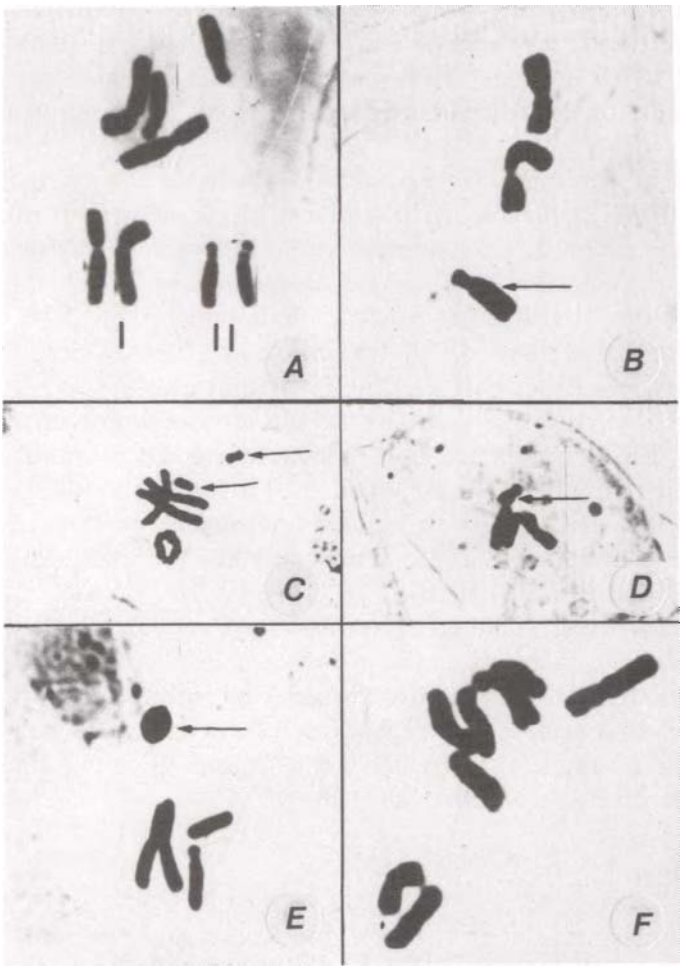

Fig. 9.8. Metaphase figures from cells of *Haplopappus gracilis* grown in suspension cultures. (A) A karyotype of a diploid cell ($2n = 4$); chromosome II has a satellite. (B) A monosomic cell for chromosome II. (C) A cell showing chromosome fragments (arrow marked). (D) A diploid cell with a deleted chromosome II (arrow marked). (E) An aneuploid cell with a ring chromosome. (F) A tetraploid cell (after Singh and Harvey, 1975a; courtesy of Dr B.D. Singh, India).

easily distinguished on the basis of their morphology. Pair 1 comprises long chromosomes (L), pair 2 has medium size satellite chromosomes (Sat) and pair 3 consists of small chromosomes (S). In long-term cultures the frequency of rearrangement varied with the chromosome: 47% for L chromosomes, 82.3% for Sat chromosomes and 64.6% for S chromosomes (Sacristan, 1971). In tissue cultures of *Haplopappus gracilis*, the occurrence of acentric fragments, microchromosomes, deleted chromosomes, dicentric chromosomes and ring chromosomes. (Fig. 9.8) was very fre-

quent (Singh, 1975a,b; Singh and Harvey, 1975a). Reciprocal and non-reciprocal translocations have been observed in potato (Shepard, 1982), ryegrass (Ahloowalia, 1976) and oat (McCoy et al., 1982). Wheat tissue cultures showed duplication or deletion of large sections of chromatin (Davies et al., 1986b).

Structural changes in chromosomes originate from breakage during the various stages of the cell cycle. While many kinds of structural changes are stably propagated through successive mitotic cycles, translocations leading to the formation of dicentric chromosomes can bring about continuing variation by initiating a breakage fusion bridge (BFB) cycle (Kao et al., 1970b; Sunderland, 1973; Toncelli et al., 1985). Lee and Phillips (1988) have proposed that culture conditions may cause delay in DNA synthesis in the heterochromatin region of the chromosomes until mitosis, resulting in the formation of non-replicated heterochromatin bridges and breakage at anaphase. Rearrangements involving breakage preferably at or close to heterochromatin regions have been reported by several workers (Murata and Orton, 1984; Johnson et al., 1987; Lee and Phillips, 1987; Benzion and Phillips, 1988).

A unique case of structural variation has been reported in triploid ($2n = 3x = 18$) *Scilla siberica* (Deumling and Clermont, 1989). Extensive chromatin dimunition during callus culture resulted in chromosomes of about one-tenth the original size. Plants regenerated from the callus possessed only 20–30% of the original DNA per cell although showing significantly increased chromosome number ($2n = 30–40$).

9.5.3. Gene mutations

In recent years several somaclonal variants due to recessive or dominant single or multiple gene mutations have been described. Recessive mutations may not express in the R_0 generation of somaclones but can be detected in their selfed progeny.

Through conventional genetic complementation tests of several tomato plants regenerated from leaf callus, 13 distinct gene mutations have been well characterized and mapped to specific loci on the chromosomes (Evans and Sharp, 1983, 1986; Evans and Bravo, 1986). The yellow fruit (chromosome 31), orange fruit (chromosome 10), jointless pedicel (chromosome 11) were single recessive gene mutations while the *Fusarium* Race 2 wilt resistance (chromosome 11) was a single dominant gene mutation. Somaclones showing single gene recessive mutations are also reported for rice (Fukui, 1983; Sun et al., 1983), tobacco (Prat, 1983) and maize (Edallo et al., 1981). In red clover, variations were due to additive effects of mutated genes (Keyes et al., 1980).

9.5.4. Gene amplification

Brown (1981) suggested that any gene that cannot modulate its expression is likely to show gene amplification, if the right selection agent is available. By subculturing alfalfa suspension in the presence of the herbicide phosphinothricin, Donn et al. (1984) selected lines showing 20- to 100-fold more resistance to the herbicide than the wild type. The resistance was caused by a 4–11-fold amplification of glutamine synthase (GS) gene and a consequential 3–7-fold increase in GS enzyme. This herbicide is a competitive inhibitor of GS. Goldsbrough et al. (1990) selected *Nicotiana tabacum* cell lines resistant to normally lethal concentrations of glyphosate and found that the level of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is inhibited by glyphosate, was elevated. Increased mRNA levels for EPSPS resulted from the amplification of at least two genes encoding this enzyme; the amplification increased with the level of glyphosate. The selected cells maintained the elevated EPSPS mRNA level even in the absence of the herbicide, suggesting that selection resulted in stable genetic modification.

9.5.5. Changes in extranuclear genes

The genetic material of cytoplasmic organelles can also be altered by in vitro culture conditions (Brown, 1991; Kane et al., 1992). Higher plant mitochondrial DNA is usually regarded as a circular molecule, containing repeated sequences which act as sites for intragenomic recombination (Palmer and Shields, 1984). Many conformational and molecular changes in mitochondria in response to tissue culture have been observed. Tobacco suspension cultures showed variability in mtDNA restriction fragment pattern. In these cases most of the mtDNA was organized as amplified circular DNA molecules derived from the main mitochondrial genome (Hanson, 1984; Newton, 1988). Hartman et al. (1989) demonstrated significant alterations in the mtDNA of wheat plants regenerated from tissue culture; the extent of change was determined by the length of the tissue culture period. The non-embryogenic calli and non-embryogenic sectors of embryogenic calli of wheat suffered a loss of about 8 kb fragment from the mtDNA found in the embryogenic cells (Rode et al., 1988). In maize, five of the six male fertile, resistant mutants obtained from male sterile populations susceptible to specific toxin of *Drechslera maydis* race T were characterized by a change in about 6000 base pairs in the mtDNA (Gengenbach and Connelly, 1981; Kemble et al., 1982; Umbeck and Gengenbach, 1983).

The chloroplast genome is relatively more conserved. One of the most frequent and noticeable manifestations of somaclonal and gametoclonal variation in grasses is the production of albino plants. Day and Ellis (1985) showed that the albino plants from anther cultures of rice may have lost up to 70% of the plastid genome. Dunford and Walden (1991) analysed five albino barley plants regenerated from microspores by direct embryogenesis. Four of the plants had undergone deletion or alterations in specific restriction fragment of the chloroplast genome. Sun et al. (1979) found that androgenic albino plants of rice contained little or no 16S or 23S RNA as well as significantly reduced levels of Rubisco protein. Since chloroplast rDNA is encoded by chloroplast DNA, any reduction in chloroplast rDNA should be due to impairment of cpDNA.

The emergence of low temperature resistant plants in anther cultures of temperature-sensitive line of *Nicotiana tabacum* has been shown to be due to changes in the genetic factors of the cytoplasmic organelles (Matzinger and Burk, 1984).

9.5.6. Activation of transposable elements

Transposable elements are genetic sequences which are capable of moving around in the genome and modify gene expression. A number of such elements (e.g. Ds, Ac, Mu) have been described in maize. Insertion of such elements at a new locus causes unstable suppression or modification of the transcriptional property of adjacent genes. Excision of the element may allow reversion to the wild phenotype. The excision is affected by several factors such as virus infection, temperature, chromosome breakage and the genetic background (Stafford, 1991).

McClintock (1984) had suggested that genomic shock can trigger transposition of these elements. There is growing evidence to suggest that the stress of tissue culture conditions can also activate previously silent transposable elements (Groose and Bingham, 1986a; Lee and Phillips, 1988; Phillips et al., 1990).

Activation of maize transposable elements following tissue culture has been reported more than once. The maize plants regenerated from tissue cultures were found to contain an active *Ac* element whereas none had been detected in the initial explant (Evola et al., 1985; Peschke et al., 1987; Phillips et al., 1990). Similarly, Evola et al. (1984) observed the activation of an *Spn* (*En*) element in half of the regenerated plants of maize. More than 20% of the alfalfa plants regenerated from tissue cultures of a white flowered somaclone exhibited the wild type purple flowered phenotype (Groose and Bingham, 1986a,b). Genetic analysis indi-

cated that while the wild type and mutated alleles were stable and sexually transmitted the culture process appeared to trigger reversion, suggesting the involvement of transposable elements.

9.5.7. DNA methylation

Phillips et al. (1990) have proposed the hypothesis that most, if not all, tissue culture-induced mutations are directly or indirectly related to alteration in the state of DNA methylation. The degree of DNA methylation of genes is inversely co-related with their expression. Accordingly, increase or decrease in DNA methylation might account for qualitative mutations, such as those controlled by single recessive genes, for increased transposable element activity, for simultaneous changes in qualitative characters and the mutations caused by chromosome bridges.

In two experiments involving *Ac* (Activator) elements, which cycle between activity and inactivity at a relatively high frequency, the elements were fully methylated when inactive but less methylated when they regained activity (Schwartz and Dennis, 1986; Chomet et al., 1987). One of these cycling with *Ac* elements put into tissue culture in inactive form showed activation of the element at 80× the normal rate for the allele (Dennis and Brettle, 1990). This increased activity was correlated with reduced methylation in the 5′ end of the element. Some correlation between tissue culture derived *Ac* genetic activity and reduced methylation of *Ac* homologous sequences has also been detected by Peschke et al. (cited in Phillips et al., 1990). Several other authors have described significant alteration in methylation status of plants regenerated from tissue cultures of maize (Brown, 1989, 1991), tobacco (Durante et al., 1989) and carrot (Palmgren et al., 1991). In maize and many other cereals the altered methylation status was stably inherited (Brown, 1991).

The occurrence of quantitative variation with extremely high frequencies in the plants regenerated from tissue cultures of maize inbred lines, led Phillips et al. (1990) to suggest the involvement of highly mutable, yet heritable DNA methylation.

9.6. ASSESSMENT OF SOMACLONAL VARIATION

The most useful somaclone would be one which conserves all the good characters of the parent cultivar with the addition of a specific desirable trait in which it was lacking. It is, therefore, extremely important to select the variants as early as possible, with minimum exposure of cells to

tissue culture environment. With prolonged culture gross abnormalities may appear (Bhojwani and Arora, 1992). While most of the deleterious changes are sieved during regeneration and field transplantation of the in vitro raised plants, some abnormalities do persist. Early detection and rejection of variants is also desirable in order to reduce losses in micropropagation.

Somaclonal variants have been mostly assessed at the phenotypic level, and in over 50% cases it is based on R_0 plants. Phenotypic screening of R_0 plants would allow detection of only dominant or homozygous traits. Moreover, the possession of normal phenotype by R_0 plants does not reflect a lack of variation. Recessive mutations in heterozygous regenerants can be recognized only in their segregating R_1 and R_2 progenies. It is, therefore, important that the variants should be assessed in the sexual progenies of the in vitro regenerated plants so that their heritability is established.

The extent of somaclonal variation at the phenotypic level is usually determined as the percentage of plants showing aberrations for one or more defined characteristics, such as plant height, heading date, tiller number, time of flowering, fertility, flower and fruit colour, yield, tolerance to salinity, disease resistance, etc. De Klerk et al. (1990) have suggested that the degree of variation in somaclonal population may be assessed by determining the value of standard deviation (SD) for a given quantitative trait. Since somaclonal variation renders the genotype more heterozygous the selfed progeny of the regenerants are expected to show increased SD for various quantitative traits (Jackson and Dale, 1989). The advantages of quantitative SD-assay over the qualitative assays are: (1) for each determination the number of plants needed is smaller (20–30 instead of 100); (2) the observation of these parameters does not depend upon the eye of the observer; (3) in a short period of time many individuals can be evaluated; and (4) the SD assay system seems to be much more sensitive.

As the main interest in somaclonal variation is a novel source of variability in plant breeding, most identifications of somaclones have been done on the basis of gross morphological features. Where this was not possible biochemical characterization was performed, usually involving protein electrophoresis. Both these techniques assess the phenotype of the plant which is affected by the environment. Assessment of genotype of the plant is a more rapid and precise method of assessing somaclonal variation. In this regard much effort has been placed on chromosome analysis (Geier, 1991). However, gross changes in chromosome number and morphology cannot account for all the observed variations at the whole plant level. RFLP offers a better method to analyse somaclones,

both from the point of view of identifying subtle changes and also in the ability to analyse plants from different environments, even while still in culture. Several reviews on the impact of this technique in plant breeding have been written (Beckman and Soller, 1986; Tanksley et al., 1989; DeVerna and Alpert, 1990). RFLP analysis for somaclonal variation assessment aims at identification of altered band pattern instead of selecting the presence or absence of alleles at different loci. For details see Potter (1991).

9.7. PRACTICAL ASPECTS

Somaclonal variation provides an additional source of genetic variability suitable for exploitation in crop improvement programmes. It is appealing to breeders because it occurs at a frequency often considerably higher than the incidence of spontaneous mutations or chemically induced mutations (Gavazzi et al., 1987; Lindsey and Jones, 1990; Phillips et al., 1994) and, thus, accelerates the process of selecting desirable genotype over the classical breeding approaches based on sexual hybridization. During tissue culture of maize the frequency of nucleotide substitution in the Adh1-1s allele was found to be 10 000 times higher than the spontaneous mutation rate (Dennis et al., 1987). Similarly, up to 15% variants were detected in regenerants of tomato against a spontaneous mutation rate of 1×10^{-6} (Evans and Sharp, 1986). Sun et al. (1983) reported 72% variants in rice regenerants. Sometimes unique mutations have been generated through tissue culture which could not be obtained through crossing or mutagenesis (Gavazzi et al., 1987; Semal and Lepoivre, 1990). For example, in potato introduction of traits such as higher yield, low colonization by aphids without disturbing the other characteristics of the parent cultivar is unique to tissue culture derived variability (Semal and Lepoivre, 1990).

The variability generated from tissue culture is already being exploited, and at least ten new varieties have been released based on somaclonal variant selection (Table 9.2). Vegisnax, comprising fresh pre-cut 'ready-to-eat celery and carrot sticks', introduced in the US market, is another example of successful commercial exploitation of somaclonal variants (Lindsey and Jones, 1990). In Hungary a somaclone variety of rice 'DAMA' has been recently released (Heszky and Simmon-Kiss, 1992). Among the existing rice varieties of that country, DAMA is most resistant to *Pyricularia* and has the best seed profile and cooking quality. DNA Plant Technology Corp., USA, developed a seedless bell pepper cv 'Bellsweet' from doubled haploids of the var. 'Golden cal wonder' produced by anther culture (Morrison and Evans, 1988). A few somaclones of

fennel obtained through somatic embryogenesis have been used in conventional breeding programmes (Desmaret et al., 1987).

As single gene mutations and organelle gene mutations have been observed in somaclonal variants, an obvious strategy to produce new cultivars through tissue culture is to introduce the best available cultivar into tissue culture and select for improvement of a specific trait amongst the regenerated plants. Hence somaclonal variation could be used to uncover new genotypes that retain all favourable characters of the existing cultivar while adding one additional trait. This approach is particularly attractive when breeding by classical sexual crossing is hampered by the lack of genetic variability to generate new hybrid classes or by lengthy hybridization and selection processes necessary to obtain a variety with new desirable traits. Potato is one such example.

On the negative side, the occurrence of uncontrolled somaclonal variation in tissue culture raised plants is at present limiting the use of in vitro methods for clonal propagation of selected genotypes and commercial production of industrial compounds from cell cultures. In the Netherlands alone losses of over US$1 million per year have occurred due to somaclonal variation (De Klerk, 1990). In most micropropagated crops somaclonal variation is avoided by adopting the axillary branching approach to shoot multiplication (see Chapter 16). However, even in these cultures, adventitious buds are often formed without notice and introduce variation which is multiplied in the subsequent cycles of shoot-proliferation (Marcotrigiano et al., 1987). In standard in vitro proliferation of chimeral strawberry, adventitious buds account for 80% of the newly formed shoots, a percentage similar to the amount of off-types (rearranged chimeras) obtained (Marcotrigiano et al., 1987). Rarely, the plants raised from the cultures of apical or lateral meristems also exhibit variation (Moyer and Collins, 1983). Elimination of cryptic or latent virus in meristem cultures may be an important cause of these variations. In addition, micropropagation via somatic embryogenesis in liquid medium, which can be automated, is a cheap alternative for the present day methods; it is hampered by, among other things, somaclonal variation. Somaclonal variation is also a potential threat for the production of genetically engineered plants without loss of any useful trait of the parent cultivar, as it involves a tissue culture step. Somaclonal variation also affects the production of secondary plant substances by cell cultures, thereby reducing the production by well established cultures (Deus-Neumann and Zenk, 1984).

Since in vitro regeneration of plants serves two diverse objectives, cloning and sub-cloning of plants, it is important to select the appropriate in vitro approach best suited to the objective.

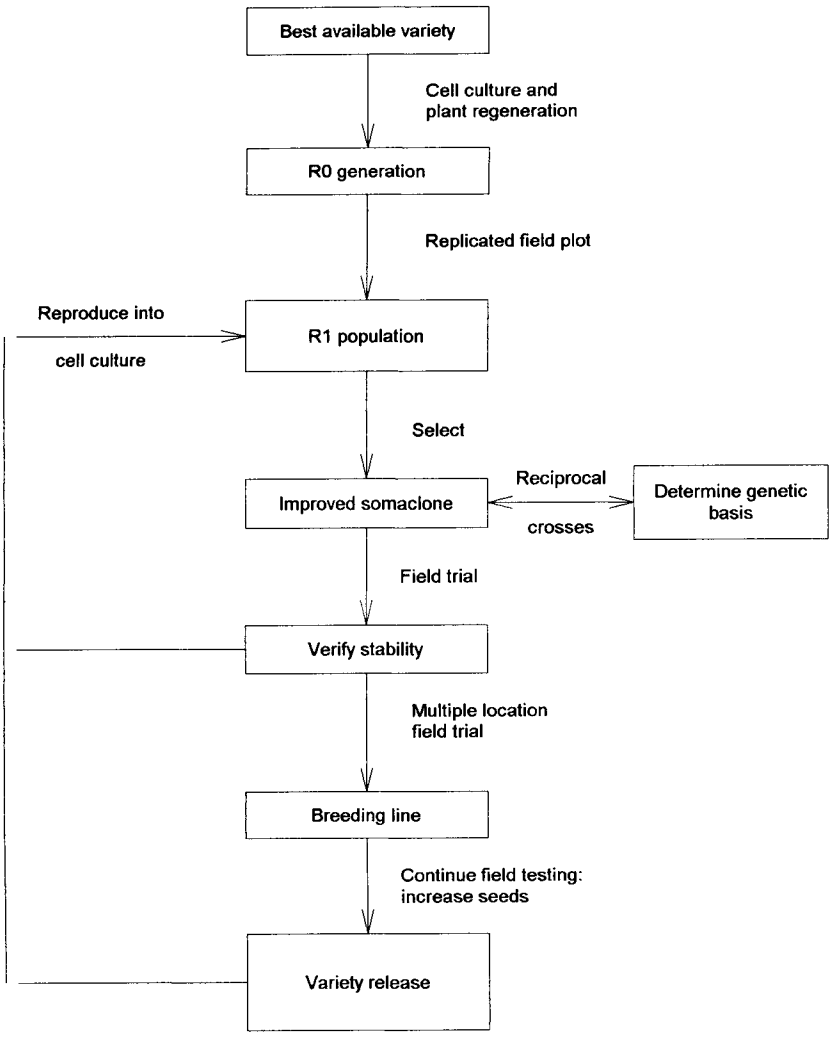

Fig. 9.9. A strategy for the production of somaclonal variants to develop new varieties (adapted from Evans et al., 1984).

9.8. CONCLUDING REMARKS

Tissue culture conditions induce a range of genetic changes, ranging from numerical and structural changes in the chromosomes to gene mutations, gene expression, and gene amplification, in cultured cells and the plants regenerated from them. Consequently, as stated by Phillips et al.

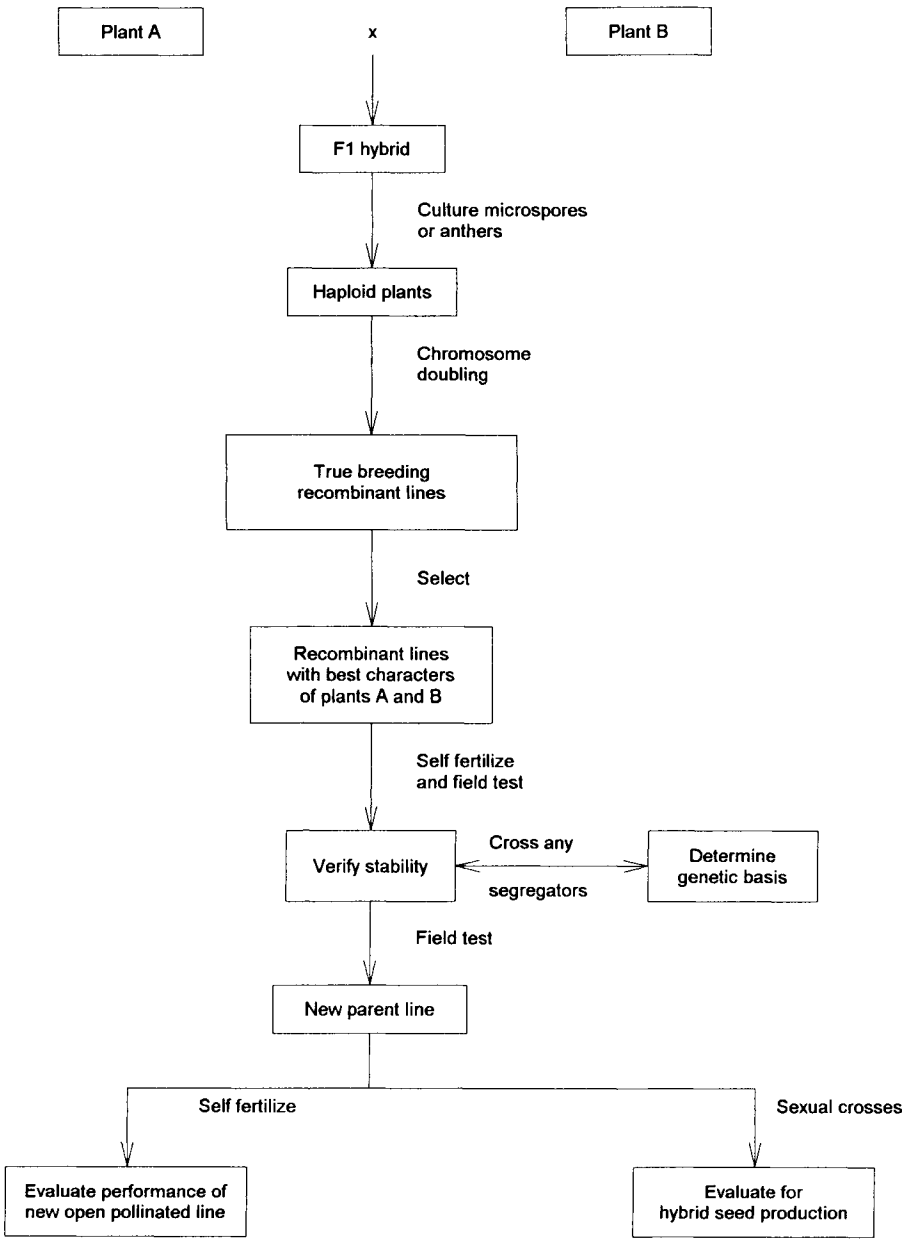

Fig. 9.10. A strategy for the production of gametoclonal variants to develop new varieties (adapted from Evans et al., 1984).

(1994), 'no two of the callus-derived plants are exactly alike and, none is just like the plant that donated the cell or cells for tissue culture'. This is proving to be a serious limitation in exploitation of the full potential of in vitro techniques for clonal plant propagation. Even when the most conservative approach of axillary bud proliferation is followed to clone plants, some adventitious buds may develop and introduce genetic variation. Therefore, to avoid variation in tissue cultures would be highly desirable. Pretreatment of leaf tissue of haploid *Petunia* with 30–100 μM BAP for 9–12 days prior to culture on regeneration medium had a stabilizing effect on the genome (Liscum and Hangarter, 1991).

Spontaneous variations generated in tissue cultures of somatic and gametic cells are being used as a novel source of heritable genetic variation suitable for upgrading the existing cultivars of crop plants. Breeders are finding it attractive because of its simplicity. During the past decade several somaclonal and gametoclonal variants have been utilized to release new varieties (Table 9.2) and some products are already in the market. Although some of the variants have been used directly as new cultivars, these variants may be more useful as breeding lines.

Since most of the mutations are recessive they cannot be detected in the R_0 somaclones, and require progeny analysis for unmasking. In this regard gametoclonal variants are more useful as they allow uncovering a recessive trait and additive characters with relatively small population. Figures 9.9 and 9.10 present breeding strategies for the use of somaclonal and gametoclonal variations for the development of new varieties.

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Chapter 10

In Vitro Pollination And Fertilization

10.1. INTRODUCTION

Sexual hybridization is a powerful tool in the hands of plant breeders for producing superior plants by combining characters distributed in different members of a species or different species of a genus. The technique involves controlled, artificial pollination of the female parent with pollen from the selected male parent. In angiosperms the female gametophyte, enclosing the egg (female gamete), is deep-seated in the ovarian cavity, well protected by the ovular tissues. The pollen grains are normally held at the stigma and there is no device for them to reach the egg. To effect fertilization the pollen grains germinate on the stigma by putting forth a tube (pollen tube) which grows through the stigma and style, finds its way into the ovule and discharges two sperms in the vicinity of the egg. One of them fuses with the egg, forming a zygote (the progenitor of the embryo), while the other fuses with the polar nuclei forming the primary endosperm nucleus.

In nature, a stigma receives a variety of pollen grains, but not all those that reach the stigma succeed in effecting fertilization. The stigma and style are equipped with devices to allow pollen of only the right mating type to function normally; others are discarded. Consequently, in hybridization programmes transferring viable pollen from one parent to the receptive stigma of another does not always lead to seed-set. Some of the barriers to fertilization are: (a) inability of pollen to germinate on foreign stigma; (b) failure of the pollen tube to reach the ovule due to excessive length of the style, or slow growth of the pollen tube, so that ovary abscises before the pollen tube reaches the base of the style; and (c) bursting of the pollen tube in the style. These are pre-fertilization or pre-zygotic barriers. In other cases fertilization may occur normally, but the hybrid embryo fails to attain maturity due to embryo-endosperm incompatibility or poor development of the endosperm (post-fertilization barriers).

From time to time various techniques have been developed to circumvent the pre-zygotic barriers to fertilization. Some of these are: (i) bud pollination; (ii) stub pollination; (iii) heat treatment of the style; (iv) irradiation; and (v) mixed pollination (see Bhojwani and Bhatnagar, 1990).

The possibility of effecting fertilization by introducing pollen grains directly into the ovary (intra-ovarian pollination) is yet another approach to bypass the pre-fertilization barriers. As early as 1926, Dahlgren (cited in Maheshwari, 1950) was able to bring about fertilization in *Codonopsis ovata* by placing pollen on the cut surface at the top of the ovary. The detailed work on developing the technique of intra-ovarian pollination was, however, done at the University of Delhi by P. Maheshwari and his associates. Kanta (1960) and Kanta and Maheshwari (1963a) obtained a good seed-set through intra-ovarian pollination in several members of the Papaveraceae (*Papaver rhoeas*, *P. somniferum*, *Eschscholtzia californica*, *Argemone mexicana*, *A. ochroleuca*). Using this technique, Kanta and Maheshwari (1963a) developed hybrids between *A. mexicana* and *A. ochroleuca*. Pollen were collected from dehiscing anthers and suspended in sterile double-distilled water containing 100 mg l^{-1} boric acid, at a density of about 100–300 grains per drop. The flowers used as the female parent were emasculated and bagged 2 days before anthesis. On the day of anthesis the ovary was surface-sterilized by wiping it with cotton soaked in ethanol, and the pollen suspension injected into the ovary through a hole using a hypodermic syringe. Another hole was made opposite the point of injection to allow the air to escape. The suspension was injected until the ovarian cavity was filled, and the liquid started to come out of the other hole. After introducing the pollen suspension the holes were sealed with petroleum jelly. Unlike *Papaver* and *Eschscholtzia*, in *Argemone* insertion of pollen through a slit proved better than the injection of suspension through a pore. While emasculating the flowers, only stamens were removed because excising the calyx and corolla led to yellowing of the ovary.

Although the technique of intra-ovarian pollination appears promising, there are no available data showing whether this technique was useful for overcoming barriers of infertility except for the cross between *Argemone mexicana* and *A. ochroleuca* (Kanta and Maheshwari, 1963a).

A still more promising and proven technique developed by Maheshwari and his students to overcome the pre-zygotic barriers to fertility is what they have described as 'test-tube fertilization'. In this technique the stigmatic, stylar and ovary wall tissues were completely removed from the path of the pollen tube, and the exposed ovules were directly dusted with pollen grains and cultured in nutrient medium until seed maturity. 'Test-tube fertilization' was first reported in *Papaver somniferum* (Kanta et al., 1962; see Fig. 10.1). The pollen grains lodged on the ovules germinated within 15 min and fertilization was effected within 1–2 days after pollination. Within 5 days the fertilized ovules enlarged, became turgid and opaque, and contained a four-celled proembryo and free nuclear en-

dosperm. Fully differentiated dicotyledonous embryo developed after 22 days. Kanta and Maheshwari (1963b) reported similar success with some other taxa belonging to the Papaveraceae (*Argemone mexicana*, *Eschscholtzia californica*) and the Solanaceae (*Nicotiana rustica*, *N. tabacum*). Since then the technique of 'test-tube fertilization' has been successfully applied to many compatible and incompatible combinations (see Table 10.1).

10.2. TERMINOLOGY

Kanta et al. (1962), Kanta and Maheshwari (1963b) and, following them, several other authors (Zenkteler, 1965, 1967, 1970; Balatkova and Tupy, 1968; Rangaswamy and Shivanna, 1969) have described the technique of seed development through in vitro pollination of exposed ovules as 'test-tube fertilization'. Seed development following stigmatic pollination of cultured whole pistils has been referred to as 'in vitro pollination'. However, in either case fertilization of the egg occurs inside the ovule by sperms delivered by the pollen tube almost in a natural fashion. Strictly speaking, the term 'test-tube fertilization' should, as in animals (Thibault, 1969), refer to in vitro fusion of excised egg and sperm cells. Therefore, in this book, in vitro application of pollen to excised ovules is referred to as 'in vitro ovular pollination', to the ovules attached to placenta as 'in vitro placental pollination' and to stigma of intact pistils as 'in vitro stigmatic pollination', under the general term of 'in vitro pollination' (Fig. 10.2). The term 'in vitro fertilization' is used only in the case of maize where fusion of isolated egg and sperm cells has been reported (Kranz and Lorz, 1990, 1993; Kranz et al., 1990, 1991).

10.3. IN VITRO POLLINATION

10.3.1. Techniques

The preliminary steps for in vitro pollination and intra-ovarian pollination are the same, viz (a) determination of the time of anthesis, dehiscence of anthers, pollination, entry of pollen tube into the ovules, and fertilization, (b) emasculation and bagging of flower buds, and (c) collection of pollen grains. In addition, for in vitro pollination a suitable nutrient medium is required that will favour the germination of pollen grains and, more importantly, the development of fertilized ovules into mature seeds.

A principal requirement for in vitro pollination is the maintenance of reasonable sterility during pollen and ovule collection. To prevent chance

pollination the buds to be used as the female partner are emasculated before anthesis and bagged. One or two days after anthesis the buds are brought to the laboratory and prepared for aseptic culture. If necessary, sepals and petals are removed and the pistil along with the pedicel, if present, is given a quick rinse in 70% alcohol, surface-sterilized with a suitable agent (see Chapter 2) and finally washed thoroughly with sterile distilled water. The stigma and style are removed and the ovary wall is peeled to expose the ovules. The whole placenta bearing the ovules and attached to a short pedicel is generally used for placental pollination. Alternatively, the placenta may be cut into two or more pieces each carrying a certain number of ovules. Balatkova and Tupy (1968, 1972, 1973) split the placenta carrying the ovules into two longitudinal halves and planted them individually with their cut ends in contact with the medium. Rangaswamy and Shivanna (1971b) further modified the technique of placental pollination. They cultured the entire pistils and exposed the placenta bearing the ovules by removing the ovary wall. Such a system would allow a study of the effect of simultaneous placental and stigmatic pollination in the same pistil (see also Wagner and Hess, 1973; Hess and Wagner, 1974). To perform in vitro stigmatic pollination the excised pistils are carefully surface-sterilized without wetting the stigma with the sterilant solution.

For collecting the pollen under aseptic conditions undehisced anthers are removed from buds and kept in sterile petri plates until they dehisce. When anthers are to be taken from open flowers they may be surface-sterilized and left in sterile petri plates containing a pre-sterilized filter paper until their dehiscence. The discharged pollen is aseptically deposited on the cultured ovules, placenta, or stigma, as the case may be. It has been reported that the pollen deposited on ovules or placenta perform better than those spread on the medium around the ovules. Free water on the surface of ovules may also inhibit pollen germination. Therefore, the film of water on the ovules should be removed with the point of a filter paper (Zenkteler et al., 1987).

Fig. 10.1. In vitro pollination in Papaveraceae. (A,B,D,F–H, *Papaver somniferum*; C, *Eschscholtzia californica*; E, *Argemone mexicana*). (A) Portion of placenta with ovules pollinated in vitro. (B) Whole mount from pollinated culture, showing pollen germination and pollen tube growth on and around the ovule. (C) Whole mount from 1-day-old pollinated culture, showing pollen tube entry into the ovule. (D) Seven-day-old culture; several young seeds are seen on the placenta. (E,F) Proembryo and globular embryo, respectively, excised from seeds developed in vitro. (G) Longisection of seed 15 DAP, showing normal embryo and endosperm. (H) Germinated seeds excised from 22-day-old pollinated culture (A,C,E,G and H after Kanta and Maheshwari, 1963b; B,D and F after Kanta et al., 1962).

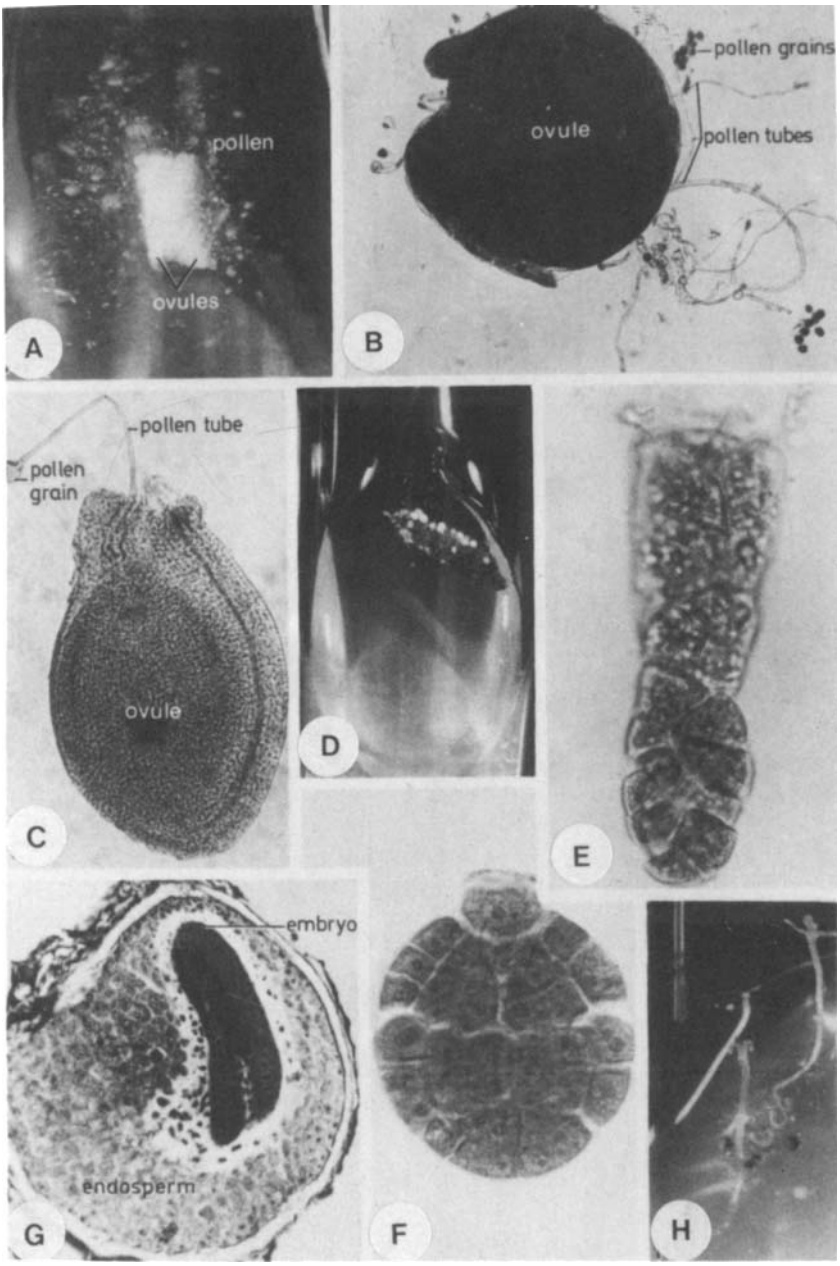
pollen
ovules
A
ovule
pollen grains
pollen tubes
B
pollen tube
pollen grain
ovule
C
D
E
embryo
endosperm
G
F
H

TABLE 10.1

Examples where viable seeds were produced after in vitro ovular or placental pollination

Species	Medium[a,b]			Reference
	Basal medium	Sucrose conc. (%)	Supplements (mg l^{-1})	
Self-pollination				
Agrostemma githago	N	5	CH (500)	Zubkova and Sladky (1975)
Argemone mexicana	N	5	CH (500)	Kanta and Maheshwari (1963b)
Brassica campestris	MS	2	–	Zenkteler et al. (1987)
B. napus	MS	2	–	Zenkteler et al. (1987)
B. oleracea	N			Kameya et al. (1966)
Dianthus caryophyllus	W	4	–	Zenkteler (1965)
Dicranostigma franchetianum	N	4	–	Rangaswamy and Shivanna (1969)
Digitalis purpurea				Zenkteler and Slusarkiewicz-Jarzina (1986)
D. lutea				Zenkteler and Slusarkiewicz-Jarzina (1986)
Diplotaxis tenuifolia				Zenkteler (1988)
Eschscholtzia californica	N	5	CH (500)	Kanta and Maheshwari (1963b)
Glycine max	MS,B_5	6		Tilton and Russell (1983, 1984)
Melandrium album	W,N	2,5	–, CH (500)	Zenkteler (1967); Zubkova and Sladky (1975)
M. rubrum	W	2	–	Zenkteler (1967)
Nicotiana alata	LS			Zenkteler (1980)
N. knightiana				Zenkteler and Slusarkiewicz-Jarzina (1986)
N. longiflora				Zenkteler and Slusarkiewicz-Jarzina (1986)
N. rustica	N	5	CH (500)	Kanta and Maheshwari (1963b)
N. tabacum	N	5	CH (500)	Kanta and Maheshwari (1963b)
Papaver nudicaule	N	5	CH (500)	Olson and Cass (1981)
P. somniferum	N	5	CH (500)	Kanta et al. (1962)
Petunia axillaris	RS	4	–	Rangaswamy and Shivanna (1971a,b)

P. hybrida	N	4	CH (500)+ IAA (0.1)	Wagner and Hess (1973)
P. parodii				Zenkteler and Slusarkiewicz-Jarzina (1986)
Primula pubescence				Zenkteler and Slusarkiewicz-Jarzina (1986)
Scopolia carniolica				Zenkteler and Slusarkiewicz-Jarzina (1986)
Sisymbrium loescli				Zenkteler (1988)
Torenia fournieri				Zenkteler and Slusarkiewicz-Jarzina (1986)
Trifolium repens	MS	3	CH (100)	Leduc et al. (1992)
Zea mays	W,N,MS	17	Yolk of hen's egg (100 drops l^{-1})	Sladky and Havel (1976)
	LS	15		Gengenbach (1977a,b)
	GP	5	GA_3 (10)	Dhaliwal and King (1978)
	MS	7	CH (500)+ IAA (1)+ Kn (0.5)	Bajaj (1979)
	MS	5	GA_3 (10.4)	Raman et al. (1980)
	MS	5	CH (500)	Dupuis and Dumas (1990)
Cross-pollination				
Brassica napus × *B. campestris*	MS	2		Zenkteler et al. (1987)
B. oleracea × *B. cretica*				Zenkteler (1992)
B. oleracea × *Diplotaxis tenuifolia*				Zenkteler (1992)
Melandrium album × *M. rubrum*	W	2	–	Zenkteler (1967)
M. album × *Viscaria vulgaris*	W	2	–	Zenkteler (1969)
M. album × *Silene schafta*	W	2	–	Zenkteler (1967, 1969)
M. rubrum × *M. album*	N,W	2	–	Zenkteler (1969)
Nicotiana alata × *N. debney*				Zenkteler (1992)
N. tabacum × *N. amplexicaulis*	N	4	CH (500)	DeVerna et al. (1987)
N. tabacum × *N. benthamiana*	N	4	CH (500)	DeVerna et al. (1987)
N. tabacum × *N. debney*	N,MS	2	–	Zenkteler (1980)
N. tabacum × *N. rependa*	N	4	CH (500)	DeVerna et al. (1987)

TABLE 10.1 (*continued*)

Species	Medium[a,b]			Reference
	Basal medium	Sucrose conc. (%)	Supplements (mg l^{-1})	
N. tabacum × *N. rustica*	N	5		Marubashi and Nakajima (1985)
Petunia parodii × *P. inflata*	W	4	–	Sink et al. (1978)
Zea mays × *Z. mexicana*	GP	5	GA_3 (10)	Dhaliwal and King (1978)

[a]GP, Green and Phillips (1975); LS, Linsmaier and Skoog (1965); MS, Murashige and Skoog (1962); N, Nitsch (1951); RS, Rangaswamy and Shivanna (1971a); W, White's medium as modified by Rangaswamy (1961).
[b]Blank spaces in columns 2–4 indicate that the information was not available.

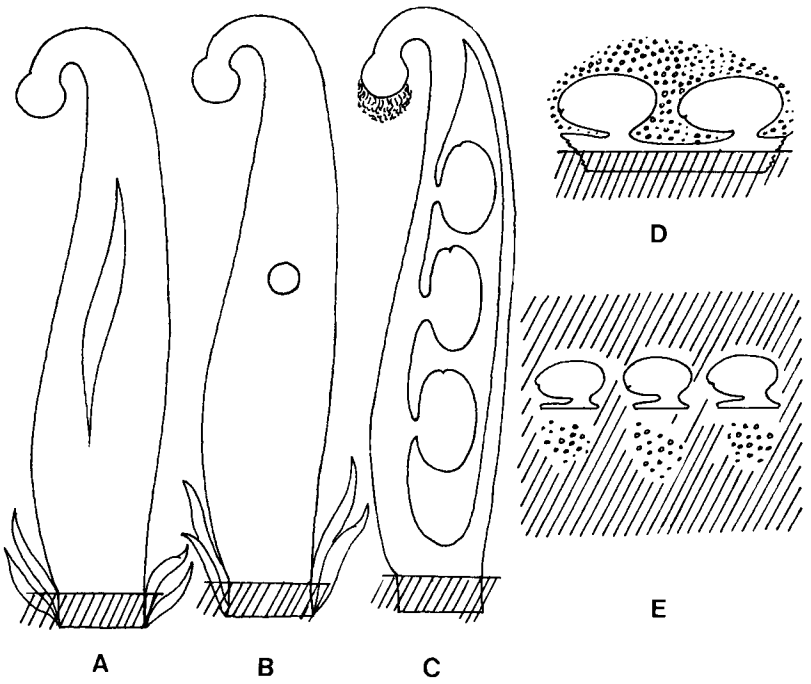

Fig. 10.2. Diagrams depicting various types of in vitro pollinations. (A,B) Intra-ovarian pollination through a slit and a pore in the ovary wall, respectively. (C) Stigmatic pollination. (D) Placental pollination. (E) Ovular pollination (modified from Tilton and Russell, 1984).

In maize, where ovaries are well protected by several layers of husks, surface sterilization has proved unnecessary (Gengenbach, 1977a, 1984). Bagged spadices are collected 2–6 days after the emergence of silk. The silks projecting from the husks are severed with a scalpel, and the outer husks removed by hand. The removal of the innermost husk and all subsequent operations are performed with sterilized instruments under aseptic conditions. Pieces of cob, each carrying a total of 4–10 ovaries in two rows, are placed on the medium in petri plates in such a way that their silks remain hanging out of the plate after putting on the lid. The silks can be pollinated with pollen not necessarily aseptic. Twenty-four hours after pollination the silks are clipped off and the petri plates sealed. Successful in vitro pollination in maize has been achieved with as few as only one pollen per silk. Generally, the fertilization rates are lower with single pollen pollination than following mass pollination (Raman et al., 1980; Hauptli and Williams, 1988) but Kranz and Lorz (1990) and Kranz and Brown (1992) reported similar fertilization rates with mass (41%) and single grain (39%) pollinations.

The most critical step of the in vitro pollination technique is the development of viable seeds from ovules (in ovular and placental pollination)

and ovaries (in stigmatic pollination) following fertilization. It would not be out of place, therefore, to discuss briefly the studies on ovule and ovary culture before dealing with the factors affecting seed-set following in vitro pollination.

(i) *Ovule culture.* Ovule culture is important not only with reference to in vitro pollination, but also because it serves as an experimental system to study the in vitro response of zygotes and very young proembryos, which are difficult to excise and culture (see Chapter 11). The first attempt to isolate ovules and culture them under aseptic conditions was made by White (1932) in *Antirrhinum majus*. However, the technique of ovule culture was developed and perfected at the University of Delhi.

In vitro development of viable seeds from ovules containing globular or older embryos is comparatively easy (*Gynandropsis* and *Impatiens*, Chopra and Sabharwal, 1963; *Nicotiana tabacum*, Dulieu, 1966; *Allium cepa*, Guha and Johri, 1966). With reference to in vitro pollination, however, it is not only desirable but absolutely essential to be able to grow very young ovules excised soon after fertilization. The first report of a successful culture of ovules containing zygote was published by Maheshwari (1958) in *Papaver rhoeas*. Maheshwari (1958) and Maheshwari and Lal (1961b) raised viable seeds of *Papaver somniferum* starting with ovules excised 6 days after pollination (DAP), when they contained a zygote or a two-celled proembryo and a few endosperm nuclei. On a rather simple medium, containing Nitsch's (1951) mineral salts, White's (1943) vitamins, and sucrose (5%) but without any growth substance, the growth of the embryo was initially slower than that in vivo but after the globular stage the embryo grew very rapidly. In 20-day-old cultures the embryos measured 0.93 mm compared to the maximum length of 0.65 mm attained by the embryos in vivo. The addition of kinetin or casein hydrolysate accelerated the initial growth of the embryo.

Poddubnaya-Arnoldi (1959, 1960) successfully grew the ovules from pollinated ovaries of several orchids simply on 10% sucrose solution. She was able to trace events from the time of the entry of the pollen tube into the ovule up to the development of the embryo.

Unlike *Papaver* and orchids, when the ovules of *Zephyranthes* containing a zygote and the primary endosperm nucleus were cultured on Nitsch's medium alone, the embryo grew up to the globular stage but failed to differentiate further (Sachar and Kapoor, 1959). The addition of growth regulators, such as kinetin, IAA, or GA_3, did not promote further growth of the embryo. However, if coconut milk or casamino acids were added to the medium the embryo developed normally and the seeds germinated in situ

(Kapoor, 1959). Individually, histidine, arginine or leucine were as effective as casamino acids. In *Trifolium repens* ovules cultured at the zygote or two-celled proembryo stage (1–2 DAP) developed into mature seeds only if the medium was supplemented with the juice prepared from young fruits (10 days after anthesis) of cucumber or watermelon (Nakajima et al., 1969). The addition of GA_3 (10 mg l^{-1}) in the presence of cucumber juice further promoted seed development from these very young ovules. Wakizuka and Nakajima (1974) noted that cucumber juice (5%) was also essential to raise fully developed, germinable seeds from *Petunia hybrida* ovules excised shortly after the entry of the pollen tube (2 DAP). Osmolarity of the culture medium has been reported to play an important role in the development of excised ovules. It is especially critical for very young ovules. In *Petunia hybrida* ovules isolated 7 DAP, enclosing a globular embryo, developed into mature seeds on a medium containing sucrose anywhere in the range of 4–10%. The optimal level of sucrose for ovules having zygote and a few endosperm nuclei was 6%, whereas for ovules just after fertilization it was 8% (Wakizuka and Nakajima, 1974).

In contrast to the reports of Maheshwari (1958) and Maheshwari and Lal (1961b), Pontovich and Sveshnikova (1966) observed that in the cultures of excised ovules (enclosing zygote or two-celled proembryo) of poppy the embryo failed to undergo differentiation even if the medium was supplemented with casein hydrolysate, coconut milk, adenine or kinetin. However, if the ovules were cultured attached to the placenta the zygote developed into a normally differentiated embryo. The beneficial effect of placental tissue on the growth and development of cultured ovules has also been observed in *Gynandropsis* (Chopra and Sabharwal, 1963). In their studies on in vitro pollination, Kanta et al. (1962) and Rangaswamy and Shivanna (1971a) recorded a similar promotive effect of placenta on seed development.

Ovule culture has proved to be a very useful technique to raise interspecific (Przywara et al., 1989; Hossain et al., 1990; Espinasse et al., 1991) and intergeneric (Hossain et al., 1988; Takahata, 1990; Ahmad and Comeau, 1991) hybrids which normally fail due to the abortion of embryo at a rather early stage when its excision and/or culture is either very tedious or impossible. The loss of a hybrid embryo due to premature abscission of fruits, as happens in many interspecific crosses in cotton, could be prevented by ovule culture (Steward and Hsu, 1978).

In the cross *Gossypium arboreum* × *G. hirsutum* the hybrid embryo develops only up to 8–10 DAP. Subsequently, numerous abnormalities occur leading to the failure of further embryo development. Beasley (1940) and Weaver (1958) cultured the immature embryos to raise full plants, but the excised embryos failed to grow. Pundir (1967) excised the

TABLE 10.2

The composition of nutrient medium for culturing young, fertilized ovules of cotton following intra- and interspecific pollination[a]

Constituents	Amount (mg l^{-1})
KNO_3	5055
NH_4NO_3	1200
$MgSO_4 \cdot 7H_2O$	493
$CaCl_2 \cdot 2H_2O$	441
KH_2PO_4	272
$FeSO_4 \cdot 7H_2O$	8.3
Na_2·EDTA	11
H_3BO_3	6.18
$MnSO_4 \cdot 4H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$NaMoO_4 \cdot 2H_2O$	0.24
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.024
Nicotinic acid	0.49
Pyridoxine·HCl	0.82
Thiamine·HCl	1.35
Inositol	180
D-Fructose	3600
Sucrose	40000
IAA	5×10^{-5} M

[a]After Steward and Hsu (1978).

ovules 3 days after cross pollination when they contained zygote or a three-celled proembryo and cultured them on Murashige and Skoog's medium containing 50 mg l^{-1} of inositol. By the 5th week in culture fully differentiated embryos were formed and by the 7th week 70–80% ovules developed hybrid seedlings. Following a similar technique, Steward and Hsu (1978) raised four different interspecific hybrids in the genus *Gossypium* which were otherwise unknown. For the composition of the medium used, see Table 10.2.

The cross *Trifolium repens* × *T. hybridum* has not been successful by the conventional or embryo rescue method. However, excising the ovules 12–14 DAP and culturing them on a medium containing 15% cucumber juice for 5–6 days provided culturable embryos (Przywara et al., 1989). Similarly, Espinasse et al. (1991) succeeded in obtaining some hybrid plants from the crosses *Helianthus annuus* × *H. maximiliani* and *H. annuus* × *H. tuberosum* by a combination of in ovulo embryo culture, for

1 week, followed by excised embryo culture. Direct embryo culture was not successful in these crosses showing post-fertilization incompatibility.

Excised ovule culture has been used by several scientists to investigate the development of cotton fibre (Beasley and Ting, 1973; Waterkeyn et al., 1975; Haigler et al., 1991). Beasley and Ting (1973) conducted detailed studies on the physiology of fibre cell elongation in cultured ovules of *Gossypium hirsutum*. The ovules excised on the day of anthesis or a day later either failed to develop or formed only a few fibres. However, most of the ovules cultured 2 days after anthesis showed the development of fibres. The ovules floating on the surface of a liquid medium produced more fibres than those which were submerged. Fibre development was better at 30°C than at 20 or 25°C. GA_3 induced marked elongation of the fibre, whereas kinetin and ABA exercised a marked inhibition. Haigler et al. (1991) found cultured ovules to be a model system to study fibre development under low temperature. Singletary and Below (1989) used ovule culture to study the effect of carbon and nitrogen supply on carbohydrate and protein accumulation in developing kernels of maize.

(ii) *Ovary culture*. LaRue (1942) was probably the first to raise aseptic cultures of angiosperm flowers. He obtained rooting of the pedicel and a limited growth of the ovaries. The technique of ovary culture was developed by Nitsch (1951) who grew detached ovaries of *Cucumis anguria*, *Fragaria* sp., *Lycopersicon esculentum*, *Nicotiana tabacum* and *Phaseolus vulgaris* on synthetic medium. The ovaries of *Cucumis* and *Lycopersicon* excised from pollinated flowers developed into mature fruits containing viable seeds. However, the fruits were smaller than those formed in nature. Similarly, the fruits of *Linaria macroccana* (Sachar and Baldev, 1958) and *Tropaeolum majus* (Sachar and Kanta, 1958) developed in vitro failed to attain a size comparable to that of fruits developed under natural conditions.

Maheshwari and Lal (1958, 1961a) succeeded in rearing the ovaries of *Iberis amara* excised from flowers 1 DAP. At this stage the ovules contained a zygote and a few free endosperm nuclei. On a simple medium containing mineral salts and sucrose, the growth of ovaries was good, but the embryo remained smaller than those formed in nature. With the addition of B-vitamins to the above medium, normal healthy fruits matching the natural fruits were obtained. In vitro formed fruits were even larger than the in vivo formed fruits when IAA was added to the mineral salts-sugar-vitamin medium (Maheshwari and Lal, 1958). In cultures, normal development of ovaries, excised at the zygote or two-celled proembryo stage, was also achieved with *Anethum graveolens* (Johri and Sehgal, 1966) and *Hyoscyamus niger* (Bajaj, 1966b). On a medium sup-

plemented with coconut milk the ovaries of *Anethum* surpassed the size of natural fruits.

In vitro studies have revealed that the floral envelopes do not function merely as protective structures for sex organs, but play an important role in the development of the fruit and the embryo. In the cultures of *Triticum aestivum* and *T. spelta* ovaries, excised soon after pollination, the embryo developed normally only if the floret envelopes (lemma and palea) were left intact. In their absence the growth of the proembryo was impaired (Redei and Redei, 1955a,b). La Croix et al. (1962) reported similar observations with barley and concluded that the 'Hull factor' is necessary for a proper development of the embryo. In the absence of the 'Hull factor' the cells of barley embryo underwent elongation and DNA synthesis but did not divide. In dicotyledonous plants removing the calyx also results in poor fruit development (Chopra, 1958, 1962; Maheshwari and Lal, 1958, 1961a; Bajaj, 1966; Guha and Johri, 1966; Richards and Rupert, 1980). It is, therefore, important that the perianth should be left intact while attempting in vitro stigmatic pollination.

Inomata (1977, 1979) raised interspecific hybrids between sexually incompatible parents *Brassica campestris* and *B. oleracea* through ovary culture. To date several other interspecific and intergeneric hybrids in the family Brassicaceae have been produced with the aid of ovary culture (Sarla and Raut, 1988; Batra et al., 1990).

10.3.2. Factors affecting seed-set following in vitro pollination

(i) *Explant.* In *Petunia axillaris* in vitro pollinated excised ovules or a group of ovules attached to a piece of placenta did not form viable seeds. The pollen grains germinated normally, but the pollen tubes failed to enter the ovules (Rangaswamy and Shivanna, 1971a). However, when intact placentae with undisturbed ovules were pollinated, normal events from pollen germination to the development of viable seeds occurred (see Fig. 10.3). Wagner and Hess (1973) reported that in *Petunia hybrida* complete removal of the style had a deleterious effect on seed-set following placental pollination. Consequently, for in vitro pollination they (Wagner and Hess, 1973; Hess and Wagner, 1974) cultured the entire pistil and exposed the ovules by simply peeling the ovary wall. In such explants if placental pollination and stigmatic pollination are made on the same pistil, the latter exhibits better fertilization.

In maize the ovaries attached to cob tissue give better results than single ovaries (Sladky and Havel, 1976; Gengenbach, 1977a; Dhaliwal and King, 1978). Reducing the number of ovaries per explant does not affect fertilization but it has a deleterious effect on kernel development

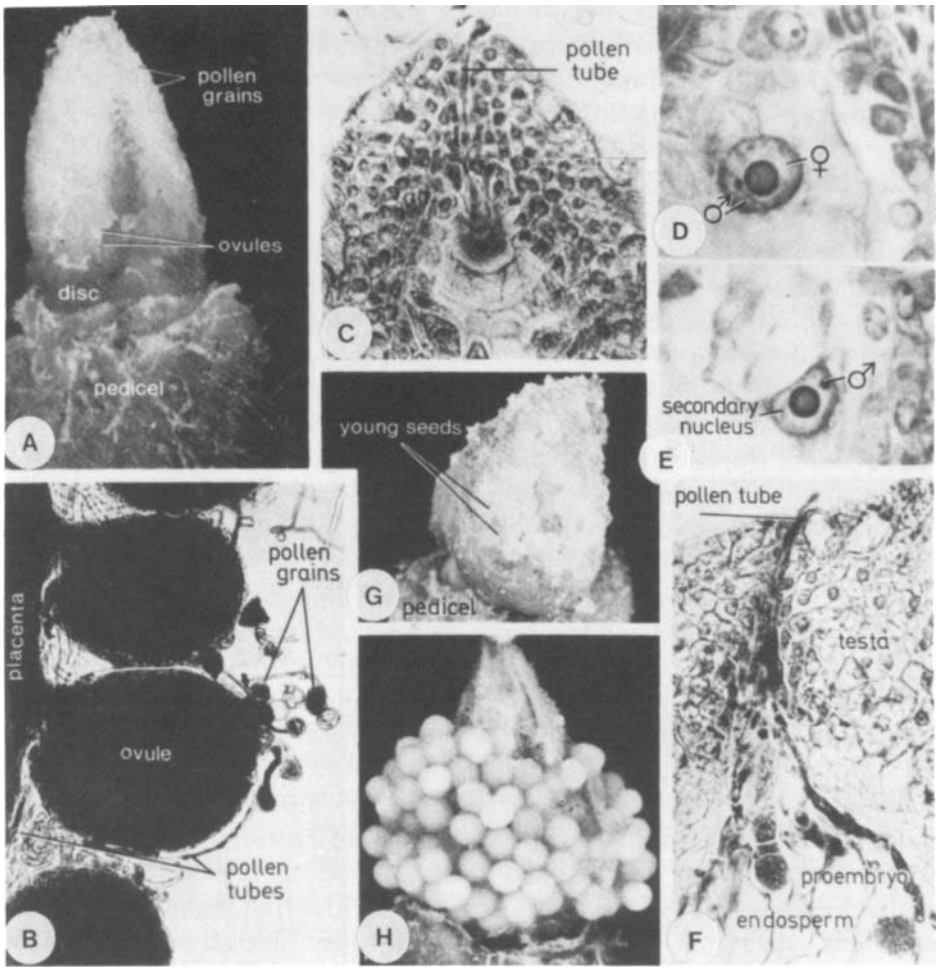

Fig. 10.3. In vitro placental pollination to overcome self-incompatibility in *Petunia axillaris*. (A) Both the placentae of an ovary, with their entire mass of ovules covered with pollen, ready for culture. (B) Free-hand section of placentae 24 h after self-pollination; the picture shows a portion of a placenta bearing 3–4 ovules. Pollen grains have germinated and the pollen tubes are seen all over and in between the ovules. (C) Longisection through micropylar region of ovule 24 h after self-pollination; note the entry of pollen tube into the embryo sac. (D,E) Sections of ovules 2 days after selfing, showing syngamy and triple fusion, respectively. (F) Micropylar end of a 7-day-old seed in longisection; note extra-micropylar part of pollen tube, remnants of pollen tube in micropyle, testa, filamentous proembryo and cellular endosperm. (G,H) Five- and 24-day-old cultures, respectively; numerous mature seeds are formed in (H). (A,B,F–H after Rangaswamy and Shivanna, 1967; C–E after Rangaswamy and Shivanna, 1971a).

(Gengenbach, 1977a; Higgins and Petolino, 1988). Gengenbach (1977a) reported that cob pieces with one or two ovaries did not form any fully developed kernel. Four ovary blocks developed only small kernels, whereas 10 ovary blocks had one or two fully developed kernels of large size. Higgins and Petolino (1988) also observed that the development of complete kernels was greatly influenced by the size of the explant and the ratio of ovule-to-cob tissue. According to these authors cob pieces bearing 30 ovaries produced twice as many fully formed kernels as with 10 ovules. Similarly, an ovule-to-cob tissue ratio of 4:24 (cob pieces with 24 ovaries were taken but only 4 ovaries per piece were pollinated; all other ovaries were removed) gave best results; depending on the genotype, 19.4–36.2% ovaries formed complete kernels.

The beneficial effect of parental tissue on ovary and ovule growth is discussed in Section 10.3.1. Role of accessory organs in seed development is also highlighted in the studies on in vitro pollination of *Trifolium* species (Richards and Rupert, 1980; Leduc et al., 1992). Besides the excision of calyx and corolla lobes, excessive injury to the ovary wall was detrimental to in vitro development of seeds. In *T. repens* even an incision along the entire length of the ovary wall, to expose the ovules for pollination, caused necrosis of the ovary. However, insertion of pollen through a pore in the ovary wall ('in vitro intra-ovarian pollination') resulted in fertilization and the formation of torpedo embryos (Leduc et al., 1992). Similarly, in *Brassica* species removal of the entire ovary wall was highly detrimental to seed-set following in vitro pollination whereas removal of only a part of the ovary wall gave the best results (Zenkteler et al., 1987). It is, therefore, recommended that in in vitro pollination studies a bare minimum of the parental tissue, necessary to pollinate the ovules, should be removed.Wetting the surface of the ovules and stigma (in stigmatic pollination) should be avoided because it may lead to poor pollen germination and/or bursting of the pollen tubes and, consequently, poor seed-set (Balatkova and Tupy, 1968; Zenkteler, 1980).

Time of excising the ovules from pistils has a definite influence on seed-set following in vitro pollination. The incidence of seed-set is higher when the ovules are excised 1–2 days after anthesis than on the day of anthesis (Kanta et al., 1962; Kanta and Maheshwari, 1963b; Balatkova et al., 1977b). The optimal stage of maize spike for in vitro pollination is 3–4 days after silking (Gengenbach, 1977b). The physiological state of the pistil at the time of excising the ovules or pistil may also influence seed-set following in vitro pollination. Balatkova et al. (1977a) observed that the unfertilized ovules of tobacco excised from the pistils pollinated with its own pollen or those of *Malus* sp. and pollinated in vitro with the pollen of tobacco gave a comparatively better seed-set than the ovules from

unpollinated pistils. Pollen germination on the stigma and pollen tube growth through the style is known to influence metabolic activities in the ovary (White, 1907; Tupy, 1961; Deurenberg, 1976). Johri and Maheshwari (1966), Sturani (1966) and Deurenberg (1976) demonstrated that pollen tube-style interaction stimulates protein synthesis in the ovary. Balatkova et al. (1977a) confirmed this observation in tobacco. These results suggest that if detailed temporal studies on pollination, pollen tube growth, entry of the pollen tube into the ovary and double fertilization are made under the conditions in which the experimental plants are grown, it should be possible to improve the chances of success of in vitro pollination by excising the ovules from pollinated pistils, but before the entry of pollen tubes into the ovary. In this context, it is interesting that in *Petunia axillaris*, a self-incompatible species, self-stigmatic pollination did not interfere with the processes of fertilization and seed development as a result of self-placental pollination simultaneously made on the same pistil (Rangaswamy and Shivanna, 1971b).

(ii) *Culture medium.* The technique of in vitro pollination involves two major processes: (a) germination of pollen grains and pollen tube growth leading to fertilization, and (b) development of the fertilized ovules into mature seeds with a viable embryo. The efficiency of the technique, to a large extent, depends on the composition of the medium which can support both processes. It is not very difficult to achieve a satisfactory germination of pollen grains and pollen tube growth. Moreover, in placental pollination, which is the most promising of the various types of in vitro pollination, pollen grains are sprinkled on the ovules where they germinate, and pollen tubes enter the ovules without even coming in contact with the medium. Where pollen grains fail to germinate on the surface of the ovules they may be separately grown on a suitable medium and pollen tubes applied to the ovules (Balatkova and Tupy, 1968). Seed development following in vitro pollination by germinated pollen has also been reported in maize (Raman et al., 1980).

Pollen grains in the Brassicaceae, which are shed at the three-celled stage, are difficult to germinate in vitro and, consequently, in vitro pollination in this group of plants is also refractory. Kameya et al. (1966) modified the technique of in vitro pollination to obtain germinable seeds in *Brassica oleracea*. They dipped the isolated ovules in a 1% solution of $CaCl_2$, planted them on a slide pre-coated with 10% gelatin solution of about 40 μm thickness and immediately pollinated them with the pollen from freshly opened flowers. The slide was stored in a covered petri plate with a moist filter paper sticking to the lid. After 24 h only the fertilized ovules were transferred to Nitsch's agar medium. Following this method,

Kameya et al. could obtain two germinable seeds from 75 ovules initially pollinated. Seed development did not occur without the calcium treatment. Promotion of pollen germination and pollen tube growth by Ca^{2+} ions is well known (see Bhojwani and Bhatnagar, 1992).

The most important role of culture medium is in supporting normal development of the fertilized ovules. It is, therefore, imperative that before attempting in vitro pollination the optimal nutritional and hormonal requirements and physical conditions for the successful culture of very young ovules (containing the zygote or a few celled proembryo) of the plant to be used as the female parent are investigated. This would improve the chances of success. Indeed, this was the secret of the first success of Kanta et al. (1962) in obtaining viable seeds through in vitro placental pollination in *Papaver rhoeas*. They used exactly the same medium as that described by Maheshwari and Lal (1961b) for the successful culture of fertilized ovules of this taxon taken 6 days after in vivo pollination.

The salt mixture commonly used for in vitro pollination is that developed by Nitsch (1951) for ovary culture. To this are added sucrose and vitamins (White, 1943). The composition of the modified Nitsch's medium widely employed to culture in vitro pollinated ovules is given in Table 10.3. Sladky and Havel (1976), who tested different basal media (White, 1943; Murashige and Skoog, 1962; Nitsch, 1969), did not find a significant difference in the response of the in vitro pollinated ovaries.

Generally, sucrose has been used at a concentration of 4–5%. Even in maize, where some authors have used as much as 15–17% sucrose (Sladky and Havel, 1976; Gengenbach, 1977a,b), Dhaliwal and King (1978) obtained viable seeds following intra- and interspecific in vitro pollination with the normal level of 5% sucrose.

The information on the effect of various growth regulators and other supplements to the basal medium on seed development from cultured ovules is very meagre. This seems to be the reason for the failure of seedsets especially when pollen germination and pollen tube entry into the ovule (Niemirowicz-Szczytt and Wyszogrodzka, 1977) and fertilization (Zenkteler and Melchers, 1978) in vitro occurred normally. Generally, the basal medium has been supplemented with 500 mg l^{-1} of casein hydrolysate (CH; see Table 10.1). Rangaswamy and Shivanna (1971a), however, did not find any beneficial effect of CH for seed development following self placental pollination in *Petunia axillaris*. Balatkova et al. (1977a) studied the effect of IAA, kinetin, tomato juice (TJ), coconut milk (CM) and yeast extract (YE) on seed development following placental pollination in tobacco. Whereas CM, TJ and YE proved inhibitory, the presence of 10 μg l^{-1} IAA or 0.1 μg l^{-1} kinetin significantly improved the number of seeds per ovary. Higher levels of kinetin (1 μg l^{-1}) were inhibitory.

TABLE 10.3

Composition of the modified Nitsch's medium widely used to culture in vitro pollinated ovules[a]

Constituents	Amount (mg l^{-1})
$CaNO_3 \cdot 4H_2O$	500
KNO_3	125
KH_2PO_4	125
$MgSO_4 \cdot 7H_2O$	125
$CuSO_4 \cdot 5H_2O$	0.025
Na_2MoO_4	0.025
$ZnSO_4 \cdot 7H_2O$	0.5
$MnSO_4 \cdot 4H_2O$	3.0
H_3BO_3	0.5
$FeC_6O_5H_7 \cdot 5H_2O$	10.00
Glycine	7.5
Ca-Pantothenate	0.25
Pyridoxine·HCl	0.25
Thiamine·HCl	0.25
Niacin	1.25
Sucrose	50000
Agar	7000

[a]After Kanta and Maheshwari (1963b).

Sladky and Havel (1976) reported the beneficial effect of hen's egg yolk (100 drops l^{-1}) on pollen germination and seed development in the cultures of in vitro pollinated ovaries of maize. However, subsequent workers did not need to add such a complex mixture for seed development in maize (see Table 10.1).

(iii) *CO_2*. In three genotypes of *Trifolium repens* treatment of florets with an elevated level of CO_2 (1%), for 24 h after in vitro pollination, increased the yield of selfed seeds (Douglas and Connolly, 1989).

(iv) *Storage of cultures*. There are hardly any data on the precise effect of light on the response of in vitro pollinated ovules. Cultures are usually stored in darkness or near darkness. Zenkteler (1969) did not find any difference in the results of in vitro pollination whether the cultures were stored in light or dark (see also Zenkteler, 1980).

Balatkova et al. (1977b) have shown that in some systems temperature may influence seed-set. They observed that in *Narcissus* storing the cultures at 15°C, instead of the usual 25°C, resulted in a dramatic increase in the number of seeds developed in each ovary. However, such a low

temperature did not improve seed-set in *Papaver somniferum*, which flowers under comparatively warmer conditions.

Dupuis and Dumas (1990) have reported that in maize the temperature during the first 4 h following in vitro pollination is very critical for fertilization. Incubation of the cultures at 40°C immediately after pollination suppressed fertilization. However, if the elevated temperature was applied 4 h after pollination at normal temperature it did not affect fertilization.

(v) *Genotype*. There is some evidence of genotypic variation in the response of in vitro pollinated ovaries of maize (Gengenbach, 1977b; Bajaj, 1979).

10.4. IN VITRO FERTILIZATION

For years animal scientists have been practising in vitro fertilization to create organisms and to study processes related to gametic fusion and early embryogenesis. This could not be done with higher plants where the egg cell is encased in several layers of ovular tissues. Therefore, the recent success of a German group in fusing isolated male and female gametes of maize (Kranz et al., 1990, 1991) and regenerating, via embryogenesis, fully fertile hybrid plants from the fusion products (Faure et al., 1993; Kranz and Lorz, 1993) is a major breakthrough in the field of plant biotechnology. It opens out the doors to study the molecular events and tissue differentiation during early stages of embryogenesis in flowering plants and offers new opportunities for the genetic engineering of crops.

Since 1987 several papers have described the isolation of male (Dupuis et al., 1987; Cass and Fabri, 1988; Wagner et al., 1989a) and female (Wagner et al., 1989a,b; Kranz et al., 1990) gametes of maize. In 1990 Kranz et al. described their maiden success with electrofusion of an isolated egg with a sperm cell of maize; the in vitro fertilized egg divided and formed mini calli with a high frequency even after being injected with plasmid DNA. More recently, normal hybrid plants have been regenerated from the zygotes formed by in vitro mating of single male and female gametes derived from different inbred lines of maize (Kranz and Lorz, 1993). The techniques of gamete isolation, their in vitro fusion and culture are summarised in the following paragraphs.

The sperms were isolated from pollen grains by osmotic shock in 540 mosmol kg^{-1} H_2O–mannitol solution. Viable egg cells were isolated by microdissection from the ovules incubated, for 40–60 min, at 24 ± 0.5°C, in an enzyme solution containing pectinase (0.75%), pectolyase Y23

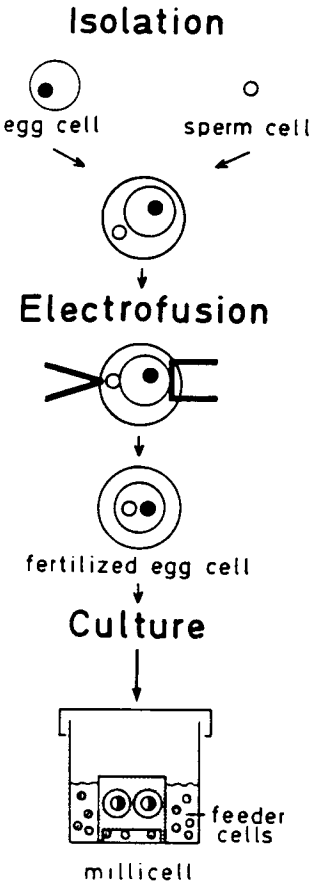

Fig. 10.4. Diagrammatized summary of the method used for electrofusion-mediated in vitro fertilization in *Zea mays*. Single isolated egg and sperm cells were transferred into a fusion droplet and fused electrically after dielectrophoretic alignment on one of the electrodes. For culture the fusion products were transferred, individually, into 'Millicell' inserts surrounded by feeder cells. For details see text (after Kranz et al., 1991).

(0.25%), hemicellulase (0.5%) and cellulase Onozuka RS (0.5%), with osmolarity adjusted to 540 mosmol kg^{-1} H_2O with mannitol, and pH set at 5. With this protocol roughly 5 intact egg cells could be obtained from every 20 ovules tried (Kranz et al., 1991). The freshly isolated gametes are naked protoplasts requiring very delicate handling.

To fuse isolated gametes (Fig. 10.4), single sperm and egg cells were selected and carefully transferred to 1 or 2 μl droplets of fusion solution (540 mosmol kg^{-1} H_2O–mannitol solution) on a coverglass with the aid of microcapillaries connected to a computer-controlled dispenser. The fusion

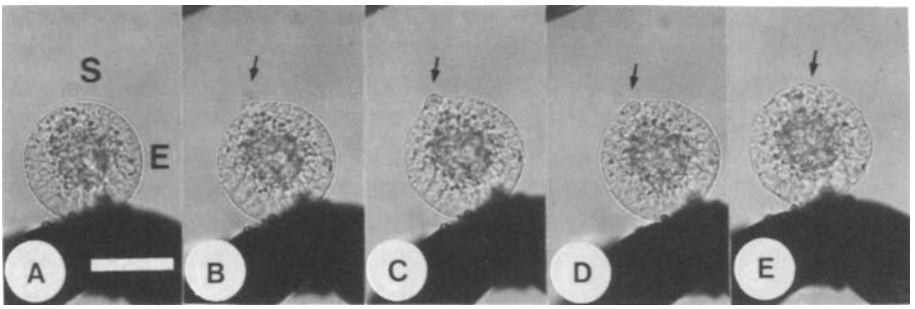

Fig. 10.5. Electrofusion sequence of a selected pair of egg (E) and sperm (S) cells of *Zea mays*. Arrows indicate the fusion site of sperm with egg cell. After the application of DC pulse the time interval from A to E was 4 s (after Kranz et al., 1991).

droplets were covered with a thin layer of mineral oil. Controlled fusion of the gametes was performed with a pair of adjustable electrodes mounted on the microscope. A single DC pulse (0.9–1.0 kV cm^{-1}) for 50 μs, after dielectrophoretic alignment (1 MHz, 71 V cm^{-1}) of the gametes on one of the electrodes for a few seconds, was sufficient to bring about high frequency 'fertilization' (Fig. 10.5). Alignment of the gametes in a manner that the egg was in contact with the electrodes favoured better fusion (Kranz et al., 1991). With this protocol up to 100% fusion could be achieved (mean fusion frequency 79%). Fusion of sperm and egg cells did not occur without an electric pulse even if they were brought into close contact.

The in vitro fertilized eggs were individually cultured on a semipermeable, transparent membrane of a Millicell-CM dish (12 mm diameter) filled with 0.1 ml of nutrient solution. This dish was inserted in the middle of a 3 cm petri plate filled with 1.5 ml of nutrient medium containing feeder cells derived from embryogenic suspension cultures of another maize inbred line. A modified MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.02 mg l^{-1} kinetin, osmolarity adjusted to 600 mosmol kg^{-1} H_2O with glucose and pH set at 5.5 was used. The cultures were maintained under a light/dark cycle of 16/8 h with approximately 50 μE m^{-2} s^{-1} irradiance. Under these conditions karyogamy occurred within 1 h of fusion (Faure et al., 1993) and 92% of the 'zygotes' showed unequal division within 3 days. Under identical conditions unfertilized eggs did not divide. About 90% of the fusion products produced mini colonies and 41% of the cells developed into globular embryos or embryo-like structures (Kranz et al., 1991; Kranz and Lorz, 1993). Full plants could be regenerated by transferring these organized structures, 10–12 days after fusion, to semisolid medium of an altered composition. From 28 fusion products 11

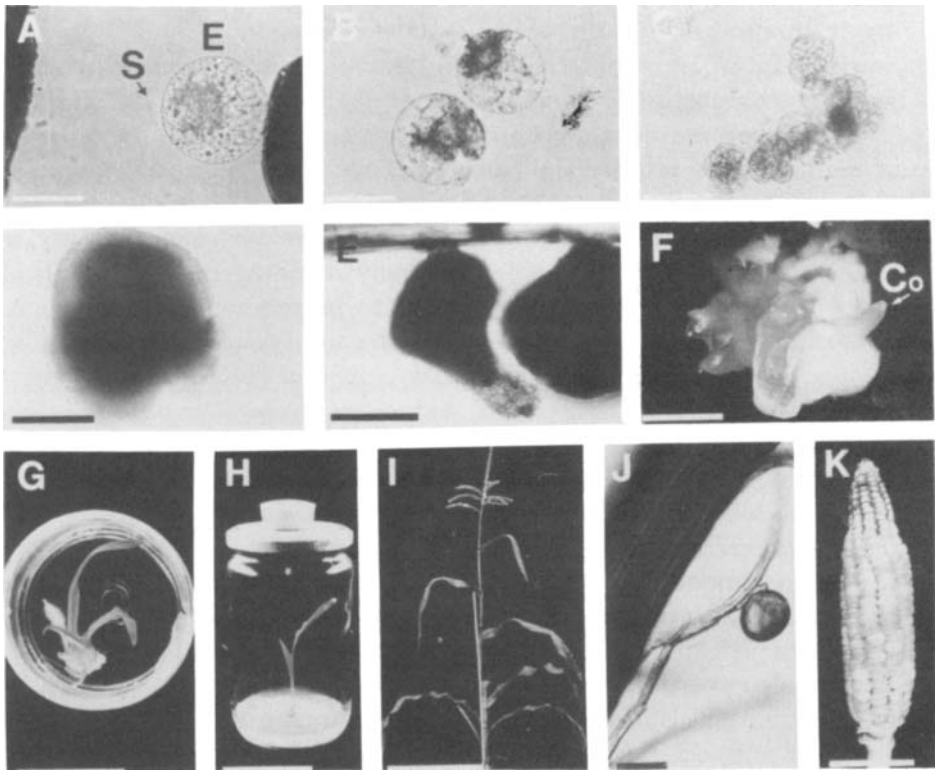

Fig. 10.6. Regeneration of fertile plants from the zygote formed by in vitro fertilization of isolated egg of *Zea mays*. (A) Alignment of an egg cell (E) with a sperm on one of the electrodes before electrofusion. The arrow is pointing at the sperm cell. (B) First polar division of the 'zygote' 42 h after 'in vitro fertilization'. (C) Multicellular structures, 5 days after fusion. (D) Polarized multicellular structure with an outer cell layer at one pole and vacuolized cells at the other end, 12 days after fusion. (E) Transition-phase embryo, 14 days after fusion. (F) A structure with compact white and green tissues, 30 days after fusion; the arrow indicates the coleoptile (Co). (G,H) Plantlets, 35 and 39 days after fusion, respectively. (I) A flowering plant, 99 days after gametic fusion. (J) Germination of a self pollen on the silk of a plant raised from in vitro fertilized egg. (K) A cob, 148 days after in vitro fertilization (after Kranz and Lorz, 1993).

plants were regenerated (a frequency of 48%; Fig. 10.6) within 86 days after fusion (Kranz and Lorz, 1993).

10.5. APPLICATIONS

Several potential applications of in vitro pollination in genetic manipulation of plants have been suggested (Tilton and Russell, 1984; Matthys-Rochon, 1992). The recent success with in vitro fertilization of excised egg of maize has added considerable importance to this area of plant tis-

sue culture. When this new technique becomes applicable to some other species, it should allow study of some novel aspects of sexual reproduction, such as intergametic attraction and gametic recognition, which has not been possible in higher plants so far. Fusion of genetically modified gametes may become another approach to genetic engineering of crop plants. Some of the proven applications of in vitro pollination are discussed here.

In vitro ovular and placental pollinations, where the stigmatic, stylar and ovary wall tissues are almost completely removed from the path of the pollen tube, are potentially very useful in inbreeding and hybridization programmes when the zone of incompatibility lies in the stigma, style or ovary. In vitro pollination has also helped in obtaining haploids.

10.5.1. Inbreeding

Petunia axillaris is a self-incompatible species. In self-pollinated pistils the pollen germination is good, but the pollen tube does not enter the ovary. Rangaswamy and Shivanna (1967, 1971a) have shown that when self-placental pollination is performed in this taxon, fertilization and seed-set occur normally. These authors (Shivanna and Rangaswamy, 1969) could also overcome self-incompatibility in *P. axillaris* through in vivo bud pollination. However, they did not comment on the comparative superiority of one technique over the other. Niimi (1970) reported the development of viable seeds on placenta pollinated by self pollen in another self-incompatible species, *Petunia hybrida*.

Production of homozygous plants of leguminous taxa through androgenesis or gynogenesis has not been successful so far. In the legumes such as *Trifolium* species, with gametophytic incompatibility, even inbreeding to achieve homozygosity is not applicable. Douglas and Connolly (1989) have reported that in *T. repens* the frequency of seed-set following in vitro self-pollination was 5–10 times greater than that in vivo.

10.5.2. Hybridization

Potentially, the most important application of in vitro placental pollination is in raising hybrids which are unknown because of the prefertilization incompatibility barriers. Zenkteler and his associates (see Zenkteler, 1992) attempted several interspecific, intergeneric and interfamily crosses (see Table 10.1) through placental pollination. In the crosses *Melandrium album* × *M. rubrum*, *M. album* × *Viscaria vulgaris*, *M. album* × *Silene schafta* and *Nicotiana alata* × *N. debney* seeds containing viable embryos developed.

Marubashi and Nakajima (1985) produced fertile hybrids with 96 chromosomes by in vitro pollination of *Nicotiana tabacum* with the pollen of *N. rustica*, followed by hybrid ovule culture. This sexually incompatible cross has also been made through protoplast fusion but all the somatic hybrids were aneuploid (Douglas et al., 1981). More recently, DeVerna et al. (1987) raised two interspecific hybrids by in vitro pollination of *N. tabacum* with *N. amplexicaulis*. This cross exhibits unilateral incompatibility. Dhaliwal and King (1978) produced interspecific hybrids by pollinating the exposed ovules of *Zea mays* with pollen grains of *Z. mexicana*.

When ovules of *Nicotiana tabacum* were pollinated with the pollen of *Hyoscyamus niger* the hybrid embryos developed up to the globular stage and well developed endosperm was formed (Zenkteler and Melchers, 1978). The embryo exhibited some budding but did not develop further normally. Excising the ovules and culturing them in a liquid medium containing 2 mg l^{-1} kinetin, 4 mg l^{-1} 2,4-D and 6% sucrose stimulated embryo growth up to the pre-heart shape stage, after which they started to callus. The point of interest in this and several other wider crosses is that one could obtain a cell(s) (zygote/young proembryo) carrying a combination of genomes from desired parents. In such cases, an elaborate attempt to culture the hybrid embryo or to induce organogenesis in the callusing hybrid proembryos would be worthwhile.

10.5.3. Haploid production

Hess and Wagner (1974) raised haploids of *Mimulus luteus* cv. *tigrinus grandiflorus* by pollinating their exposed ovules with the pollen of *Torenia fournieri*. The authors have concluded that the haploids developed parthenogenetically. However, in the absence of detailed anatomical and cytological investigations the alternative origin of the haploids through selective elimination of chromosomes of *Torenia* following fertilization cannot be ruled out (see Section 7.7). Hess and Wagner could not obtain haploids of *M. luteus* through anther culture. Parthenogenetic development of haploid plants in the cultures of unfertilized ovules and ovaries is well known (see Section 7.6).

10.5.4. Production of stress tolerant plants

In vitro pollination at high temperature (38°C) resulted in the production of heat stress-tolerant plants of maize (Petolino et al., 1990). At the elevated temperature only heat stress tolerant pollen grains were able to effect fertilization, and the resulting sporophytes expressed the gameto-

phytic trait. These plants exhibited better agronomic performance (increased seedling vigour, reduced stalk and root lodging, and high grain yield) at high temperatures compared to the plants produced through in vitro pollination at normal temperature (28°C). This approach to gametic selection during in vitro pollination may prove useful to produce plants resistant to other environmental stresses.

10.6. CONCLUDING REMARKS

The technique of in vitro pollination appears very promising to overcome pre-fertilization barriers to compatibility, and raising new genotypes. Although the feasibility of the technique was demonstrated almost three decades ago, for long the plant breeders did not show much interest in applying it to a specific desirable cross. Probably the more fascinating areas of plant tissue culture, such as haploid production through anther and pollen culture and protoplast fusion and culture overshadowed the potential of this simple technique. However, the last decade has witnessed fresh interest in the application of this technique to produce hybrids. Very extensive studies have been made on in vitro pollination and fertilization in maize.

One of the proposed uses of isolated plant protoplasts is in producing hybrids through somatic cell fusion, which involves several tedious steps. One of the most important of these is the selection and identification of the hybrid components from amongst millions of other types of cells, tissues and plants. Although comparative data are not available, it should be easier to identify the hybrid cell in in vitro pollination than in somatic hybridization. In several interspecific and intergeneric combinations in vitro pollination resulted in fertilization and development of young hybrid proembryos but the embryos failed to mature, reflecting our inadequate knowledge of culture requirements of young embryos. In such cases, a detailed search for a suitable nutrient medium to promote the normal development of the hybrid embryo in ovulo or ex ovulo may allow full hybrid plants to be raised. Alternatively, it may be possible to induce organogenesis or embryogenesis in the callusing hybrid embryo; after all, this is the only way of obtaining a hybrid through somatic cell fusion. As a rule, the embryonal callus is more likely to exhibit plant regeneration than the callus derived from somatic cells.

Considerable success has been achieved with isolation of viable male and female gametes of higher plants (Yang and Zhou, 1992). The recent report of plant regeneration from the fusion product of isolated single egg and sperm cells of maize has opened up an entirely new approach to crop improvement (Kranz and Lorz, 1993). Faure et al. (1994) have reported

high frequency (79.7%) fusion of isolated male and female gametes in the presence of 5 mM $CaCl_2$. In this chemical fusion method the gametes preserved the cell recognition system so that the fusion was largely restricted to male–female gamete pairs. They also observed that the egg fertilized in vitro did not fuse with additional sperm, suggesting that a block to polyspermy exists.

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Chapter 11

Zygotic Embryo Culture

11.1. INTRODUCTION

Embryo culture is an in vitro technique that has been practised by plant breeders for over half a century now, and is described in most plant-breeding texts. The first systematic attempt to grow the embryos of angiosperms in vitro, under aseptic conditions, was made by Hannig (1904), who cultured mature embryos of two crucifers, *Cochleria* and *Raphanus*. Subsequently, many workers raised plants by culturing embryos excised from mature seeds. Dieterich (1924) pointed out that on a semi-solid medium containing Knop's mineral salts and 2.5–5% sucrose, the mature embryos grew normally but those excised from immature seeds failed to achieve the organization of a mature embryo. Instead, they grew directly into seedlings, skipping the stages of normal embryogenesis. Dieterich described this phenomenon of precocious germination of excised immature embryos as 'Kunstliche Fruhgeburt' (see Section 11.5).

A stimulus for further progress in the field of embryo culture was provided by Laibach (1925, 1929), who demonstrated the most important practical application of this technique. In the interspecific cross *Linum perenne* × *L. austriacum*, Laibach noted that the seeds were greatly shrivelled, very light, and incapable of germination. By excising embryos from such seeds and growing them on moist filter paper or on cotton wadding containing sucrose he was able to raise the hybrid plants. This led Laibach to suggest that in all those crosses where viable seeds are not formed it may be appropriate to excise their embryo and grow them in an artificial nutrient medium. Since then the technique of embryo culture has been widely used to produce hybrids which were otherwise not possible due to embryo abortion (see Section 11.10.1).

The possibility of growing embryos outside the environment of the ovule (ex-ovulo) provides an excellent opportunity to study the nutrition of the embryos at various stages of development (see Section 11.3). Using this technique the regeneration potential of whole embryos and their segments have also been investigated.

TABLE 11.1

Progressive embryogenesis in *Capsella bursa-pastoris*[a]

Developmental stage	Length of embryo (μm)	Nutritional requirements
Early globular	20–60	Unknown for embryos smaller than 40 μm
Late globular	61–80	Basal medium (macronutrient salts[c] + vitamins[d] + 2% sucrose) + IAA (0.1 mg l^{-1}) + kinetin (0.001 mg l^{-1}) + adenine sulphate (0.001 mg l^{-1})
Heart-shaped	81–450	Basal medium alone
Torpedo-shaped	451–700	Macronutrient salts[b] + vitamins[d] + 2% sucrose
Walking-stick-shaped and mature embryos	700 and larger	Macronutrient salts[b] + 2% sucrose

[a]After Raghavan (1966).
[b]Macronutrient salts (mg l^{-1}): 480 $Ca(NO_3)_2 \cdot 4H_2O$, 63 $MgSO_4 \cdot 7H_2O$, 63 KNO_3, 42 KCl, and 60 KH_2PO_4.
[c]Micronutrient salts (mg l^{-1}): 0.56 H_3BO_3, 0.36 $MnCl_2 \cdot 4H_2O$, 0.42 $ZnCl_2$, 0.27 $CuCl_2 \cdot 2H_2O$, 1.55 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 3.08 ferric tartrate.
[d]Vitamins (mg l^{-1}): 0.1 thiamine hydrochloride, 0.1 pyridoxine hydrochloride, 0.5 niacin.

11.2. TECHNIQUES

From technical considerations the two most important aspects of embryo culture are: (a) excision of the embryo and (b) composition of the culture medium. The choice of the plant material may become important when the objective is to introduce the technique to a beginner. The composition of the medium and the preparation of the explant for aseptic culture varies with the plant and the age of the embryo to be cultured. A detailed discussion on the constituents of various media is given in Section 11.3. For compositions of some successfully used embryo culture media, refer to Tables 11.1–11.5.

11.2.1. Plant material

The selection of plant to be used for embryo culture is normally dictated by the problem in hand. However, if a choice exists, as in introductory demonstrations, it would be advisable to start with a plant material whose embryos can be easily dissected out. Mature embryos of seed leg-

TABLE 11.2

Composition of the two media used in different parts of the same petri dish to obtain uninterrupted growth of *Capsella bursa-pastoris* embryos from the globular stage (ca. 50 μm) to maturity[a]

Constituents	Amount (mg l^{-1})	
	Medium 1 (external ring)	Medium 2 (central zone)
KNO_3	1900	1900
$CaCl_2 \cdot 2H_2O$	484	1320
NH_4NO_3	990	825
$MgSO_4 \cdot 7H_2O$	407	370
KCl	420	350
KH_2PO_4	187	170
Na_2·EDTA	37.3	–
$FeSO_4 \cdot 7H_2O$	27.8	–
H_3BO_3	12.4	12.4
$MnSO_4 \cdot H_2O$	33.6	33.6
$ZnSO_4 \cdot 7H_2O$	21	21
KI	1.66	1.66
$Na_2MoO_4 \cdot 2H_2O$	0.5	0.5
$CuSO_4 \cdot 5\ H_2O$	0.05	0.05
$CoCl_2 \cdot 6\ H_2O$	0.05	0.05
Glutamine	–	600
$B_1 = B_6$	0.1	0.1
Sucrose	–	180000
Agar (Difco)	7000	7000

[a]After Monnier (1976).

umes and crucifers, possessing large seeds, are good starting materials. Consideration must also be given to the possibility of obtaining a large number of genetically uniform embryos and at the same stage of development. Plants grown under controlled conditions would normally provide uniform material for each experiment. When embryos of specific stages of development are required it is best to select plants which flower and fruit regularly in order to ensure a sufficient supply of material needed. Shepherd's purse (*Capsella bursa-pastoris*) satisfies some of these requirements (Raghavan, 1967). In this plant the inflorescence is a raceme. In each raceme ovules at various stages of development are present. Generally the younger embryos are arranged at the top and the older ones are towards the base along the inflorescence axis. Each capsule contains about 20–25 ovules which are more or less at the same stage of development (Raghavan, 1967; Torrey, 1973).

TABLE 11.3

Improved nutrient medium for *Capsella* embryo culture[a]

Constituents	Amount (mg l^{-1})
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	880
NH_4NO_3	825
$MgSO_4 \cdot 7H_2O$	370
KCl	350
KH_2PO_4	170
Na_2·EDTA	14.9[b]
$FeSO_4 \cdot 7H_2O$	11.1[b]
H_3BO_3	12.4
$MnSO_4 \cdot H_2O$	33.6
$ZnSO_4 \cdot 7H_2O$	21
KI	1.66
$Na_2MoO_4 \cdot 2H_2O$	0.5
$CuSO_4 \cdot 5H_2O$	0.05
$CoCl_2 \cdot 6H_2O$	0.05
Glutamine	400
Vitamin B_1	0.1
Vitamin B_6	0.1
Sucrose	120000
Agar	7000

[a]After Monnier (1976, 1978).
[b]2 ml of a stock solution containing 5.57 g $FeSO_4 \cdot 7H_2O$ and 7.45 g Na_2·EDTA l^{-1}.

Artificial pollination of freshly opened flowers may be necessary when the embryos are to be cultured at specific stages of development. In such studies it would be helpful to first prepare a calendar showing the relationship between the stages of embryo development and days after pollination (DAP).

When the goal is to obtain plants from otherwise abortive seeds, the embryos should be excised for culture prior to the onset of abortion.

11.2.2. Sterilization

Zygotic embryos, being enclosed within the sterile environment of the ovular and ovarian tissues, do not require surface sterilization. Entire ovules are disinfected following the standard methods described in Chapter 2, and embryos are dissected out and transferred to the culture medium under strictly aseptic conditions. In orchids, where the seeds are minute and lack a functional endosperm, and the seed-coat is highly reduced, whole ovules are cultured (in ovulo embryo culture; see Section

TABLE 11.4

Composition of the medium used for the culture of globular embryos of *Brassica juncea* (after Liu et al., 1993a)[a]

Constituents	Amount (mg l^{-1})	Constituents	Amount (mg l^{-1})
Macroelements			
NH_4NO_3	200	$KH_2PO_4 \cdot 2H_2O$	100
KNO_3	1500	$Na_2EDTA \cdot 2H_2O$	37
$CaCl_2 \cdot 5H_2O$	850	$FeSO_4 \cdot 7H_2O$	28
$MgSO_4 \cdot 7H_2O$	400		
Microelements			
KI	0.75	$Na_2MoO_4 \cdot 2H_2O$	0.25
H_3BO_3	3	$CuSO_4 \cdot 5H_2O$	0.025
$MnSO_4.H_2O$	10	$CoCl_2 \cdot 6H_2O$	0.025
$ZnSO_4.7H_2O$	2		
Sugar			
Sucrose	4000	Mannose	100
Glucose	2000	Rhamnose	100
Fructose	100	Cellobiose	100
Ribose	100	Sorbitol	100
Xylose	100	Mannitol	100
Organic acids[b]			
Sodium pyruvate	20	Malic acid	40
Citric acid	40	Fumaric acid	40
Vitamins and amino acids			
Inositol	500	Nicotinic acid	0.1
Glutamine	200	Pyridoxine·HCl	0.1
Thiamine·HCl	1	D-Biotin	0.01
Other supplements			
Coconut water[c]	300 (ml l^{-1})	Casein hydrolysate	100
Agarose (low gelling temperature Sea Plaque)		6000	

[a]Medium was filter sterilized.
[b]pH adjusted to 5.5 with NH_4OH.
[c]Obtained from green coconuts.

10.3.1). In such cases whole fruits can be surface-sterilized, and the seeds excised under aseptic conditions and spread as a monolayer on the surface of the agar medium using a sterilized needle.

TABLE 11.5

Composition of media used in embryo culture of barley[a]

Constituents	Amount (mg l^{-1})			
	B–II[b]	C–17[c]	C–21[d]	C–45[e]
Macronutrients				
KNO_3	–	300	300	900
$CaCl_2 \cdot 2H_2O$	740	250	–	400
$MgSO_4 \cdot 7H_2O$	740	325	300	300
$(NH_4)_2SO_4$	–	–	–	60
$NaH_2PO_4 \cdot H_2O$	–	100	–	75
KCl	750	150	300	–
KH_2PO_4	910	150	500	170
$Ca(NO_3)_2$	–	–	500	300
NH_4NO_3	–	200	–	500
Micronutrients				
KI	–	0.10	–	–
H_3BO_3	0.5	0.5	15.0	1.0
$MnSO_4 \cdot 4H_2O$	3.0	0.5	–	5.0
$ZnSO_4 \cdot 7H_2O$	0.5	0.25	–	5.0
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.012	–	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.012	–	0.012
$CoCl_2 \cdot 6H_2O$	0.025	0.012	–	0.012
Ferric citrate	10	3	20	20
Fe-EDTA	–	17.5	10	28
Vitamins				
Nicotinamide	–	–	–	1.0
Thiamine·HCl	0.25	0.25	10	10
Pyridoxine·HCl	0.25	0.25	–	10
Inositol	50	50	150	100
Ca-pantothenate	0.25	0.25	–	–
Glycine	–	0.75	–	–
Ascorbic acid	–	0.5	–	1.0
Amino acids				
Glutamine	400	–	–	600
Glutamic acid	–	150	300	–
Alanine	50	30	–	100
Cysteine	20	–	–	–
Arginine	10	20	50	–
Leucine	10	10	–	–
Phenylalanine	10	20	–	–
Tyrosine	10	–	–	–
Aspartic acid	–	30	100	100
Proline	–	50	50	–

TABLE 11.5 (*continued*)

Constituents	Amount (mg l^{-1})			
	B–II[b]	C–17[c]	C–21[d]	C–45[e]
Valine	–	10	–	–
Serine	–	25	25	50
Threonine	–	10	–	100
Lysine	–	10	–	–
Sucrose	34000	60000	45000	45000
Agar (Difco)	6000	–	–	–
pH	5	5.5	5.5	5.8

[a]In addition to the constituents mentioned in the table, the four media contain the following additives per litre of medium: B-II, 1 g malic acid dissolved in 50 ml of water and pH set to 5 with NH_4OH; C-17, 500 mg citric acid dissolved in 50 ml of water and pH adjusted to 5.3 with NH_4OH; 300 mg Tris-potassium citrate added to final medium and pH of medium adjusted to 5.5 with KOH (filter sterilized); C-21, 50 mg citric acid dissolved in 50 ml of water, pH adjusted to 5 with NH_4OH, added to medium and final pH brought to 5.5 with KOH (filter-sterilized); 250 mg Tris-potassium citrate added to the medium and pH of medium adjusted to 5.5; C-45, 300 mg malic acid dissolved in 50 ml of water containing 300 mg citric acid and pH brought up to 5 with NH_4OH.
[b]After Norstog (1973).
[c]Used by Jensen (1976, see Jensen, 1977) for small and non-uniform monoploid embryos.
[d]Used by Jensen (1976, see Jensen, 1977) for uniform, well-developed embryos.
[e]Used by Jensen (1976, see Yeung et al., 1981) for 1–2-week-old embryos.

11.2.3. Excision of embryo

For the in vitro culture of embryos, generally it is necessary to free them from their surrounding tissues. The mature embryos can be isolated with relative ease by splitting open the seed. Seeds with a hard seed-coat are dissected after soaking them in water. Smaller embryos require careful dissection with the aid of a stereoscopic microscope and quick transfer to the culture vial. Liu et al. (1993a) found it necessary to carry out dissections of globular embryos of *Brassica juncea* in a solution of 9% glucose to reduce osmotic shock to the proembryos. These scientists have recommended that during the dissection of very young embryos, care should be taken not to touch the embryo proper with forceps or needles. They used Gilson micropipettes to pick and transfer globular embryos from dissection plate to culture medium.

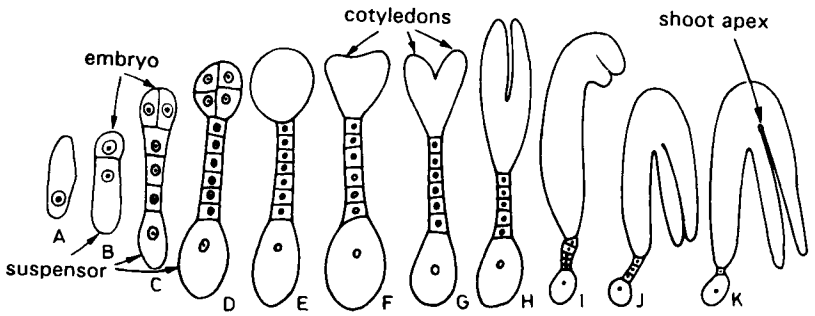

Fig. 11.1. Stages in the normal development of embryo in *Capsella bursa-pastoris*. (A) Zygote; (B) two-celled proembryo; (C–E) globular; (F) heart-shaped; (G) intermediate stage; (H) torpedo-shaped; (I) walking-stick-shaped; (J) inverted U-shaped; (K) mature (after Raghavan, 1966).

Raghavan and Torrey (1963) adopted the following procedure to isolate embryos at varying stages of development (see Fig. 11.1) from the ovules of *Capsella bursa-pastoris*. Sterile capsules (Fig. 11.2A) were kept in a few drops of the sterile culture medium. Outer walls were removed by an incision in the region of the placenta and the two halves pulled apart with forceps to expose the ovules (Fig. 11.2B). Torpedo-shaped and younger embryos are confined to one longitudinal half of the ovule (Fig. 11.2D) and are clearly visible through the chalaza either because of their green colour (intermediate and torpedo-shaped) or because of the transparent vesicle of their suspensor. To excise these immature embryos a single ovule, removed from the placenta, was placed in the depression of a new slide with a drop of medium. With the help of a sharp mounted blade the ovule was split longitudinally (Fig. 11.2D) to isolate the half containing the embryo. By carefully teasing apart the ovular tissues the entire embryo, along with the attached suspensor, could be removed. For excising older embryos a small incision was made in the ovule on the side lacking the embryo (Fig. 11.2C) and a slight pressure applied with a blunt needle. This was enough to release the intact embryo into the surrounding fluid. The whole operation, especially when dealing with immature embryos, must be performed with utmost care so that there is no apparent injury to the embryo.

In monocots, a well-studied plant for embryo culture is barley (*Hordeum vulgare*). Norstog (1965b) has described the following procedure for the isolation of immature barley embryos. The caryopsis were placed on a sterilized slide under a dissecting microscope at 20–50× magnification. Using watchmaker's forceps or fine-tipped needles embryos as small as 0.2 mm could be readily excised and transferred to

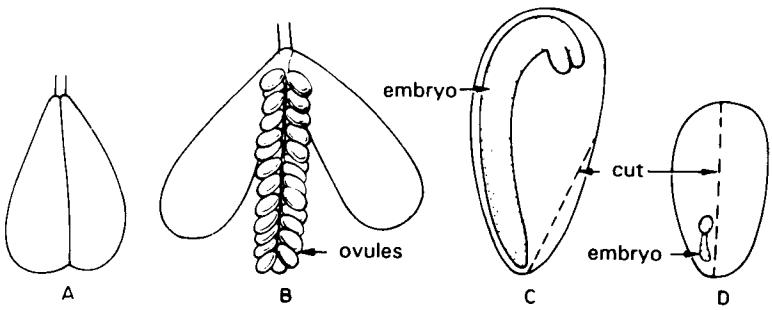

Fig. 11.2. Isolation of the embryos of *Capsella bursa-pastoris*. (A) A capsule; (B) the capsule has been opened to expose the ovules; (C) an ovule with walking-stick-shaped embryo inside; the dotted lines show the region of incision of the ovule to release the embryo; (D) an ovule with globular embryo. A cut along the dotted lines exposes the embryo (after Raghavan, 1966).

the culture medium. However, for smaller embryos a modified procedure was required because they tend to dry out during dissection and planting on the medium. In barley, the region of the ovule that contains the embryo is beak-like. The beak was excised and transferred to a drop of sterile paraffin oil. Tissues of the beak were carefully teased apart to release the embryo. The isolated embryo was lifted out in the oil film using a microspatula and transferred to the nutrient medium. The oil appeared to float free and it was possible to push the embryo to an oil-free area on the medium. The oil did not seem to interfere with the embryonic growth.

11.2.4. Embryo-nurse endosperm transplant

As a rule very young embryos are difficult to culture on artificial culture medium. Despite considerable progress in the improvement of embryo culture media, one is still often confronted with the problem of rescuing the hybrid embryos where abortion occurs at a very early stage of development (Williams and De Lautour, 1980). Ziebur and Brink (1951) had shown that in vitro growth of excised immature embryos (300–1100 μm long) of *Hordeum* was considerably promoted by surrounding it, on the culture medium, with excised endosperm from another seed of the same species. Kruse (1974) reported that implanting immature embryos on cultured barley endosperm showed significant improvement in the frequency of hybrid plant development in certain intergeneric crosses. For example, in the cross *Hordeum* × *Secale* the survival rate with the implantation technique was 30–40% compared to 1% with the traditional

method of embryo culture. Ziebur and Brink (1951) and Kruse (1974) have emphasized that endosperm older than the embryo by 5 days was more efficient as nurse tissue than the endosperm of the same age as the embryo.

A modified endosperm transplant technique for young embryo culture has been described by De Lautour et al. (1978), Williams (1978, 1980) and Williams and De Lautour (1980). They inserted an excised hybrid embryo into a cellular endosperm dissected out from a normally developing ovule of one of the parents or a third species and cultured the nurse endosperm with the transplanted embryo on an artificial medium. Using this technique these workers have produced many interspecific hybrids in the genus *Trifolium* which could not be reared by growing embryos directly on the medium. The procedure described by Williams and De Lautour (1980) is as follows. Transplant manipulation was carried out in a laminar air-flow cabinet with the aid of a stereoscopic microscope. A heat-absorbing glass filter was placed in front of the light source in order to protect the embryo from desiccation. Surface-sterilized pods carrying hybrid embryos or normally developing embryos (for nurse endosperm) were placed in sterile petri-dishes lined with moist filter paper until dissection. Two ovules, one carrying a hybrid embryo and the other with a normal embryo were removed from their pods and placed on a small (2 × 2 mm) pre-sterilized 'carrier' square of moist filter paper on the sterilized microscope stage. For the following text refer to Fig. 11.3. After making a shallow cut (c_1) across the back of each ovule, opposite the funiculus, the ovule wall was lifted back as a flap (f_1). The ovule was held steady with a needle inserted into the bar of the structural tissues (*) near the funiculus. A second shallow cut (c_2) towards the hilum allowed the ovule wall to be peeled back as two flaps (f_2) and expose the central embryo sac region. If the hybrid embryo was small a third shallow cut (c_3) was made around the top of the ovule so that the ovule wall could be peeled back further for the removal of the embryo. In the normal ovule, containing nurse endosperm, gentle left-to-right pressure with the aid of a needle along the line p-p detached the embryo suspensor from the maternal tissues and forced the embryo and the surrounding endosperm out on the filter paper through the previously cut holes (c_1 and c_2). However, to remove the hybrid embryo (without endosperm) its suspensor was gently detached from the ovule directly with the point of a needle. At this stage the normal embryo was removed from inside the endosperm (for use as a nurse tissue) and the hybrid embryo inserted in its place through the exit hole (see Fig. 11.4). The nurse endosperm enclosing the hybrid embryo was then transferred to the surface of the artificial medium.

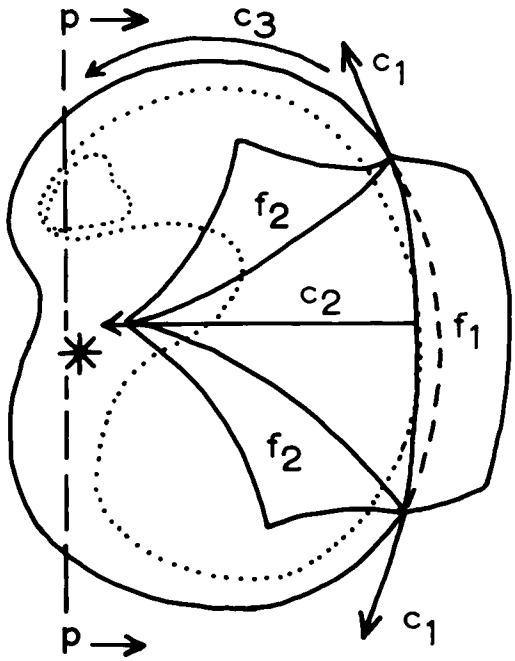

Fig. 11.3. Dissection procedure for 1.0–1.5 mm long ovules of *Trifolium, Lotus* or *Ornithopus*. c_1, c_2 and c_3 represent portions of sequential needle cuts, f_1 and f_2 are the flaps of the ovule wall peeled back after cutting to expose embryo sac contents. p– – –p is the line along which pressure (left to right) is applied with the side of a needle to expel the embryo and endosperm from the embryo sac. *Marks the position where a needle can be inserted into structural tissues to hold the ovule steady without damage to the embryo sac (after Williams and De Lautour, 1980).

11.3. CULTURE REQUIREMENTS

The most important aspect of embryo culture is the selection of the right culture medium that would support progressive and orderly development of embryos excised at different stages of development. Early reports of embryo culture were generally concerned with the development of plants from mature embryos (post-germinal development) on a simple medium. Hannig (1904) used a mineral salts–sucrose solution to culture mature embryos (2 mm long) of crucifers. Laibach (1925) reared full plants from excised hybrid embryos (1 mm long) using only 15% glucose solution. In contrast, immature embryos generally fail to grow on such a simple medium. Their nutritional requirements are more elaborate than those of the mature embryos. The persisting interest of workers to elucidate problems such as the control of embryogenesis, and the desirability of obtaining hybrids from non-viable crosses led to extensive investiga-

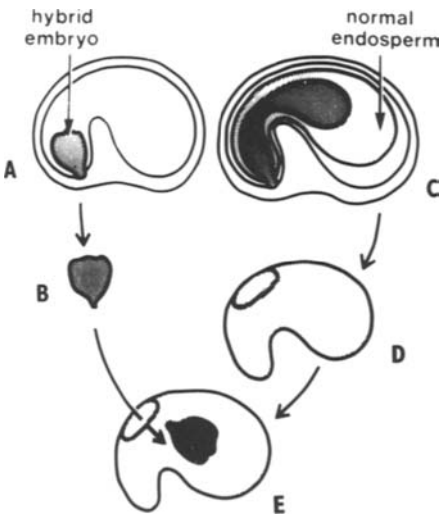

Fig. 11.4. Endosperm transplant for the culture of hybrid embryos in *Trifolium*, *Lotus* or *Ornithopus*. (A,B) The hybrid embryo is removed from the ovule in which endosperm development has failed. (C) To provide transplant endosperm a normally developing intraspecifically pollinated ovule is dissected at a stage when it contains cellular endosperm enclosing a heart-shaped to torpedo-shaped embryo. (D) The normal embryo is pressed out of the sac of endosperm leaving an exit hole. (E) The hybrid embryo is inserted into the normal endosperm through the exit hole (after Williams and De Lautour, 1980).

tions on the culture requirements of immature embryos (pre-germinal development). Continual efforts have been made to improve the culture media to rear younger embryos and duplicate, as far as possible, the in ovulo pattern of embryogenesis in the excised embryos. The two angiosperm systems studied in greatest details for embryo culture are *Capsella* and *Hordeum*. Appreciable work has also been done with *Citurs*, *Datura* and *Phaseolus*.

With respect to its nutrition, two phases of embryo development have been recognized by Raghavan (1966): (a) the heterotrophic phase: during this early phase the embryo is dependent and draws upon the endosperm and the surrounding maternal tissues; and (b) the autotrophic phase: during this the embryo is metabolically capable of synthesizing substances required for its growth from the basic mineral salts and sugar and is, thus, fairly independent for its nutrition. The critical stage at which the embryo comes out of the heterotrophic phase and enters the autotrophic phase, however, varies with the species (Raghavan, 1976a). In *Capsella bursa-pastoris* the embryos are definitely heterotrophic until the globular stage, and only in the late heart-shaped stage they turn autotrophic (Raghavan, 1976a). Even within the two phases the exoge-

nous requirements of the cultured embryos become progressively simpler with the age of the embryo. This is clearly brought out by the observations of Van Overbeek et al. (1941, 1942) on *Datura* and those of Raghavan and Torrey (1963, 1964; see also Raghavan, 1966) on *Capsella* (see Table 11.1).

Changing growth requirements of the embryos growing in ovulo is also observed in the cultures of immature embryos. This necessitates the transfer of embryos from one medium to another in order to achieve their optimal growth. Monnier (1976, 1978) described a new culture method which allows complete development of 50 μm long embryos (early globular stage) of *Capsella* up to germination in the same culture plate (Table 11.2), without moving the embryo from its original position (for details of this method see Fig. 11.5). The composition of the two media used in the culture dish is given in Table 11.2. It may be noted that the media used in this method are different from Monnier's medium for traditional embryo culture, described in Table 11.3. Similarly, a double-layer culture system, involving two semi-solid media (Table 11.4), differing only in their osmolarity, was used by Liu et al. (1993a) for successful culture of *Brassica juncea* proembryos as small as 35 μm in size (8.36 cells). The proembryos were planted on the bottom-layer medium (O.P. 0.45 mol l^{-1}) and overlaid with molten top-layer medium (O.P. 0.63 mol l^{-1}) held at 38°C. The proembryos developed into normal mature embryos with 65–95% efficiency.

Refinement of nutrient medium for the culture of heterotrophic embryos has involved, as for any other type of plant tissue cultures, considerations of mineral salts, organic nutrients, and growth regulators. In addition, osmotic pressure of the medium is a critical factor for successful culture of proembryos. Finally, there is an intimate relationship between embryo culture and the use of the plant extracts in culture media.

11.3.1. Mineral salts

Many different formulations of mineral salts have been used for embryo culture without much critical evaluation of the role of individual elements. In his studies on the effects of various standard inorganic solutions (Knop's, Heller's, Murashige and Skoog's) on cultured embryos of *Capsella*, Monnier (1976) observed that there was no correlation between the growth and survival of the immature embryos on a particular medium (see Fig. 11.6). In Murashige and Skoog's (MS) medium which supported maximum growth of embryos the survival frequency of the embryos was very low, whereas in Knop's medium, which was least toxic, the growth of the embryos was very poor. This prompted Monnier to for-

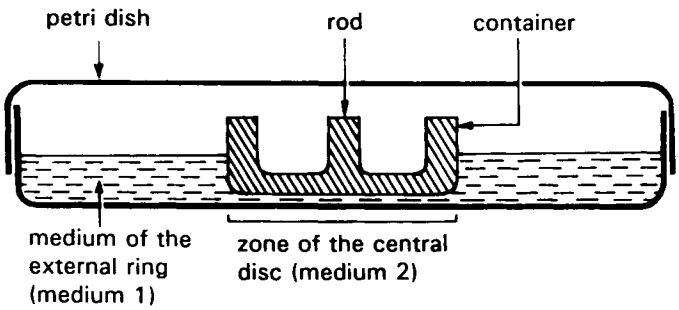

Fig. 11.5. Device allowing the juxtaposition of two media with different compositions (see Table 11.2). The first agar medium is liquified by heating and then poured around the central glass container. This medium gives the external ring. After cooling and solidification of the medium the container is removed. In the central ring, thus formed, a second medium of a different composition is poured. The embryos are cultivated on the second medium, in the central part of the petri dish. As a result of diffusion the embryos are subjected to the action of variable medium with time (after Monnier, 1976, 1978).

mulate a new mineral solution that would favour both good growth and high survival. He altered the concentration of every salt in the MS medium to study the effect of such variations, and on the basis of these experiments developed a medium on which the growth of embryos was as good as on MS but survival was high (see Fig. 11.6). The composition of the new medium is given in Table 11.3. Compared with the inorganic composition of MS medium, Monniers medium has high concentrations of K^+ and Ca^{2+}, and a reduced level of NH_4^+ ions.

Umbeck and Norstog (1979) reported that NH_4^+ in the medium was essential for proper growth and differentiation of immature barley embryos. With NO_3^- as the sole source of inorganic nitrogen, 500 μm long barley embryos (at culture) showed very little growth, that was largely due to cell elongation. For the differentiation of scutellum, 'ligule' and 'sheath' 4.3–8.6 mM NH^+_4 was required. In *Datura stramonium* (Paris et al., 1953) and *D. tatula* (Matsubara, 1964) NH_4^+ was either essential or a preferred source of inorganic nitrogen, especially for immature embryos. Embryos of jute (*Corchorus capsularis*; Mitra and Datta, 1951a,b), on the other hand, showed an absolute requirement for nitrate nitrogen.

11.3.2. Carbohydrate and osmotic pressure of the culture medium

A suitable source of carbon energy is generally required for the cultivation of excised mature and immature embryos. Sucrose is by far the

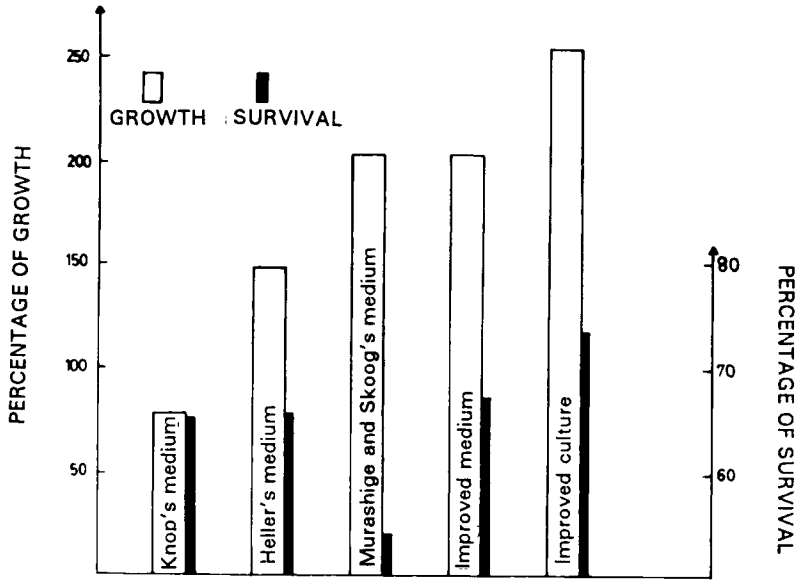

Fig. 11.6. Growth and survival of immature *Capsella* embryos in various culture media. Composition of the 'improved medium' is given in the Table 11.3. For 'improved culture' Monnier's medium (1976, 1978) (see Table 11.3) was used, and it involved agar-coating of the embryos and tyndallization of the medium (after Monnier, 1976, 1978).

best form of carbohydrate and has been most commonly used for embryo culture (Van Overbeek et al., 1944; Lofland, 1950; Rijven, 1952; Mauney, 1961; Matsubara and Nakahira, 1965; Burghardtova and Tupy, 1980).

Sucrose is added to the embryo culture media not only as a source of energy but also to maintain a suitable osmolarity which is extremely important for immature embryos (Liu et al., 1993a). For this latter function the optimum concentration of sucrose varies with the stage of embryo development. Mature embryos grow fairly well with 2% sucrose but younger embryos require higher levels of the carbohydrate. This is in harmony with the observations that in situ the proembryos are surrounded by a fluid of high osmolarity (Ryczkowski, 1960; Mauney, 1961; Smith, 1973). Eight to twelve percent sucrose is generally adequate for the culture of proembryos (*Datura*; Rietsema et al., 1953; *Hordeum*; Ziebur and Brink, 1951; Norstog, 1961; *Capsella*; Rijven, 1952; Veen, 1963; Monnier, 1978). In long-term experiments, with the age of the cultures the embryos need to be transferred to media with progressively lower levels of sucrose, or a culture system as that of Monnier (1976) or Liu et al. (1993a), as described in Section 11.3, should be used.

A high concentration (60 mmol l^{-1}) of NH_4NO_3 has been reported to promote the growth of immature embryos (200–300 μm) of *Datura tatula* by increasing the osmotic value of the medium (Matsubara, 1964).

Raghavan and Torrey (1964) also noted a stimulatory effect of high concentrations (12–18%) of sucrose on in vitro development of excised globular embryos (smaller than 80 μm) of *Capsella*. However, a combination of IAA (0.1 mg l^{-1}), kinetin (0.001 mg l^{-1}) and adenine sulphate (0.001 mg l^{-1}) added to the basal medium with only 2% sucrose provided the best medium for continued division and growth of the proembryos. Based on these observations, Raghavan and Torrey suggested that in vitro growth and differentiation of excised embryos is not so much dependent on the osmotic value as on the availability of specific growth factors. The effect of high osmoticum in preventing precocious germination of excised immature embryos is effectively reproduced by ABA (Crouch and Sussex, 1981). Whether the beneficial influence of the high osmotic pressure is mediated through its effect on the endogenous pool of growth regulators remains to be demonstrated.

11.3.3. Amino acids and vitamins

The addition of amino acids, singly or in combination, to the culture medium may stimulate embryo growth. Hannig (1904) reported that asparagine was very effective in enhancing embryo growth. Generally, however, glutamine has proved to be the most effective amino acid for the growth of excised embryos (Paris et al., 1953; Rijven, 1955; Matsubara, 1964; Monnier, 1978). Rijven (1955) reported that glutamine promoted the growth of embryos from nine different families of flowering plants, and the promotion was considerably more than that by asparagine. For some of these species (*Capsella, Arabidopsis, Reseda*) asparagine was even inhibitory. Matsubara (1964) tested 18 amino acids and two amides for the culture of young *Datura tatula* embryos and observed that all except glutamine were inhibitory.

Casein hydrolysate (CH), an amino acid complex, has been widely used as an additive to the embryo culture media. Sanders and Burkholder (1948) tried it for the culture of pre-heart-shaped (100–250 μm long) embryos of two species of *Datura*. When added to the basal medium (containing mineral salts, vitamins, and sucrose) with cysteine and tryptophan, CH increased embryogenic differentiation and the size of the embryo. In an attempt to identify the growth-promoting factor(s) in CH, Sanders and Burkholder tested a mixture of 20 amino acids in the proportion in which they occur in CH. In addition, 10 of these amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, lysine,

phenylalanine, proline, tyrosine), which had been earlier found to be beneficial for plant growth, were also tested individually and collectively. The mixture of 20 amino acids proved as effective as CH for embryo growth and development, suggesting that the promotive effect of CH for embryo growth was due to amino acids. The mixture of 10 amino acids promoted embryo growth similar to CH in *D. innoxia* but not in *D. stramonium*. Individually none of the amino acids matched either CH or the mixture of 20 amino acids in its effect. These observations suggest a synergistic interaction between amino acids. CH was shown by Kent and Brink (1947) to inhibit precocious germination and promote pre-germinal embryo development in *Hordeum vulgare*.

Rangaswamy (1961) reported that CH was essential for the culture of very young globular embryos (14–28 × 14–28 μm) of *Citrus microcarpa*. On a modified White's medium these proembryos collapsed within 3 days of culture. Increasing the level of sucrose up to 10% did not support normal embryogenic development. However, when White's medium was supplemented with 400 mg l^{-1} CH, the proembryos passed through all pre-germinal stages of embryo development, and in 3–4 weeks fully organized embryos were formed in 80% of the cultures.

The sensitivity of embryos to concentrations of CH may vary with the species. The optimum level of CH for *Hordeum vulgare* was 500 mg l^{-1} (Kent and Brink, 1947). The embryos of *Datura innoxia* and *D. stramonium* grew best with 400 mg l^{-1} CH (Sanders and Burkholder, 1948). The growth of embryos of *D. tatula* was promoted by CH at 50 mg l^{-1} but at 100 mg l^{-1} it proved toxic. Ziebur et al. (1950) concluded that the effect of high levels of CH on excised embryos of barley was largely due to its osmotic effect which could be reproduced by a high concentration of sucrose.

Vitamins have been used in embryo culture media but their presence is not always essential. In some cases a vitamin may even inhibit normal morphogenesis (Raghavan, 1980). It is pertinent, therefore, that vitamins are included in the media only after their desirability has been established through empirical experimentation for the system in question.

11.3.4. Natural plant extracts

From his experience of culturing embryos of several plant species LaRue (1936) remarked '...embryos 0.5 mm or slightly less in length can be grown successfully ... those of still smaller size have not yet been brought under control ... at a length of 0.5 mm we may have reached a new lower threshold which will be difficult to cross'. Obviously, the relationship between the size of the embryo and its stage of development would vary with the species. For large-seeded plants 500-μm long em-

bryos would be fairly young whereas for small-seeded plants it would represent a comparatively older stage. However, the main point of LaRue's statement is that at that time it was difficult to culture younger embryos. Van Overbeek et al. (1942) observed that post-torpedo-shaped embryos of *Datura* formed normal seedlings in a medium containing 1% dextrose, 1% agar, mineral salts, glycine, thiamine, ascorbic acid, nicotinic acid, vitamin B_6, adenine, succinic acid and pantothenic acid. However, heart-shaped and younger embryos failed to develop on this medium. Since immature embryos in the ovule are surrounded by endosperm, Van Overbeek et al. (1942) added non-autoclaved coconut milk (CM; liquid endosperm of coconut) to the above medium in an attempt to culture younger embryos. On this modified medium normal seedlings developed from embryos as small as 150–200 μm long (heart-shaped) and as young as 10 DAP. The growth-promoting factor in the coconut milk was referred to as 'embryo factor' (Van Overbeek et al., 1942), and this discovery proved a turning point in the history of embryo culture; it enabled the successful culture of young embryos of a number of species (Warmke et al., 1946; Norstog, 1956b, 1961; Matsubara, 1962). Coconut milk was indispensable for the culture of 35–80 μm proembryos of *Brassica juncea* (Liu et al., 1993a).

In order to find a suitable substitute for CM for the culture of pre-heart-shaped embryos of *D. tatula* (<150 μm long), Matsubara (1962) tested a range of natural substances including CH, dried brewer's yeast, skimmed milk, and diffusate from the endosperm of *Ginkgo* and the seeds of several angiosperms. The alcohol diffusates from young seeds of *Lupinus* and mature seeds of *Sechium* proved as effective as CM.

Kent and Brink (1947) reported promotion of the embryogenic growth and inhibition of precocious germination of immature barley embryos by extracts of dates and bananas, wheat glutein hydrolysate, milk and tomato juice (TJ). Of these TJ was most effective. On the basal medium containing 22–66% TJ, 7–9-day-old embryos underwent normal embryogenic development. Without TJ these embryos grew irregularly.

With an urge to develop purely synthetic media for the culture of immature embryos attempts have been made to substitute the 'embryo factor' of coconut milk by chemically defined substances. For the growth of barley proembryos, the requirement for CM could be replaced by phosphate-enriched White's medium fortified with glutamine and alanine as the major amino acids and five other amino acids as the minor sources of N_2, at pH 4.5. The survival rate of the embryos was considerably increased when the concentration of KCl, KNO_3 and certain organic components was increased 5–10 times (Norstog, 1967, 1973). This modified Norstog's medium was found to be the best of the various recipes tested

by Cameron-Mills and Duffus (1977) for the culture of 250-μm long embryos of five varieties of *Hordeum distichum*.

11.3.5. Growth regulators

For the callusing of embryos and embryo segments generally an auxin or cytokinin, or both, are required. However, there is not enough evidence to suggest that exogenous growth regulators are necessary for the normal development of excised mature or immature embryos apart from breaking dormancy in some plants. In fact, auxin is generally inhibitory for embryo growth (Raghavan, 1980). Monnier (1978) suggested that hormones should not be added to the embryo culture media because they bring about structural abnormalities. He felt that embryos are autonomous for most of the growth regulators. This has been clearly shown at least for GA_3 in *Phaseolus*, where the suspensor fulfils the requirement of excised embryos for this hormone (see Section 11.4).

Raghavan and Torrey (1964) reported that for cultivating globular embryos of *Capsella* on a mineral salts–vitamin medium with only 2% sucrose, it was necessary to supplement it with IAA, kinetin, and adenine sulphate. Precocious germination of cultured immature barley embryos (500 μm long), which is promoted by GA_3 and kinetin, could be suppressed, and orderly embryogenic development induced by the application of ABA, especially in combination with NH_4^+ ions (Norstog, 1972; Umbeck and Norstog, 1979). The role of ABA in supporting morphologically and biosynthetically normal embryogenesis in cultured embryos is dealt with in Section 11.5.

11.3.6. pH of medium

The pH of the ovular sap of *Capsella* is about 6.0, and its excised embryos grow equally well in the medium with pH 5.4–7.5 (Rijven, 1952). The optimal pH for early heart-shaped embryos of *Datura tatula* ranged from 5.0 to 7.5 (Matsubara, 1962). Rice embryos (8-day-old) showed best growth at two pH values, viz. 5 and 9 (Sapre, 1963). Norstog and Smith (1963) have reported that for organogenic differentiation of immature barley embryos the pH of the medium was critical. The optimum value was about 4.9. At a pH above 5.2 embryo growth occurred without any appreciable differentiation.

11.3.7. Culture storage

Embryos of most plants grow well at temperatures between 25 and

30°C (Narayanaswamy and Norstog, 1964). The optimum temperature of *Datura tatula* is reported to be 35°C (Matsubara and Nakahira, 1965). According to Narayanaswamy and Norstog light is not critical for embryo growth. Matsubara and Nakahira also concluded that the growth of young *Datura* embryos was not influenced by light. However, in barley light suppresses precocious germination of immature embryos (Norstog, 1972).

11.4. ROLE OF THE SUSPENSOR IN EMBRYO CULTURE

The suspensor is an ephemeral structure found at the radicular end of the proembryo; it usually attains its maximum development by the time the embryo reaches the globular stage (see Fig. 11.1). Detailed structural (Yeung and Clutter, 1979), cytological (Nagl, 1962; Avanzi et al., 1970), physiological (to be discussed below), and biochemical studies (Clutter et al., 1977) suggest an active involvement of the suspensor in the development of the young embryos (see also Yeung and Meinke, 1993).

Generally, embryos have been cultured without an intact suspensor because the small size and delicate structure of the suspensor make it difficult to excise it along with the embryo proper. Some painstaking studies have, however, shown that, in cultures, the presence of a suspensor is critical for the survival of young embryos (Corsi, 1972; Cionini et al., 1976; Yeung and Sussex, 1979). Cionini et al. (1976) observed that the older embryos (500 μm or over in length) of *Phaseolus coccineus* grew equally well whether cultured with the intact suspensor or without it. However, in the culture of young embryos removal of the suspensor significantly reduced the frequency of plantlet formation. Confirming the importance of the suspensor for the growth of young embryos of *P. coccineus*, Yeung and Sussex (1979) stated that the suspensor, when intact with the embryo proper or when detached from it but placed in its close proximity on the culture medium, strongly stimulated the further development of the embryo in comparison to the embryo cultured in its absence (see Table 11.6). According to these authors, the growth-promoting activity of the suspensor is maximal at the early heart-shaped stage of the embryo (see Table 11.6). Of the various growth regulators tested, gibberellin at a concentration of 5 mg l^{-1} most effectively substituted for the requirement of the suspensor (Yeung and Sussex, 1979; also see Cionini et al., 1976). This is in accordance with the observations of Alpi et al. (1975) that in *P. coccineus*, at the heart-shaped stage gibberellin activity in the suspensor is about 30 times higher than that in the embryo proper. After the formation of cotyledons, when the suspensor is in its initial stages of degeneration, it shows a sharp decline in the level of GA_3 with a

TABLE 11.6

The effect of suspensor on in vitro growth and development of *Phaseolus coccineus* embryos[a]

Initial[b] stage (fresh weight)	Treatment	Fresh weight[c] ± SE (*N*)	% Embryos[e] forming plants (no. of embryos cultured)
Early heart-shaped (0.87–0.02 mg)	Embryo proper only	3.19 ± 0.52 (10)	41.5 (89)
	Embryo proper with suspensor attached	8.91 ± 1.16[d] (10)	88.4 (95)
	Embryo proper with detached suspensor in direct contact	6.22 ± 0.78[d] (10)	72.5 (51)
	Embryo proper with heat-killed detached suspensor in direct contact	4.10 ± 0.43 (5)	37.0 (43)
	Embryo proper with suspensor 1 cm away		33.3 (30)
Late heart-shaped (1.07 ± 0.07 mg)	Embryo proper only	17.2 ± 2.84 (5)	94.4 (18)
	Embryo proper with suspensor attached	15.4 ± 1.41 (6)	94.4 (18)
Early cotyledon (3.92 ± 0.19 mg)	Embryo proper only	20.3 ± 2.5 (7)	100 (18)
	Embryo proper with suspensor attached	24.4 ± 2.75 (11)	100 (19)

[a]After Yeung and Sussex (1979).
[b]Seed sizes: early heart-shaped stage - 4.5 mm; late heart-shaped stage - 6.5 mm; early cotyledon stage - 7 mm.
[c]Fresh weight was taken 10 days after culture; (*N*) represents the sample size.
[d]Significant at the 1% level.
[e]Assessed 8 weeks after culture.

concomitant increase in its level in the embryo proper. Kinetin also promoted the growth of young embryos in the absence of the suspensor and was effective over a wide range of concentrations (0.001–1 mg l^{-1}) but at no level did its effect match that of gibberellin.

Recently, Liu et al. (1993a) have reported that removal of suspensor had only a small effect on in vitro development of 55 μm or smaller embryos of *Brassica juncea* and no effect on the embryos larger than 55 μm.

However, these authors used a very complex culture medium rich in organic nutrients and was supplemented with 30% coconut water, which may contain sufficient plant hormones to substitute for the suspensor.

11.5. PRECOCIOUS GERMINATION

To an embryologist, embryo development is almost synonymous with embryogenesis which embraces developmental stages from the zygote to the fully formed embryo characteristic of the species. However, plant physiologists and biochemists conceive embryo development as a linear progression from zygote formation to germination (Dure, 1975; Sussex, 1978; Walbot, 1978). Walbot (1978) has classified embryo development into five stages (see Table 11.7). Initially the growth of the embryo is predominantly by cell division resulting in the formation of a proembryo which comprises small meristematic cells. This is followed by tissue differentiation during which the meristematic activities become localized. After its full-term development the embryo becomes dehydrated and enters a phase of metabolic quiescence and developmental arrest. During this phase (dormancy) which, depending on the genotype and the environmental conditions, may last from a few days to several months or even years, the embryo is normally incapable of germination. Thus, embryo development is a highly regulated process.

Embryos of mangroves and some viviparous varieties of cultivated plants germinate while still attached to the parent without any diminution of its rate of growth. Excised immature embryos of even other plants when cultured on nutrient medium not only bypass the stage of dormancy but also cease to undergo further embryogenic mode of development. They do not exhibit normal biosynthetic activities characteristic of late embryogeny (Choinski and Trelease, 1978; Crouch and Sussex, 1981; Dure and Galau, 1981). Instead, the embryos develop into weak seedlings displaying only those structures already present at the time of embryo excision (see Fig. 11.7). This phenomenon of seedling formation without completing normal embryogenic development is called precocious germination. One of the aims of culturing immature embryos has been to stimulate normal embryogenic development in order to understand the factor(s) that regulate the orderly in ovulo development of embryos in nature.

The extension of embryogenic development and delayed germination by CH in the cultures of excised immature embryos of barley was reported by Kent and Brink (1947). Later, Ziebur et al. (1950) found that, although not as effective as CH, a high sucrose level (12.8%) in the medium also inhibited precocious germination of cultured immature em-

TABLE 11.7

Major stages in the development of *Phaseolus* embryos[a]

Stage	Characteristics
1. Cleavage and differentiation	Cell division with little growth; differentiation of all major tissues
2. Growth	Rapid cell expansion and division
3. Maturation	Little or no cell division or expansion, synthesis and storage of reserve materials
4. Dormancy	Developmental arrest
5. Germination	Renewed cell expansion and division; embryo growth

[a]After Walbot (1978).

bryos of barley. Precocious germination of *Capsella* embryos was similarly inhibited by the presence of a high concentration of sucrose (12–18%) in the medium (Rijven, 1952). These observations are in agreement with the findings that both in monocots and dicots the values of osmotic pressure and sugar concentration of the ovular sap are high during the early stages of embryo development and they progressively decrease with the age of the ovule (Ryczkowski, 1974). Norstog (1972) and Norstog and Klein (1972) have shown that exogenous factors, besides high osmolarity, which suppress precocious germination of excised immature barley embryos are reduced O_2 tension, elevated temperature and high light intensity.

Andrews and Simpson (1969) observed that naked embryos from freshly harvested mature seeds of a highly dormant strain of *Avena fatua* failed to germinate on a medium that supported the germination of naked embryos from non-dormant (after-ripened) seeds of the same strain. However, the dormant embryos germinated in the same medium if they were supplied with exogenous GA_3 or cultured after they had been leached on an agar medium. Seeds of *Taxus baccata* require an after-ripening period to complete embryogeny. During this period the embryo is normally incapable of germination. Culturing the dormant embryos in liquid medium for 8 days rendered them germinable. Apparently some leachable inhibitor(s) residing in the embryo is(are) responsible for preventing precocious germination (Le Page-Degivry and Garello, 1973). One of the inhibitors of germination seems to be abscisic acid (ABA) which is known to accumulate in high concentrations during the later stages of seed development in some plants (Dure, 1975; King, 1976; Hsu, 1979). The hypothesis that ABA like substance(s) is(are) involved in preventing

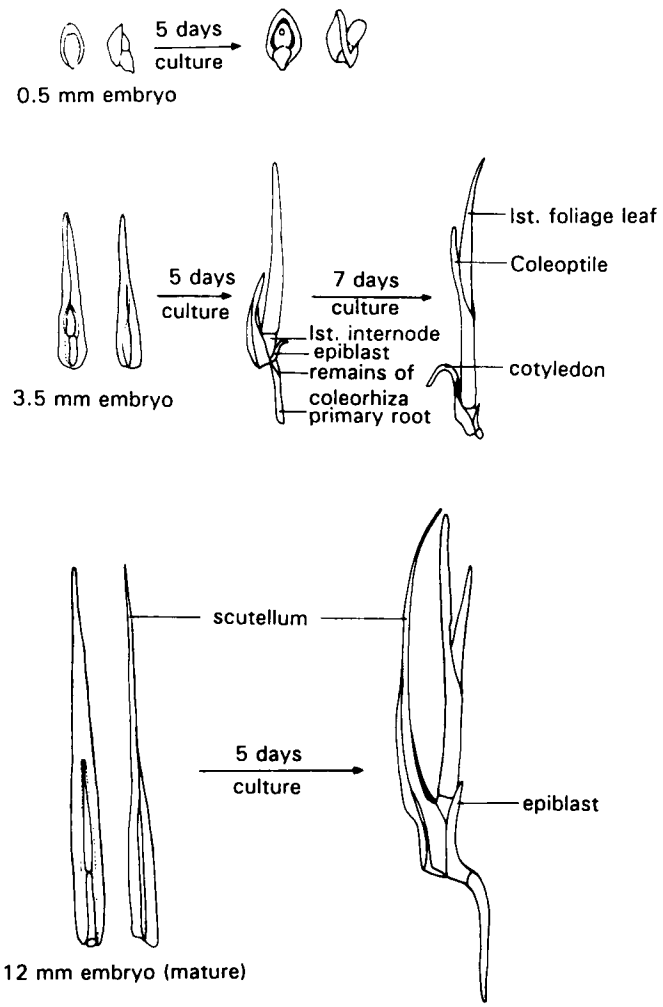

Fig. 11.7. In vitro growth of *Zizania aquatica* embryos excised from ovules at different stages of development. Embryos 0.05 mm long showed very little in vitro growth. Older, immature embryos (3.5 mm long) germinated precociously and formed seedlings which were not as well developed as those formed by the mature embryos in the same culture period (after LaRue and Avery, 1938).

precocious germination in nature gains support from the work of Norstog (1972), who noted that precocious germination of immature barley embryos may be induced under culture conditions which normally inhibit it (high osmolarity of the medium, high light intensity and elevated temperature) by GA_3 application. This GA_3-induced precocious germination was suppressed by ABA treatment.

In cotton, mRNA for the synthesis of enzymes carboxypeptidase C, isocitrase and protease, required to hydrolyze the reserve material during first few hours of germination, are transcribed much before the seed attains maturity and the embryo enters dormancy (Ihle and Dure, 1969, 1972a,b). However, translation of the mRNA remains inhibited during the dormancy period due to the presence of high levels of ABA in the tissues surrounding the embryo (Dure, 1975). Walbot (1978) has proposed that ABA may be preventing precocious germination by suppressing water uptake by the embryo which is essential for germination. Temporal separation of transcription and translation of the germination-specific mRNA in cotton is prevented during precocious germination of the young embryos excised at a stage before the mRNA is normally transcribed (Ihle and Dure, 1972a,b). The germination of the excised embryos and the appearance of the enzymes are inhibited by the aqueous extract of the ovules as well as exogenously applied ABA, suggesting that in cotton the factors suppressing the precocious germination of the embryo reside in extra-embryonal tissues of the seed.

11.6. MORPHOGENESIS IN THE CULTURES OF SEEDS WITH PARTIALLY DIFFERENTIATED EMBRYOS

In many flowering plants (belonging to 19 families) fully developed seeds on the plant contain embryos that lack differentiation into radicle, plumule and cotyledons (Rangaswamy, 1967). In *Eranthis* (Ranunculaceae) seeds at shedding enclose a pear-shaped embryo (comprising only a few hundred cells) with a long suspensor. After the seed has fallen on the ground the embryo undergoes intraseminal growth and achieves the usual morphology of a mature dicotyledonous embryo (Maheshwari and Rangaswamy, 1965). In orchids and root parasitic members of the Orobanchaceae also the seed harbours an unorganized embryo but in these plants the intraseminal growth is absent and the seedling is formed by the globose embryo without undergoing further embryogenic differentiation. In such plants the embryonal end proximal to the micropyle is regarded as the 'radicular pole' and that distal to the micropyle is termed the 'radicular pole'. Interestingly, both in orchids and the Orobanchaceae members, only one of the poles of the embryo is involved in the development of the seedling.

During seed germination in orchids, the plumular pole of the embryo enlarges to form a spherule-like structure, called the protocorm. Initially the protocorm is non-chlorophyllous but gradually it turns green. After attaining a certain size the protocorm differentiates roots and shoot. In this way in orchids the entire seedling develops exclusively by the divi-

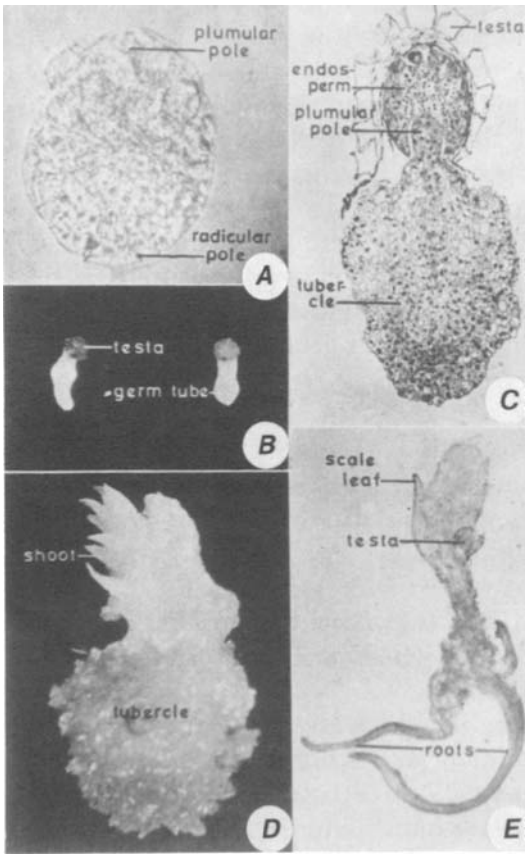

Fig. 11.8. Embryo morphogenesis in seed cultures of two members of the Orobanchaceae, with reduced embryos. (A,E) *Orobanche aegyptiaca* and (B–D) *Cistanche tubulosa*. (A) Whole mount of excised, mature embryo; it lacks a radicle and a plumule. (B) Three-week-old culture on White's medium containing CM and CH, showing the formation of the germ tube. (C) Longisection of a seed at the stage shown in (B); the plumular pole of the embryo has remained quiescent inside the seed whereas the radicular pole has formed a massive holdfast. (D) Differentiation of a shoot from the proliferated embryo after transfer to Tepfer's H-medium and storage in the dark. (E) Five-week-old culture on medium containing strigol. The seedling is formed by bipolar activity of the embryo. (A) After Rangaswamy, 1963; (B–D) after Rangan and Rangaswamy, 1968; (E) after Kumar and Rangaswamy, 1977.

sion of the plumular pole of the embryo (Carlson, 1943; Rao and Avadhani, 1963). Almost the same pattern of seedling development occurs in the seed cultures of orchids (Rao and Avadhani, 1963).

Unlike orchids, in the root parasitic members of the Orobanchaceae normally a seedling is derived by the sole activity of the radicular pole of the embryo (see Fig. 11.8A). According to Kadry and Tewfic (1956) during

seed germination in *Orobanche crenata* the radicular pole of the embryo forms a 'radicular cylinder' (see Kumar and Rangaswamy, 1977) whose tip penetrates the host root. The portion of radicular cylinder that remains outside the host root proliferates into an irregular mass of tissue, called the tubercle, from which the shoot differentiates. Rangan and Rangaswamy (1968) germinated the seeds of *Cistanche tubulosa*, another member of the Orobanchaceae, in vitro and recorded a similar pattern of germination (see Fig. 11.8B–D) as described for *O. crenata* by Kadry and Tewfic (1956). Rangaswamy (1963) also reported monopolar seedling development in seed cultures of *O. aegyptiaca*. However, Kumar and Rangaswamy (1977) have shown that the pattern of seedling development in this parasite can be modified from monopolar to bipolar by modifying the composition of the culture medium. On TB medium (salts after Tepfer et al., 1963, glycine, niacin, thiamine·HCl, calcium pantothenate and sucrose) supplemented with CM or yeast extract the germination was monopolar (formation of seedling by the sole activity of the radicular pole). The addition of IAA (0.1 mg l^{-1}), kinetin (0.5–10 mg l^{-1}), GA_3 (5–30 mg l^{-1}) or strigol (0.01 μg l^{-1}) to the TB medium induced a bipolar pattern of seedling development (see Fig. 11.8E); the plumular pole differentiated into a shoot bud and the radicular pole produced roots. Substitution of sucrose in the TB medium by glucose, mannose or raffinose also favoured bipolar germination (Usha, 1968). The highest frequency of bipolar seedling development occurred in TB medium + GA_3 (7.5 mg l^{-1}).

The mature embryos of *Cuscuta* have a distinct plumule but lack a radicle (Haccius and Troll, 1961; Truscott, 1966). According to Truscott (1966) there is no indication of the existence of a protoderm, root meristem or hypocotyl either during embryogenesis or in the mature embryos of *C. gronovii*. During in vivo seed germination the plumule forms a shoot but the radicular end of the embryo starts to die off 4–5 days after the initiation of germination. Thus, in this plant a root system is absent altogether. Truscott (1966) cultured mature and immature embryos of *C. gronovii* to test if roots can be artificially induced by applying growth substances, such as adenine, kinetin and GA_3, or complex nutrient mixtures, such as CM, and CH. However, none of the treatments induced rooting. This led Truscott to conclude that *C. gronovii* lacks a root growth potential. Similarly, root differentiation never occurred in the cultures of embryos or shoot apices of *C. reflexa* (Maheshwari and Baldev, 1962).

11.7. MICROSURGICAL EXPERIMENTS

A typical plant embryo is a bipolar structure, possessing the basic organization of the adult plant. During seed germination different parts of

the embryo perform their specialized functions and follow pre-determined mode of development. In vitro microsurgical experiments with embryos have thrown light on, at least, two aspects of growth and development: (a) interaction between different parts of the embryo during seed germination, and (b) the morphogenetic potentials of the various constituent parts of the embryo.

Studies to-date suggest that the cotyledon(s) plays an important role in the normal development of the embryonal axis into a seedling. This is true for dicots, monocots and, at least, one gymnosperm (Raghavan, 1980). Kruyt (1952) and Kester (1953) demonstrated the necessity of intact cotyledons for normal growth of seedlings in pea and peach, respectively. In *Zea mays* (Andronescu, 1919), *Avena sativa*, *Hordeum vulgare* and *Pennisetum typhoideum* (Narayanaswamy, 1963) removing the scutellum (equal to the single cotyledon of other monocots) inhibited the development of root and shoot. Tilton (1981) has reported that in *Zea mays*, hypocotyl-scutellum explants were more rhizogenetic than were epicotyl explants. Since the hypocotyl segments, in culture, showed complete lack of root growth and total deformity of the explant, it is suggested that in maize the scutellum provides the growth factor(s) which promotes root growth and normal root morphology during germination. For epicotyl growth, however, the presence of the scutellum was slightly inhibitory (Tilton, 1981). A quantitative relationship between the amount of cotyledon removed and the extent of growth of root, hypocotyl and shoot in embryo cultures of *Vigna sesquipedalis* has been noted by Hotta (1957); the larger the portion of cotyledons severed the greater was the suppression of seedling development.

Rangaswamy and Rangan (1971) made similar observations with *Cassytha filiformis* (a stem parasite); these are summarized in Table 11.8 (see also Fig. 11.9). It is evident from the data that in *C. filiformis* the growth factor(s) for shoot morphogenesis resides in the 'radicular-halves' of the cotyledons. If the 'plumular-halves' of both the cotyledons are severed (treatment D) the seedling is well developed but if 'radicular-halves' are removed (treatment E) the plumule does not grow. Further, the 'radicular-half' of only one cotyledon alone (treatment G) or along with the 'plumular-half' of one of the cotyledons (treatment C,F) is not sufficient to support the growth of the plumule. The minimum fraction of the cotyledons necessary to permit normal morphogenesis of the plumule is the 'radicular-half' of one cotyledon together with 'plumular-half' of both the cotyledons (treatment A) or the 'radicular-halves' of both the cotyledons (treatment D).

In the embryo cultures of *Phaseolus vulgaris* an increase in the weight of embryonal axis was directly proportional to the amount of cotyledonary tissue left on it (Monnier, 1978).

TABLE 11.8

Effect of cotyledons on seedling formation in *Cassytha filiformis*[a,b]

Portion of the cotyledons severed	Response
A. Radicular-half of one cotyledon	Seedling well developed
B. Plumular-half of one cotyledon	Seedling well developed
C. One entire cotyledon	Hypocotyl and radicle inhibited
D. Plumular-halves of both cotyledons	Seedling well developed
E. Radicular-halves of both cotyledons	Plumule quiescent
F. Radicular-half of one cotyledon and plumular-half of the other	Plumule quiescent
G. One entire cotyledon and plumular-half of the other	Plumule quiescent
H. One entire cotyledon and radicular-half of the other	Plumule quiescent
I. Both cotyledons; leaving behind the embryonal axis	Plumule quiescent

[a]After Rangaswamy and Rangan (1971).
[b]Decotylated embryos were cultured on medium after Rangaswamy (1961).

Thevenot and Come (1971, 1973) observed that the growth of partially after-ripened apple (*Pyrus malus*) embryos was much better when they were planted with the cotyledons in contact with the medium than in any other orientation, suggesting that the cotyledons are the chief structures involved in the absorption of nutrients from the medium and transferring them to the other parts of the embryo. In gymnosperms, Bulard (1952) showed that cultured mature embryos of *Ginkgo* displayed normal shoot growth only when the cotyledons were in contact with the medium. Paradoxically, completely decotylated embryos of *Cajanus cajan* (Kanta and Padmanabhan, 1964) and *Dendrophthoe falcata* (Bajaj, 1966a) have been reported to grow into normal seedlings. In *Pinus* normal root development occurred irrespective of whether the cotyledons were in contact with the medium or away from it (Sacher, 1956) or when completely removed before culturing the embryos (Berlyn and Miksche, 1965). However, an appreciable shoot growth never occurred in these cultures.

In some genotypes of maize (Harms et al., 1976) and barley (Dale and Deambrogio, 1979) embryo callusing was much better when the embryo was planted with the scutellum in contact with the medium than when the scutellum was away from it.

Existing studies related to the interaction between different constituent tissues of the embryo cannot be critically evaluated especially when the media used by different authors are not identical. For example,

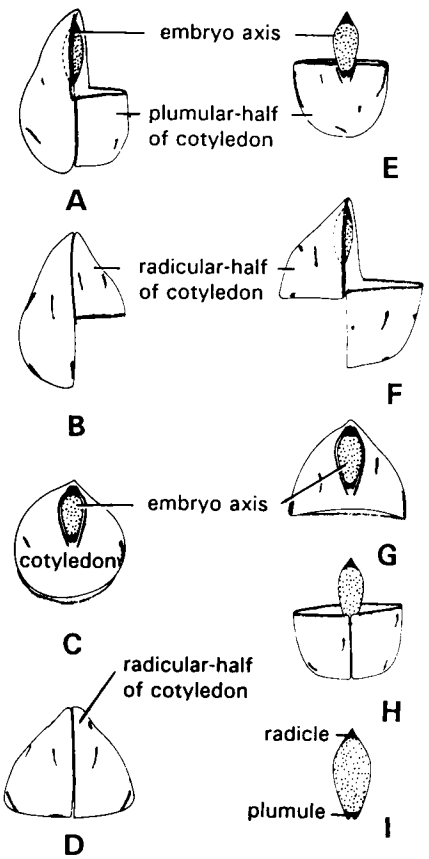

Fig. 11.9. Decotylation patterns of *Cassytha* embryos in microsurgical experiments. For the responses of various treatments refer to Table 11.8 (after Rangaswamy and Rangan, 1971).

Cassytha embryo segments were cultured on a simple medium comprising mineral salts, vitamins, amino acids, and sucrose whereas for *Dendrophthoe* embryos the medium was enriched with casein hydrolysate.

11.8. EMBRYO AND SEED CULTURE OF PARASITIC ANGIOSPERMS

From the viewpoint of morphogenesis, parasitic angiosperms constitute an interesting group of plants. Depending on the mode of their attachment to the host plant these parasites fall into two broad categories: root parasites (establishing contact through host roots) and stem parasites (establishing contact through aerial parts of the host plant).

Root parasites are dependent on the host stimulus to various degrees. In nature most of them require a host contact for normal seedling morphogenesis, especially shoot development (Kuijt, 1969). Some root parasites, including *Cistanche, Orobanche* and *Striga*, are also dependent on the host plant for seed germination (Shivanna and Rangaswamy, 1976). In cultures the seeds of parasites belonging to the second category will not germinate in a salts–sugar–vitamins–agar medium. However, the addition of extract or exudate of the host roots to the medium may stimulate seed germination independent of the host (Saunders, 1933; Brown and Edwards, 1944; Cezard, 1965; Okonkwo, 1966). Many different chemically defined substances have been shown to substitute the stimulus provided by the host for seed germination in obligate root parasites: cytokinins for *Striga euphrasioides* (Rangaswamy and Rangan, 1966), gibberellins for *Orobanche ludoviciana*, *O. ramosa* (Nash and Wilhelm, 1960) and *O. aegyptiaca* (Kumar and Rangaswamy, 1977), scopotelin for *Striga asiatica* (Worsham et al., 1962) and strigol for *S. lutea* (Cook et al., 1966) and *O. aegyptiaca* (Kumar and Rangaswamy, 1977).

Seeds of *Alectra vogelii* germinate without a host stimulus but seedling formation does not proceed beyond the stage of radicle emergence unless the radicle establishes contact with the roots of a suitable host (Okonkwo, 1975). Similarly, in *Sopubia delphinifolia* a germinating seed normally fails to develop a shoot without a host contact. In seed cultures of these two obligate root parasites full seedling development occurred independent of a host stimulus when an exogenous carbohydrate, such as sucrose and glucose, was supplied through the medium (Okonkwo, 1975; Shivanna and Rangaswamy, 1976). In seed cultures of *S. delphinifolia* shoot development without a host stimulus was also favoured by high light intensity (3000 lx) (Sahai, 1978).

In vitro seed germination and shoot development independent of host stimulus has also been observed in the root parasites, *Osyris wightiana* (Bhojwani, 1968), *Exocarpus cupressiformis* (Johri and Bhojwani, 1965; Bhojwani, 1969a) and *Santalum album* (Rao and Rangaswamy, 1971). However, most of the root parasites did not form haustoria in cultures without a host. Aseptic seedlings of *Striga senegalensis* formed haustoria only if grown with seedlings of *Sorghum bicolor* (Okonkwo, 1966). Cultured seedlings of some scrophulariaceous root parasites have been reported to develop haustorial structures without a host stimulus when treated with chemically undefined substances, such as aqueous extracts of gum tragacantha (*Agalinis purpurea,* Riopel and Musselman, 1979; *S. delphinifolia,* Sahai and Shivanna, 1981), cotton strings and soya beans (*Orthocarpus purpurascens,* Atsatt et al., 1978). None of the common growth hormones or sugars was effective in haustorial induction.

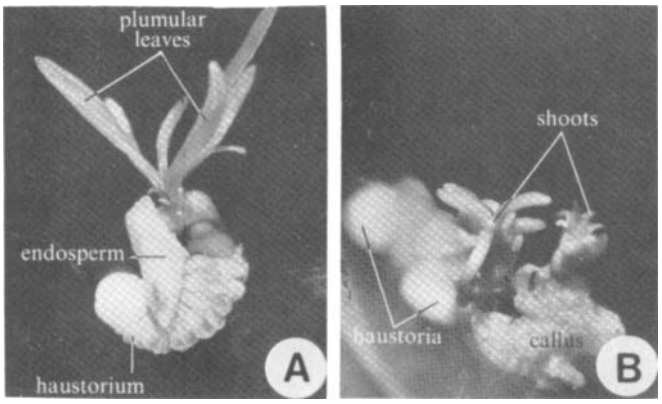

Fig. 11.10. *Scurrula pulverulenta.* (A) Seedling formed in vitro independent of a host, showing a worm-like haustorium and several plumular leaves. (B) Differentiation of shoot-buds and haustoria from the embryo callus (after Johri and Bhojwani, 1970).

Unlike root parasites, loranthaceous stem parasites frequently form distinct haustorial structures, comparable to those formed in nature, without a host or a plant extract (Johri and Bajaj, 1963; Bhojwani, 1969b; Johri and Bhojwani, 1970; Nag and Johri, 1976; see Fig. 11.10A). Even callused embryos (Johri and Bhojwani, 1970; Nag and Johri, 1976; see Fig. 11.10B), and endosperm (Bhojwani and Johri, 1970) of leafy mistletoes differentiated haustorial structures. These mistletoes are also distinct from root parasites and other stem parasites in the ability of their seeds to germinate on simple nutrient media lacking growth hormones or a complex nutrient mixture like casein hydrolysate and coconut milk (Johri and Bhojwani, 1971).

11.9. MORPHOGENIC POTENTIAL OF THE EMBRYO CALLUS

One of the most critical needs of applying methods of somatic cell genetics to crop improvement has been to establish cell and callus cultures from which complete plants can be regenerated. Embryo calluses have been shown to possess a high regeneration capacity in relation to those derived from mature organs like leaf, stem and root (Merkle et al., 1990).

Dale and Deambrogio (1979) cultured meristem tips, mesocotyl, leaf sheath, immature embryos and mature embryos of barley. Best growing calluses originated from root and embryo explants but shoot differentiation occurred only from the immature embryo callus. Green and Phillips (1975) reported that in four different genotypes of maize the embryos excised 18 DAP gave the highest frequency of differentiating calluses. No differentiation occurred in calluses of mature embryo origin (see Table

TABLE 11.9

Influence of embryo age and genotype on percent cultures showing plant regeneration from the scutellar callus in maize[a,b]

Genotype	Embryo age (DAP)					
	14	16	18	20	22	24
A188	18	15	21	14	9	0
A619	0	5	6	5	0	0
A632	0	0	0	0	0	0
B9A[c]	0	5	8	0	0	0
W64A	0	0	11	0	0	0

[a]After Green and Phillips (1975).
[b]Each value represents the % of 60 embryos.
[c]These cultures died during the fourth month of growth (fourth subculture).

11.9). That the age of the embryo in maize considerably influences the regeneration ability of their calluses was confirmed by Freeling et al. (1976). Immature embryos of *Avena* (Cummings et al., 1976) and *Sorghum* (Gamborg et al., 1977; Thomas et al., 1977) have also been found to be good explants for initiating calluses capable of plant regeneration. Several other crop plants, such as most of the cereals, grain legumes, cotton and several tree species, which were once regarded as recalcitrant for plant regeneration in tissue cultures have become amenable to improvement by the biotechnological methods simply because highly embryogenic cultures could be established from immature zygotic embryos.

11.10. PRACTICAL APPLICATIONS

11.10.1. Obtaining rare hybrids

The most useful and popular application of zygotic embryo culture has been in raising rare hybrids. In many interspecific and intergeneric crosses fertilization occurs normally and the embryo shows early development, but the poor or abnormal development of the endosperm causes premature death of the hybrid embryo, and germinable seeds are not formed. In 1925 Laibach made a very significant contribution when he demonstrated that in such unsuccessful crosses the embryo is often potentially capable of normal growth, and full hybrid plants can be raised by excising the embryos before the onset of abortion and culturing them on nutrient medium. In this context, Raghavan (1976a) has remarked

that 'The triumph of the embryo culture method is nowhere better seen than in the dramatic demonstration of the ability of the embryos removed from the seeds of inviable crosses to grow in cultures.' Since the time of Laibach (1925) the technique of embryo culture has been extensively used to produce agriculturally and horticulturally useful hybrids from crosses which normally fail due to the post-fertilization barriers to crossability. In this section only some selected examples are mentioned. Raghavan (1976a, 1977) has dealt with many more examples.

All attempts to cross *Lilium henryi* and *L. regale* were unsuccessful until the embryo culture technique was adopted (Skirm, 1942). Emsweller and Uhring (1962) crossed *L. speciosum-album* with *L. auratum*. The seeds obtained in this cross were larger in size due to a large amount of endosperm. The embryo was also very long. During the storage of seeds or soaking them in water the embryo underwent degeneration and no seedlings could be obtained. This has been ascribed to some sort of embryo-endosperm incompatibility. By growing excised immature embryos on nutrient medium Emsweller and Uhring (1962) could raise full hybrid plants from this cross.

Embryo culture has been successfully applied to produce many interspecific hybrids in the family Fabaceae. *Melilotus officinalis* is an agronomically important species but the plants have high levels of coumarin which is harmful to cattle. Attempts have been made to introduce into this species the genes for low coumarin content by crossing it with another species (*M. alba*) which possesses the trait. In the reciprocal cross between *M. officinalis* and *M. alba* the hybrid embryo grows for some time but fails to mature because the ovules abort 8–10 DAP. Only through embryo culture could Webster (1955) and Schlosser-Szigat (1962) obtain some hybrid plants from this cross. Interspecific hybridization in the genus *Trifolium* has often proved difficult due to the occurrence of both pre- and post-fertilization barriers. Embryo culture was successfully applied to many such crosses where hybridization was normally impossible due to endosperm abortion and consequent abnormal differentiation and starvation of the hybrid embryo (Williams, 1978, 1980; Williams and De Lautour, 1980).

The interspecific cross *Phaseolus vulgaris* × *P. lunatus* is normally unsuccessful due to failure of the hybrid embryo to grow beyond the heart-shaped stage. Kuboyama et al. (1991) obtained a full hybrid plant from this cross with the aid of embryo culture but it was fully sterile.

Crossing cultivated tomato (*Lycopersicon esculentum*) with wild tomato (*L. peruvianum*) has been considered desirable from the point of view of transferring pest and disease resistance from the latter to the former. The cross *L. peruvianum* × *L. esculentum* does not succeed due to pre-

fertilization barriers. In the reciprocal cross, however, fertilization occurs but the cross fails because of embryo abortion, and no viable seeds are formed (Cooper and Brink, 1945; Hogenboom, 1972; Thomas and Pratt, 1981). Smith (1944), Chowdhury (1955) and Alexander (1956) reported that they were able to produce hybrid plants in the cross with *L. esculentum* as the female parent with the aid of embryo culture. However, Thomas and Pratt (1981) did not obtain even a single culturable hybrid embryo out of 401 under-developed seeds formed in the cross *L. esculentum* var. VENT × *L. peruvianum* var. LA 1283-4. These workers followed an alternative approach of 'embryo-callus culture' to obtain full hybrid plants. The poorly developed hybrid embryos were dissected out from ovules 35 DAP and cultured to form a callus followed by plant differentiation. The hybrid plants thus obtained were diploid or tetraploid. The embryo-callus culture approach has also yielded hybrids from the crosses *Lycopersicon esculentum* × *L. chilense* and *L. esculentum* × *Solanum lycopersicoides* in which embryos capable of direct plant formation do not develop (Scott and Stevens, cited in Thomas and Pratt, 1981).

Numerous laboratories have attempted interspecific crosses in the genus *Oryza* with the objective of developing high yielding rice varieties resistant to diseases and pests and capable of withstanding adverse environmental conditions. Embryo abortion, however, has been a serious stumbling block in achieving this goal, and embryo culture has enabled this hurdle to be bypassed. By culturing 10–25-day-old hybrid embryos from several interspecific crosses of rice, which normally failed, Iyer and Govilla (1964) were able to raise full hybrids. On an agar medium containing 10% CM and 0.1% malt extract, 88% of the excised hybrid embryos germinated. After the seedlings had grown fully in the test tubes, they were transplanted to the soil.

In the intergeneric cross *Hordeum jubatum* × *Secale cereale* the hybrid seeds collapse 13–16 days after fertilization, leading to the failure of the cross. Histological studies revealed that in this cross although the hybrid embryos showed considerable growth they ceased to grow prematurely due to incompatible endosperm. Brink et al. (1944) excised embryos from 9 to 12-day-old seeds and cultured them in White's medium. Of the 81 embryos cultured only one developed into seedling; most of the other surviving embryos showed undifferentiated growth. Regeneration of plants from the hybrid embryo callus was not attempted.

A significant improvement in the frequencies of hybrid production from the intergeneric crosses *Hordeum* × *Secale*, *Hordeum* × *Triticum* and *Hordeum* × *Agropyrum* was achieved with the aid of embryo culture (Kruse, 1974). Embryo culture methods have also been used successfully for raising intergeneric hybrids from the crosses *Triticum* × *Aegilops*

(Chueca et al., 1977) and *Triticum* × *Secale* (Taira and Larter, 1977ab, 1978).

In certain intergeneric crosses it is essential to give special treatment to the female parents, following pollination, to obtain embryos suitable for in vitro culture. When tetraploid *Triticum turgidum* (female parent) is crossed with diploid *Secale cereale* fertilization occurs with a high frequency but embryos abort 14–20 DAP. Depending on the strains of the parent used the number of culturable embryos formed may be very low. Taira and Larter (1977a) observed that treatment of the ovules with ε-amino-*n*-caproic acid or its analogue L-lysine daily for 10 days, beginning 3 DAP, significantly promoted the yield of normally differentiated embryos capable of forming normal seedling when cultured in vitro. Optimum day/night temperatures during the treatment was 17°C (Taira and Larter, 1977b). Cooper et al. (1978) reported that spraying the spikes, following pollination, with a mixture of GA_3, NAA and 2-ip was required to obtain culturable embryos in the cross barley × rye. In the non-treated spikes fruit development ceased before the embryo attained a size suitable for their culture.

11.10.2. Haploid production

A novel application of embryo culture technique has been in the production of haploids through directional elimination of chromosomes following distant hybridization (see Section 7.7). Briefly, in the cross *Hordeum vulgare* × *H. bulbosum* fertilization proceeds readily but the chromosomes of *H. bulbosum* are preferentially lost during the first few divisions of embryogenesis. As a result, the haploid embryos show slow growth. This, coupled with the disintegration of the endosperm 2–5 days after fertilization, necessitates culture of the excised embryo to raise haploid *H. vulgare* plants.

11.10.3. Shortening the breeding cycle

Occasionally the breeding work on horticultural plants is delayed due to long dormancy periods of their seeds. By growing excised embryos in nutrient medium this period may be reduced. For example, using embryo culture Randolph and Cox (1943) could shorten the life cycle of *Iris* from 2 or 3 years to less than 1 year. *Rosa* normally takes a whole year to come into flowering; through embryo culture it has been possible to produce two generations in a year and, thus, shortening its breeding cycle (Lammerts, 1946; Asen, 1948). Nickell (1951) reported that in cultures the excised embryos of weeping crabapple (*Malus* sp.) start germi-

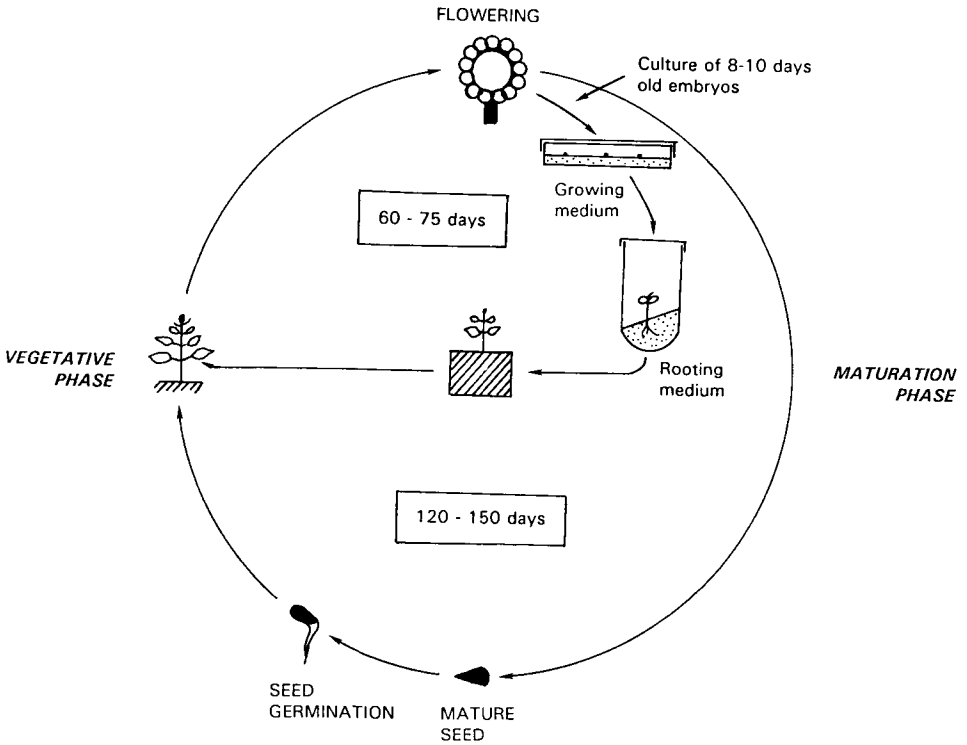

Fig. 11.11. Diagrammatized summary of immature embryo (8–10 DAP) culture to shorten the life cycle duration of sunflower from 120–150 days to 60–75 days (after Serieys, 1992)

nation within 48 h, and within 4 weeks transplantable seedlings are formed. By the end of 5 months the seedlings attain a height of about 1 m. On the other hand, seeds planted in the soil take about 9 months to germinate.

Seed maturation in soybean and sunflower takes 50–60% of the life cycle duration (120–150 days) (Serieys, 1992. By in vitro culture of 10-day-old immature embryos of sunflower Plotnikov (1983) could reduce the duration of the life cycle by half. Similarly, Alissa et al. (1986) and Aspiroz et al. (1988) could raise four generations of sunflower in a year by culturing 7 and 10–18-day-old embryos, respectively (Fig. 11.11).

11.10.4. Rapid seed viability test

The possibility of breaking seed dormancy by embryo culture also allows the use of this technique for rapid testing of the viability of a par-

ticular batch of seeds. Germination of excised embryos is regarded as a more exact and reliable test than the commonly used staining methods for seed viability (Barton, 1961). A good correlation has been shown between the growth of excised embryos of non-after-ripened seeds and the germination of after-ripened seeds of peach (Tukey, 1944).

11.10.5. Propagation of rare plants

Seeds of *Musa balbisiana*, a wild relative of the commercial banana, do not germinate in nature, However, seedlings can be readily obtained by culturing their excised embryos (Cox et al., 1960).

As an abnormality, some coconuts develop soft, fatty tissue in place of the liquid endosperm (Mohan Ram, 1976). Such nuts are called 'makapuno'. Being rare, makapunos are very expensive and served only at special banquets in the Philippines. It had been felt that plants propagated from makapuno nuts would be truly makapuno-bearing (Zuniga, 1953). However, under normal conditions the coconut seeds fail to germinate. Using the technique of in vitro culture of excised embryos, De Guzman (1969) and De Guzman and Del Rosario (1974) succeeded in raising plantlets from makapuno nuts. Eighty-five percent of the field-grown plants obtained with the aid of embryo culture bore makapuno nuts (De Guzman et al., 1976).

11.10.6. Others

Embryo culture is a useful technique to study basic aspects of embryogenesis, such as the effect of nutrients, phytohormones and other chemical and physical factors on embryonal growth and differentiation (Monnier, 1990; Liu et al., 1993b).

In desiccated zygotic embryos the plasma membranes are perforated with large pores, making them permeable to molecules of certain size. However, during inbibition the membrane rapidly becomes intact and uniform. Taking advantage of this unique feature of the embryos, Topfer et al. (1989) transformed some cereals and legumes by imbibing their mechanically isolated embryos in a solution containing plasmid DNA with a chimeric NPT-II gene, and chemicals which enhance the permeability of the membrane.

Establishment of highly regenerable cultures from immature embryos of a large number of agronomic, horticultural and forest species has considerably expanded the scope of application of biotechnological methods to crop improvement.

11.11. CONCLUDING REMARKS

Embryo culture technique has already established its credibility as an invaluable tool for obtaining hybrid plants in some difficult crosses. Considerable progress has also been made in understanding the various aspects of embryo morphogenesis. However, factors regulating embryo development inside the ovules (in ovulo) are still not fully understood. The ovules excised at the zygote stage have been reported to show normal embryogenesis in cultures (see Chapter 10) but embryos excised prior to the differentiation of organ primordia either fail to survive in culture or exhibit callusing. In this context the recent success of Liu et al. (1993a) to culture 35 μm early globular embryos of *Brassica juncea*, using a double layer culture system and a complex nutrient medium is noteworthy. Even more remarkable is the recovery of full fertile plants from in vitro fertilized egg (in vitro formed zygote) of maize (see Chapter 10). In zygotic and non-zygotic embryogenesis an attachment of the proembryo to the parent tissue seems to be essential for normal embryogenic development. This attachment may be necessary to maintain the gradient of nutrients and other growth factors supplied by the parent tissue for proper polarized differentiation of the embryos. Liu et al. (1993b) have demonstrated that the transition from radial symmetry of globular embryos to bi-lateral symmetry of heart-shaped embryos of dicotyledonous plants is brought about by the polar transport of auxin. Interference with the polar transport of auxin resulted in the formation of embryos with fused cotyledons. More such studies are likely to enhance our understanding of the process of embryogenesis.

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Chapter 12

Protoplast Isolation and Culture

12.1. INTRODUCTION

In eukaryotes the transfer of genetic material from one individual to another is conventionally achieved through sexual breeding, the scope of which is extremely limited, particularly in animals. Even in plants, where fairly distant species could be crossed, it has not always been possible to obtain full hybrids between desired individuals because of sexual incompatibility barriers (see also Chapters 10 and 11). This has often proved a serious handicap in crop improvement programmes through hybridization. In this respect cell fusion offers a novel approach to distant hybridization (somatic hybridization). Fusion of cells, whether in plants or animals, must occur through the plasma membrane. Unlike animals, in plants the plasma membrane is bound by a rigid cellulosic wall, and the adjacent cells are cemented together by a pectin-rich matrix. It is mainly for this reason that somatic cell genetics is more advanced with animal than with plant systems. It is only since 1960, when E.C. Cocking at the University of Nottingham demonstrated the feasibility of enzymatic degradation of plant cell walls to obtain large quantities of viable naked cells, hereafter called protoplasts[1], that real interest in genetic modification of somatic cells in higher plants has developed. Actually, active contributions in this area started appearing after 1970.

Besides being able to fuse with each other, higher plant protoplasts can also take up foreign DNA, through their naked plasma membrane, under specific chemical and physical treatments. However, the importance of an isolated protoplast system in genetic transformation of plants has been somewhat eclipsed by more recent techniques of gene insertion into intact plant cells such as co-cultivation of explants with disarmed vectors of *Agrobacterium* and particle gun (see Chapter 14).

Protoplasts also provide an experimental system for a wide range of biochemical and molecular studies ranging from investigations into the growth properties of individual cells to membrane transport.

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[1] Torrey and Landgren (1977) have defined higher plant protoplasts as 'cells with their walls stripped off and removed from the proximity of their neighbouring cells.'

Essential ingredients of the technique of genetic modification of plant cells through the protoplast system are: (a) isolation of protoplasts, (b) culture of protoplasts to raise whole plants, (c) cell fusion, and (d) introduction of foreign genetic material into the protoplasts. This chapter deals with the techniques of isolation and culture of protoplasts. Cell fusion in relation to somatic hybridization and genetic transformation of plants using isolated protoplasts are discussed in Chapters 13 and 14, respectively.

12.2. ISOLATION OF PROTOPLASTS

The isolation of protoplasts from higher plants was pioneered by Klercker in 1892. The procedure followed by him was largely mechanical; the cells were kept in a suitable plasmolyticum and cut with a fine knife. In this process some of the plasmolyzed cells were cut only through the cell wall, releasing intact protoplasts. This procedure for protoplast isolation is applicable only to vacuolated cells. Another limitation of this mechanical method is that the yields are extremely low.

In 1960, Cocking demonstrated the possibility of enzymatic isolation of a large number of protoplasts from cells of higher plants. He used a concentrated solution of cellulase enzyme, prepared from cultures of the fungus *Myrothecium verrucaria*, to degrade the cell walls. However, real progress in this area was made after 1968 when cellulase and macerozyme enzymes became available commercially.

The commercial preparations of the enzymes for protoplast isolation were first employed by Takebe et al. (1968). In the scheme followed by these workers to isolate mesophyll protoplasts of tobacco, the two enzymes were used sequentially. The leaf pieces were first exposed to macerozyme to liberate single cells which were then treated with cellulase to digest the cell walls and release the protoplasts. Power and Cocking (1968) demonstrated that the two enzymes can be used together. This 'simultaneous' or 'one step' method is faster than the sequential method and reduces the chances of microbial contamination by cutting down a few steps. Most workers now use this simplified one-step method (for example see Appendix 12.I). A range of enzyme preparations are now available commercially (Table 12.1), and, depending on the nature of the tissue these are used in different combinations.

The use of commercially available enzymes has enabled the isolation of protoplasts from virtually every plant tissue, as long as the cells have not acquired lignification. Protoplast isolation has been reported from mesophyll cells of in vivo and in vitro growing plantlets, aseptic seedlings, microspore mother cells, young microspores, pollen grain calli, and embryo-

TABLE 12.1

Some commonly used commercially available enzymes for protoplast isolation

Enzyme	Source	Supplier
Cellulases		
Onozuka RS	*Trichoderma viride*	Yakult Honsha, Japan
Cellulase R-10	*T. viride*	Yakult Honsha, Japan
Cellulysine	*T. viride*	Calbiochem, USA
Driselase	*Irpex lactes*	Kyowa Hakko Kogyo, Japan
Meicelase-P	*T. viride*	Meiji Seik Kaisha, Japan
Hemicellulase		
Hemicellulase	*Aspergillus niger*	Sigma, USA
Rhozyme HP-150	*Aspergillus niger*	Rohm and Hass, USA
Zymolyase	*Arthrobacter luteus*	Sigma, USA
Pectinase		
Macerozyme R-10	*Rhizopus* sp.	Yakult Honsha, Japan
Macerase	*Rhizopus* sp.	Calbiochem., USA
Pectinase (purified)	*A. niger*	Sigma, USA
Pectolyase Y23	*A. japonicus*	Seishin Pharmaceutical, Japan
Pectinol	*A. niger*	Rohm and Hass, USA

genic and non-embryogenic suspension cultures (Bhojwani and Razdan, 1983). More recently, viable protoplasts have been obtained from male and female gametes (see Chapter 10).

Isolation of viable and culturable protoplasts in large quantities is affected by several factors, and optimum conditions for a system are established empirically. The work of Uchimiya and Murashige (1974) with cultured cells of tobacco should serve as a model when attempting to isolate protoplasts from a new system (see Table 12.2).

Protocols for isolation of protoplasts from mesophyll cells, seedling explants and cultured cells of some plants are given in Appendix 12.1 (see also Fig. 12.1).

12.2.1. Factors affecting yield and viability of protoplasts

(i) *Source of material.* Leaf has been the most favourite source of plant protoplasts because it allows the isolation of a large number of relatively uniform cells without the necessity of killing the plants. Since the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. When protoplasts are prepared from leaves the age of the plant

TABLE 12.2

Optimal conditions for the isolation of protoplasts from cultured cells of tobacco[a]

Parameter	Optimum condition
Plant material	4–5-day-old subculture
Cellulase	1% Onozuka R-10
Macerozyme	0.1–0.2% Onozuka R-10
pH of enzyme solution	4.7–5.7
Volume of enzyme solution/fresh weight of tissue	10 ml g^{-1}
Incubation period	2–3 h
Incubation temperature	22–37°C
Rate of agitation	50 rev. min^{-1}
Osmoticum	300–800 mmol l^{-1} mannitol

[a]After Uchimiya and Murashige (1974).

and the conditions under which it has been grown may be critical. To achieve maximum control on the growth conditions of source plants several workers have used in vitro growing shoots. Leaves from such plants also do not require exposure to surface sterilants. The leaves from in vitro rooted shoots of *Pyrus communis* released twice as many viable protoplasts as the leaves from field grown material (Ochatt and Caso, 1986). When the leaves are derived from glasshouse- or growth room-grown plants, it would be desirable to optimize the growth conditions (light, humidity, temperature and supply of nutrients) for the donor plants. For *Brassica* species, hypocotyl segments from aseptic seedlings have been widely used to isolate protoplasts (Glimelius, 1984; Chuong et al., 1985; Barsby et al., 1986; Chuong et al., 1987a,b; Yamashita and Shimamoto, 1989).

Owing to the difficulty in isolating culturable protoplasts from leaf cells of cereals and some other species their cultured cells have been used as an alternative source material. The yield of protoplasts from cultured cells depends on the growth rate and growth phase of the cells. Frequently sub-cultured (every 3–7 days) suspension cultures, and cells taken from the early log phase are most suitable. To obtain totipotent protoplasts generally embryogenic suspension cultures are used.

(ii) *Pre-enzyme treatments*. To facilitate the penetration of enzyme solution into the intercellular spaces of leaf, which is essential for effective digestion, various methods are followed. A most commonly practised method is to peel the lower epidermis and float the stripped pieces of leaf on the enzyme solution in a manner that the peeled surface is in contact

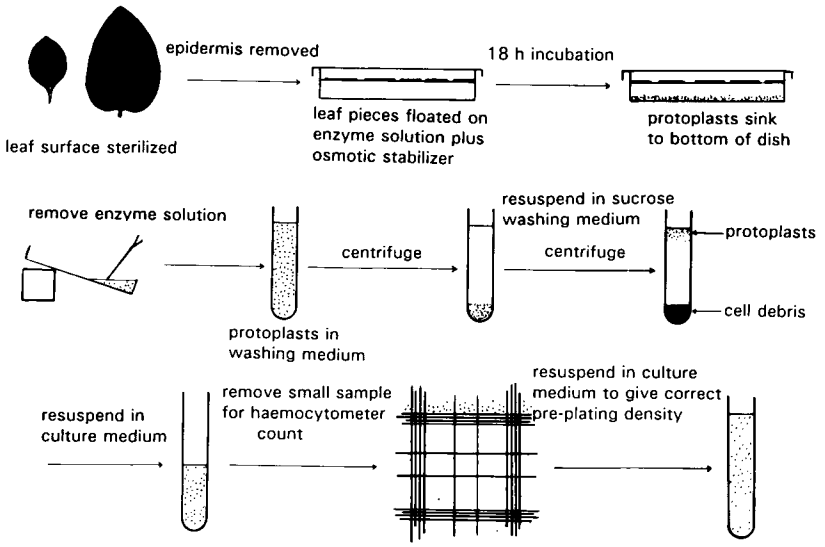

Fig. 12.1. Flow diagram for the isolation of mesophyll protoplasts (courtesy of E.C. Cocking, UK).

with the solution. Where it is not possible or is inconvenient to peel the epidermis, cutting the leaf or tissue into small strips (1–2 mm wide) has been found useful. When combined with vacuum infiltration the latter approach has proved very effective. Mesophyll protoplasts of cereals could be isolated within 2 h by infiltrating the leaf pieces with enzyme solution under a partial vacuum for 3–5 min (Scott et al., 1978). The criterion used to check adequate infiltration is that leaf pieces will sink when the vacuum is removed. Brushing the leaf with a soft brush or with the cutting edge of a scalpel may also improve enzymatic action, and cutinase has been used to remove leaf epidermis (Power et al., 1989). Large calli are chopped into small pieces before transfer to enzyme mixture.

Agitation of the incubation mixture during enzyme treatment improves protoplast yield from cultured cells.

(iii) *Enzyme treatment.* The release of protoplasts is very much dependent on the nature and concentration of the enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulase and pectinase. The latter degrades mainly the middle lamella and the former is required to digest the cellulosic cell wall. The first commercially available enzymes of fungal origin were Onozuka Cellulase SS and Onozuka Macerozyme SS. Due to increasing demands for these

enzymes many other companies are now producing these enzymes and marketing under different trade names (see Table 12.1). Driselase, having a number of zymolytic activities, such as cellulase, pectinase, laminarinase and xylanase (Kao et al., 1974), has proved especially useful for isolating protoplasts from cultured cells. Even purified enzymes, like cellulase R-10, seem to carry adequate pectinase (Okuno and Furusawa, 1977; Slabas et al., 1979). Pectolyase Y-23, a highly powerful macerozyme, in combination with cellulase released protoplasts from mesophyll cells of pea within 30 min (Nagata and Ishii, 1979).

Some tissues may also require hemicellulase in addition to cellulase and macerozyme. Aleurone cells of barley treated with cellulase did not liberate protoplasts. A thin cellulase-resistant wall was left around them. Such cells, called spheroplasts, had to be treated with glusulase to digest the remaining wall (Taiz and Jones, 1971).

The crude commercial enzymes carry nucleases and proteases as impurities which may be harmful to protoplasts viability. Therefore, some workers prefer to purify the enzymes by eluting them through biogel or Sephadex G-25 filtration (Constabel, 1982). However, mostly the enzymes have been employed in their crude forms with satisfactory results. Indeed, Arnold and Eriksson (1976) observed that purification of enzymes resulted in fewer surviving protoplasts, and the crude enzymes were more effective.

The activity of enzymes is pH dependent. The optimal pH values of Onozuka cellulase R-10 and macerozyme R-10, as given by the manufacturers, are 5–6 and 4–5, respectively. In practice, however, the pH of the enzyme solution is mostly adjusted anywhere between 4.7 and 6.00.

The optimal temperature for the activity of these enzymes is 40–50°C which happens to be too high for the cells. Generally 25–30°C is found adequate for isolation of protoplasts. The concentration of the enzymes and the duration of enzyme treatment is to be decided after several trials. The incubation period in the enzyme solution may be as short as 30 min (Nagata and Ishii, 1979). Another factor that may affect the yield of protoplasts is the relative volume of the enzyme solution to the amount of tissue. Generally 10 ml solution for 1 g tissue is satisfactory.

The cells that are damaged or lysed during isolation may release hydrolytic enzymes capable of damaging the healthy protoplasts. To counter this problem, addition of potassium dextran sulphate (0.5% w/v) to the enzyme solution has been recommended (Ochatt and Power, 1992). Addition of antioxidants, such as PVP-10 (average MW 10 000) to the enzyme mixture has proved essential for isolation of large numbers of viable protoplasts of recalcitrant plants such as deciduous tree species (Revilla et al., 1987).

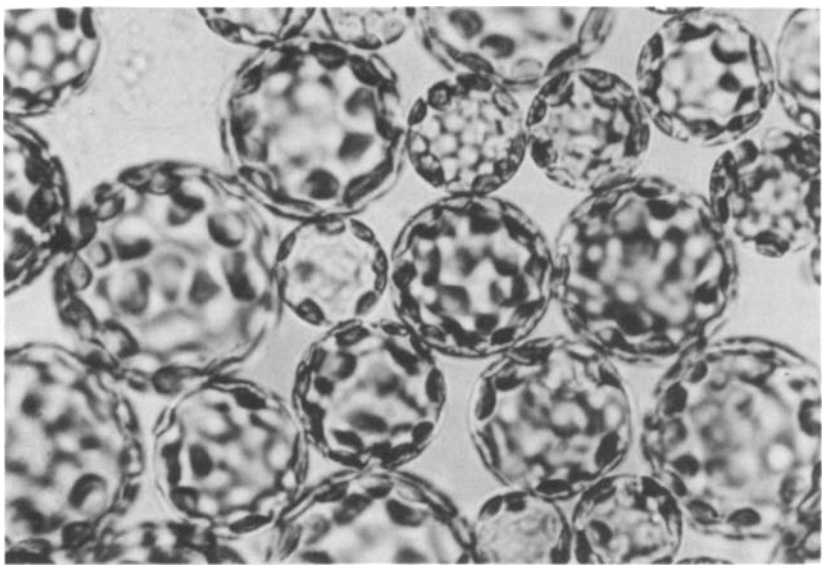

Fig. 12.2. Freshly isolated mesophyll protoplasts (courtesy of J.B. Power, UK).

(iv) *Osmoticum*. A fundamental property of isolated protoplasts is their osmotic fragility and, hence, the need for a suitable osmotic stabilizer in the enzyme solution, the protoplast washing medium, and the protoplast culture medium. In a solution of proper osmolarity freshly isolated protoplasts appear completely spherical (see Figs. 12.2 and 12.4A). On a quantitative basis protoplasts are more stable in a slightly hypertonic rather than isotonic solution. A higher level of the osmoticum may prevent bursting and budding but, at the same time, it may inhibit the division of the protoplasts.

A variety of solutes, ionic and non-ionic, have been used for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture but the most widely used osmotica are sorbitol and mannitol in the range 450–800 mmol. Uchimiya and Murashige (1974) observed that for isolating protoplasts from tobacco suspension cultures several soluble carbohydrates, including glucose, fructose, galactose, sorbitol and mannitol, were equally effective. When non-ionic substances are used as osmotic stabilizer the enzyme solution is often supplemented with certain salts, especially $CaCl_2$ (50–100 mmol l^{-1}). This improves the stability of the plasma membrane.

Meyer (1974) and Bohnke and Kohlenbach (1978) reported that the use of ionic osmotica (335 mmol l^{-1} KCl and 40 mmol l^{-1} $MgSO_4 \cdot 7H_2O$) im-

proved the viability of the protoplasts and yielded cleaner preparations. However, adjusting the osmotic pressure of the culture medium with salts proved detrimental.

12.2.2. Purification of protoplasts

After the material has been incubated in enzyme solution for an adequate period the incubation vessel is gently swirled or the leaf pieces are gently squeezed to release the protoplasts held in the original tissue. The digestion mixture at this stage would consist of subcellular debris, especially chloroplasts, vascular elements, undigested cells and broken protoplasts, besides intact and healthy protoplasts. It is, therefore, necessary to remove these contaminants. The large debris is removed by passing the digestion mixture through a metal or nylon sieve (30–100 μm pore size). For further purification one of the following two methods has been generally followed. Generally, the filtrate is sedimented in a centrifuge tube at 100 × g for about 5 min, and the supernatant containing small debris is discarded. The pellet is resuspended in the washing medium and washed three times by repeated centrifugation at 50 × g for 3–5 min and resuspension. Alternatively, the pelleted protoplasts and debris suspended in a small volume of the enzyme mixture or the washing medium is loaded at the top of a sucrose pad (21%) in a centrifuge tube and spun at 100 × g for 10 min. The debris moves down to the bottom of the tube and a band of clean protoplasts appears at the junction of the sucrose pad and the protoplast suspension medium. The protoplasts are gently removed with a Pasteur pipette and transferred to another centrifuge tube. Following the repeated centrifugation and resuspension, as in the first procedure, the protoplasts are washed three times and finally suspended in the culture medium at an appropriate density.

Hughes et al. (1978) purified the protoplasts using 450 mmol l^{-1} sucrose (bottom) and 450 mmol l^{-1} mannitol (top) discontinuous gradient. Piwowarczyk (1979) modified the density gradient in a way that intact protoplasts, free of the enzyme and debris, can be obtained by a single spinning. The gradient is prepared by sequentially filling the centrifuge tube with 500 mmolv sucrose in the culture medium, a layer of 140 mmol l^{-1} sucrose and 360 mmol l^{-1} sorbitol in the culture medium and, finally, a layer of protoplast suspension in the enzyme solution which contains 300 mmol l^{-1} sorbitol and 100 mmol l^{-1} $CaCl_2$. After spinning at 400 × g for 5 min a clean layer of protoplasts is formed just above the sucrose layer and the debris moves down to the bottom of the tube. Larkin (1976) and Scowcroft (1977) found the commercial density buffer Lymphoprep (Nyegaard A/S, Oslo, Norway) to be excellent for removing debris. The

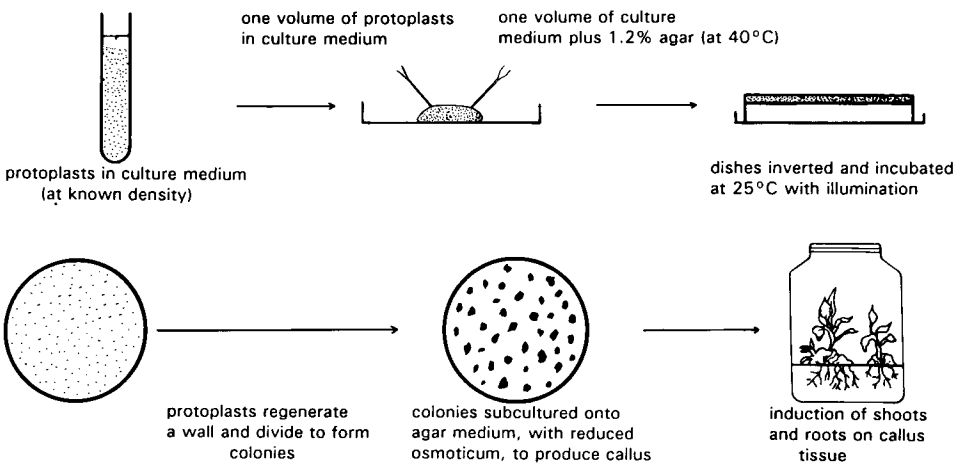

Fig. 12.3. Flow diagram for the culture of protoplasts (courtesy of E.C. Cocking, UK).

buffer comprises 9.6% (w/v) sodium metrizoate and 5.6% (w/v) Ficoll, with a specific gravity of 1.077 ± 0.001 g ml^{-1}.

12.2.3. Viability of the protoplasts

Viability of the freshly isolated protoplasts can be checked by a number of methods: (a) observation of cyclosis or cytoplasmic streaming as an indication of active metabolism. This method is not very helpful with mesophyll protoplasts which carry a large number of peripheral chloroplasts; (b) oxygen uptake measured by an oxygen electrode which indicates respiratory metabolism (Taiz and Jones, 1971); (c) photosynthetic activity (Kanai and Edwards, 1973); (d) exclusion of Evan's blue dye by intact membranes (Kanai and Edwards, 1973; Glimelius et al., 1974); and (e) staining with fluroescein diacetate. The last method is most commonly used. Some of the methods to test cell/protoplast viability are described in Section 4.3.6.

12.3. PROTOPLAST CULTURE

The culture methods and the culture requirements of isolated protoplasts are often similar to those of single cells. Protoplasts may be cultured in agar plates (see Fig. 12.3) following the Bergmann's technique of cell plating (see Chapter 4). An advantage in using semi-solid medium is that the protoplasts remain stationary which makes it convenient to follow the development of specific individuals. However, liquid medium has

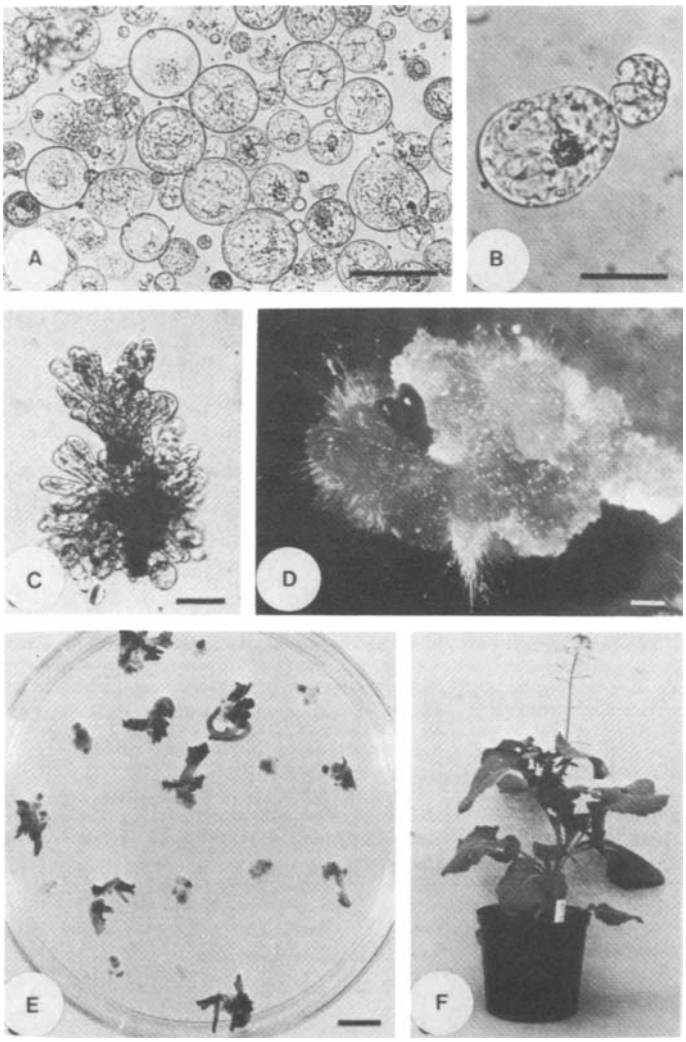

Fig. 12.4. Plant regeneration from hypocotyl protoplasts of *Brassica napus*. (A) Freshly isolated protoplasts of different sizes. (B) One-day-old protoplasts; both the protoplasts have regenerated wall but only the small one has divided. (C) Callus obtained from the protoplasts after 14 days of culture. (D) Differentiation of shoot buds from the protoplast-derived callus. (E) Calli with regenerated shoots as in (D), transferred to regeneration medium containing zeatin and IAA. (F) Plant regeneration from protoplast-derived callus (reprinted by permission from K. Glimelius, 1984, Physiol. Plant., 61: 38–44).

been generally preferred for the following reasons: (a) protoplasts of some species would not divide if plated in agarified medium (Gosch et al., 1975; White and Bhojwani, 1981), (b) the osmotic pressure of the medium can be effectively reduced after a few days of culture, (c) if the degenerating

component of the protoplast population produces some toxic substances which could kill the healthy cells it is possible to change the medium (Schenck and Hoffmann, 1979; Bhojwani and White, 1982), and (d) the density of cells can be reduced or cells of special interest may be isolated after culturing them for a few days at a high density (Kao, 1977; Gleba, 1978; Gleba and Hoffmann, 1978; White and Bhojwani, 1981). In liquid medium protoplasts have been cultured variously. The protoplast suspension is plated as a thin layer in petri plates, incubated as static cultures in flasks (about 5 ml of the protoplast suspension in 50–100 ml flasks) (Takebe and Nagata, 1973; Coutts and Wood, 1975), or distributed in 50–100 μl drops in petri plates and stored in a humidified chamber.

Embedding protoplasts in agarose beads or discs is reported to improve plating and regeneration efficiency in many species (see Dons and Colijn-Hooymans, 1989). The protoplasts of several recalcitrant species of Magnoliaceae and Liliaceae divided and regenerated when their protoplasts were trapped in agarose droplets in such a way that streaks of locally high cell densities were obtained (Binding et al., 1988). In practice, the protoplasts suspended in molten (40°C) agarose medium (1.2% w/v agarose) are dispensed (4 ml) into small (3.5–5 cm diameter) plates and allowed to solidify. The agarose layer is then cut into 4 equal sized blocks and transferred to larger dishes (9 cm diameter) containing liquid medium of otherwise the same composition (Shillito et al., 1983). Alternatively, protoplasts in molten agarose medium are dispensed as droplets (50–150 μl) on the bottom of petri plates and after solidification the droplets are submerged in the same liquid medium.

Alginate is another gelling agent used for culture of protoplasts, particularly of the species which are heat sensitive, such as *Arabidopsis thaliana* (Damm and Willmitzer, 1988).

Even a healthy protoplast preparation, under the most favourable culture conditions, shows bursting of some protoplasts during the first 24 h in culture. The stable protoplasts rapidly resume recovery from the trauma of being stripped off their wall. They exhibit an active increase in the number of cell organelles, cytoplasmic streaming, respiration, and synthesis of RNA, protein, and polysaccharides, suggesting an active cellular metabolism.

12.3.1. Cell wall formation

Within 2–4 days in culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new wall regeneration. More reliable and direct demonstration of wall regeneration has been through staining with Calcofluor White ST (American Cyanamide

Co., Wayne, NJ, USA)[2] and from the use of a variety of electron microscopic techniques (Willison, 1976; Fowke, 1978).

The regularity of cell wall regeneration and the lag period prior to the onset of wall formation depends partly on the plant species and the degree of differentiation of the cells used for protoplast isolation (Ochatt and Power, 1992). Mesophyll, callus and cell suspension protoplasts of most solanaceous and many *Brassica* species form cell wall very quickly (within 24–40 h of culture) (Gamborg et al., 1981; Evans and Bravo, 1983; Power et al., 1989). In contrast, cereal protoplasts (Vasil, 1987, 1988) and mesophyll protoplasts of legumes (Davey and Power, 1988) may require up to 4 days for cell wall regeneration. An even longer lag phase (7 days or more) is required for wall formation by the protoplasts of the woody plants (McCown and Russell, 1987; Vardi and Galun, 1988).

Horine and Ruesink (1972) reported that the cell wall regeneration in *Convolvulus* protoplasts required an exogenous supply of a readily metabolizable carbon source, such as sucrose. In its absence cell wall formation did not occur. Ionic osmotic stabilizers in the culture medium suppress the development of a proper wall (Meyer, 1974; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978). The protoplasts of carrot cell suspensions developed a wall faster and more uniformly if polyethylene glycol 1500 was added to the culture medium (Wallin and Eriksson, 1973).

There is a direct relationship between wall formation and cell division. Protoplasts which are not able to regenerate a proper wall fail to undergo normal mitosis (Bawa and Torrey, 1971; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978). Protoplasts with a poorly developed wall often show budding and may enlarge several times their original volume. They may become multinucleate because karyokinesis is not accompanied by cytokinesis. Among other reasons, inadequate washing of the protoplasts prior to culture leads to these abnormalities.

12.3.2. Cell division and callus formation

While the presence of a proper wall is essential for regular division, not all such cells regenerated from protoplasts embark upon division. The

[2] To test the presence of a wall, protoplasts are incubated in 0.01 or 0.1% Calcofluor solution, in an appropriate osmotic stabilizer, for 5 min. The protoplasts are then washed to remove any excess dye and mounted on a slide, in an osmotically suitable solution. Calcafluor binds to the wall material and fluoresces when observed using a mercury vapour lamp, with an excitation filter BG12, and suppression filter K 510. Tinapol Solution B.O.P.T (Geigy U.K. Ltd., Dye Stuff and Textile Chemicals Div. Simonsway, Manchester, UK) behaves in a similar way to Calcafluor (Evans and Cocking, 1975).

plating efficiency of protoplasts varies considerably with the experimental material; it may range from as low as 0.1% to as high as 80%.

The protoplasts capable of dividing, undergo the first division within 2–7 days (see Fig. 12.4B). Rarely, the lag phase before the first division lasts as long as 7–25 days (Bhojwani et al., 1977b; Khasanov and Butenko, 1979; Ochatt, 1990). Protoplasts from actively dividing cell suspensions, as a rule, enter the first division faster than those from highly differentiated cells of the leaf. The cells which continue dividing develop multicellular colonies after 2–3 weeks in culture (see Fig 12.4C). After another 2 weeks macroscopic colonies are formed which can be transferred to osmoticum-free medium and treated as standard tissue cultures (see Fig. 12.4D–F).

In protoplast cultures, the cell divisions are asynchronous. The first division may be equal or unequal. Mitosis is normal. Several factors influence divisions in protoplast cultures.

(i) *Nutritional requirements*. Mostly the salts of MS (Murashige and Skoog, 1962) and B_5 (Gamborg et al., 1968) media and their modifications have been used. Kao et al. (1973) reported that the addition of 1 mmol l^{-1} $CaCl_2$ to B_5 medium improved the percentage of dividing cells in protoplast cultures of *Vicia hajastana* and *Bromus inermis*. However, supplementing the medium with 20 mmol l^{-1} NH_4NO_3 reduced the frequency of dividing cells. Ammonium ions proved detrimental to protoplast survival of many other species, and media have been devised that either have a reduced concentration of ammonium (paper mulberry, Oka and Ohyama, 1985) or lack it (potato, Upadhya, 1975; tomato, Zapata et al., 1981; tobacco, Caboche, 1980; *Pyrus*, Ochatt and Caso, 1986; Ochatt and Power, 1988a,b).

Vitamins used for protoplast culture are the same as those used in standard tissue culture media. The 8p medium developed by Kao and Michayluk (1975) for low density protoplast culture contains several vitamins, organic nutrients, sugar alcohols and undefined nutrients such as casamino acids and coconut water (Table 12.3). This medium and modifications thereof have been used successfully on a broad range of species including cereals (Thompson et al., 1986b), legumes (Gilmour et al., 1987), ornamentals (Power et al., 1989) and fruit trees (Patat-Ochatt et al., 1988).

Growth hormones, particularly auxins and cytokinins, are almost always required. For cereal protoplasts, however, 2,4-D alone is either sufficient or better than in combination with cytokinin. The type of auxin and cytokinin and their ratios in the medium required to induce divisions at optimum rate may vary considerably with the plant material. The

TABLE 12.3

A medium for culturing protoplasts at low density[a,b]

Constituents	Amount (mg l^{-1})	Constituents	Amount (mg l^{-1})
Mineral salts			
NH_4NO_3	600	KI	0.75
KNO_3	1900	H_3BO_3	3.00
$CaCl_2 \cdot 2H_2O$	600	$MnSO_4 \cdot H_2O$	10.00
$MgSO_4 \cdot 7H_2O$	300	$ZnSO_4 \cdot 7H_2O$	2.00
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
KCl	300	$CuSO_4 \cdot 5H_2O$	0.025
Sequestrene 330 Fe[c]	28	$CoCl_2 \cdot 6H_2O$	0.025
Sugars			
Glucose	68400	Mannose	125
Sucrose	125	Rhamnose	125
Fructose	125	Cellobiose	125
Ribose	125	Sorbitol	125
Xylose	125	Mannitol	125
Organic acids (adjusted to pH 5.5 with NH_4OH)			
Sodium pyruvate	5	Malic acid	10
Citric acid	10	Fumaric acid	10
Vitamins			
Inositol	100	Biotin	0.005
Nicotinamide	1	Choline chloride	0.5
Pyridoxine·HCl	1	Riboflavin	0.1
Thiamine·HCl	10	Ascorbic acid	1
D-Calcium pantothenate	0.5	Vitamin A	0.005
Folic acid	0.2	Vitamin D_3	0.005
p-Aminobenzoic acid	0.01	Vitamin B_{12}	0.01
Hormones	Soybean × barley	Soybean × pea or *N. glauca*	
2,4-D	1	0.2	
Zeatin	0.1	0.5	
NAA	–	1	
Vitamin-free casamino acid[d]	125 mg l^{-1}		
Coconut water (from mature fruits; heated to 60°C for 30 min and filtered)	10 ml l^{-1}		

[a]After Kao and Wetter (1977).
[b]Sterilized by filtration.
[c]Geigy Chemical Corp., Ardsley, NY.
[d]Difco Laboratories, Detroit, MI.

most commonly used auxin is 2,4-D, but Uchimiya and Murashige (1976) reported that NAA was superior to 2,4-D or IAA for the culture of protoplasts from cell suspensions of tobacco. The cytokinins commonly used are BAP, kinetin and 2-ip. Whereas the protoplasts from actively growing cultured cells may find a high auxin/kinetin ratio suitable for their division, those derived from highly differentiated cells, such as leaf cells, often require a high kinetin/auxin ratio for dedifferentiation.

Sometimes culture requirements of intact cells and tissues may give clues to the composition of the medium suitable for their protoplast culture, but the simple concept of the cultural behaviour of protoplasts being equivalent to that of cells without a cell wall is not always valid. For example, the growth regulator autonomy of cultured-crown gall tumour cells is lost upon removing the cell wall and is restored at the multicellular stage (Scowcroft et al., 1973). Similarly, culture requirements of pea shoot-tip protoplasts are different from those of its cells (Gamborg et al., 1975). Scott et al. (1978) observed that freshly isolated protoplasts of cereals were sensitive to phytohormones in the medium but cells regenerated from them could be transferred to a medium containing auxin and cytokinin to induce divisions.

The low viability of protoplasts of *Lycopersicon pennellii* could be correlated with high ethylene production and increased cell osmolality (Rethmeier et al., 1991). Cell wall degrading enzymes also influence the release of ethylene. Addition of ethylene-inhibitor, silver thiosulphate to the culture medium improved yield, viability and regeneration of protoplasts of *L. pennellii* (Rethmeier et al., 1991) and potato (Perl et al., 1988; Mollers et al., 1992).

Antioxidants in the medium are either essential or improve the response of cultured protoplasts in some cases. For protoplast culture of sweet cherry (*Prunus avium*) addition of the antioxidants, glycine and/or PVP-10, to the culture medium was essential to counter the phenolic browning of protoplasts and protoplast derived tissues at all culture stages (Ochatt, 1991). In *Beta vulgaris* addition of the antioxidant n-propylgallate (n-PG) to the medium proved essential for successful culture of protoplasts and shoot regeneration (Krens et al., 1990). A combination of the antioxidants glutathione, glutathione-peroxidase and phospholipase increased the plating efficiency and growth of microcalli from protoplasts of *Lolium perenne* (Creemers-Molenaar and Van Oort, 1990).

Addition of 2% Ficoll to the culture medium more than doubled the cell division frequency in mesophyll protoplast cultures of *Brassica napus* (Millam et al., 1988). The colonies on this medium were larger and greener than those on the control, probably because of better aeration of

the cells; in the presence of Ficoll protoplasts and microcolonies float on the medium.

(ii) *Osmoticum.* Isolated protoplasts require osmotic protection in the culture medium until they regenerate a strong wall. Osmolarity of the medium is generally adjusted with 500–600 mmol l^{-1} mannitol or sorbitol, as in the enzyme solution. Scott et al. (1978) and Arnold and Eriksson (1976) reported that for mesophyll protoplasts of cereals and pea, respectively, sucrose or glucose could not replace mannitol or sorbitol as the osmotic stabilizer in the medium. However, some authors have noted the superiority of glucose over other osmotic agents (Gamborg et al., 1975; Michayluk and Kao, 1975; Evans et al., 1980). Shepard and co-workers (Shepard and Totten, 1977; Bidney and Shepard, 1980; Shahin and Shepard, 1980) routinely used sucrose as the osmotic stabilizer for the culture of protoplasts of potato, sweet potato, and cassava. For brome grass, sucrose proved better than glucose or mannitol (Michayluk and Kao, 1975). The use of an ionic osmoticum in the culture medium suppresses the regeneration of a proper wall, leading to the failure of normal mitosis (Horine and Ruesink, 1972; Meyer, 1974; Meyer and Abel, 1975; Bohnke and Kohlenbach, 1978).

Seven to ten days after initial culture, by which time most of the viable protoplasts have regenerated a good wall and undergone a few divisions, the osmolarity of the medium is gradually reduced by periodic addition of a few drops of fresh medium lacking in the osmoticum or containing it at a fairly low level. In the presence of the original high level of the osmoticum the cells may stop dividing after some time (Kao and Michayluk, 1980). Macroscopic colonies are finally transferred to a fresh medium lacking the osmoticum.

(iii) *Plating density.* As in cell cultures, the initial plating density of protoplasts has a profound effect on plating efficiency. Protoplasts are generally cultured at a density of 1×10^4 to 1×10^5 protoplasts ml^{-1} of the medium. At such high densities the cell colonies arising from individual protoplasts tend to grow into each other at a fairly early stage in culture. This would result in the formation of chimeral tissue if the protoplast population was genetically heterogeneous. Cloning of individual cells, which is highly desirable in somatic hybridization and mutagenic studies, can be achieved if protoplasts or cells derived from them can be cultured at a low density (100–500 protoplasts ml^{-1}). It may also allow the development of individual cells to be followed, thus enabling the solation of hybrid colonies in the absence of a stringent selection system.

Kao and Michayluk (1975) developed a complex culture medium (see Table 12.3) in which individually cultured protoplasts of *Vicia hajastana* regenerated a wall, underwent sustained divisions and formed callus. In this medium (8p) mesophyll protoplasts of alfalfa, pea, and *Vicia* divide faster at lower population densities (less than 100 protoplasts ml^{-1}) than at higher densities. The 8p medium and its various modifications have been successfully used to culture protoplasts of a range of other species. While using 8p medium the cultures should be stored in the dark or under very low light intensity (50 lx) because in strong light the medium becomes phytotoxic (Kao and Wetter, 1977).

The feeder cell layer technique, developed by Raveh et al. (1973) for plant protoplasts, is another approach to culture protoplasts at low densities. Tobacco protoplasts normally do not divide at a plating density below 10^4 protoplasts ml^{-1} but with the feeder cell layer they could be cultured at a density as low as 10–100 protoplasts ml^{-1} (Raveh et al., 1973; Raveh and Galun, 1975). The feeder cell layer was prepared by exposing the protoplasts (10^6 cells ml^{-1}) to an X-ray dose of 5×10^3 R which inhibited cells from dividing but allowed them to remain metabolically active. Protoplasts were then washed two to three times (it is important to wash properly to remove any toxic substances produced due to irradiation) and plated in soft agar. Non-irradiated protoplasts in agar medium were layered over the feeder cell layer. The optimal density of cells in the feeder layer was the same as the optimal plating density of the protoplasts cultured without the feeder layer (2.4×10^4 ml^{-1}). The feeder layer can also be prepared with cells from suspension cultures (Cella and Galun, 1980).

The importance of the feeder layer or nurse culture has now been demonstrated for various crops (Jain et al., 1995). Individual protoplasts of barley, tobacco and rape could be successfully cultured using feeder systems (Eigel and Koop, 1989; Schaffler and Koop, 1990). A simple versatile feeder layer system for *Brassica oleracea* protoplasts has been described by Walters and Earle (1990). Kyozuka et al. (1987) used actively growing nurse cells in liquid medium to support the regeneration of rice protoplasts embedded in agarose beads (Fig. 12.5). Jain et al. (1995) have shown that feeder layers with *Oryza ridleyi* or *Lolium multiforum* were able to induce division in protoplasts of two indica rice varieties which did not divide otherwise. The latter was four times more effective than the former. Feeder layers with cells of both the species gave maximum plating efficiency.

Some cross-feeding is known to occur between protoplasts of different species (Vardi, 1978; Butenko and Kuchko, 1980; Cella and Galun, 1980). However, for tobacco and orange protoplasts the feeder layer prepared with their own protoplasts was more effective than that with alien cells

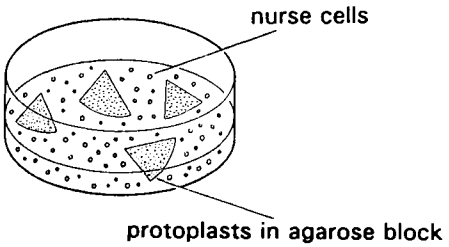

Fig. 12.5. Nurse culture of rice protoplasts. The protoplasts are embedded in nutrient agarose blocks and suspended in liquid medium containing nurse cells (reprinted by permission from: J. Kyozuka et al., 1987, Mol. Gen. Genet., 206: 408–413; © Springer-Verlag).

(Vardi, 1978). Similarly, in oat feeders from graminaceous plants promoted protoplast proliferation while feeders from dicotyledonous species suppressed protoplast division (Hahne et al., 1990).

The microdroplet technique was used by Kao (1977), Gleba (1978) and Gleba and Hoffmann (1978) to culture individual protoplasts and cells regenerated from them. They used special Cuprak dishes which have two chambers, a small outer chamber and a large inner chamber. The latter carries numerous numbered wells each with a capacity of 0.25–25 μl of nutrient medium. The protoplast suspension is transferred to the wells as microdroplets. The outer chamber is filled with sterile distilled water to maintain the humidity inside the dish. After covering it with the lid the dish is sealed with parafilm. Following this method, Gleba (1978) obtained whole plants of tobacco from protoplasts cultured individually in 0.25–0.5 μl droplets. The size of the droplets is critical for the division of single protoplasts. One protoplast per 0.25–0.5 μl droplet gives a ratio of cell/volume of culture medium equal to a cell density of 2–4 $\times$ 10^3 ml^{-1}. An increase in the size of the droplet would decrease the effective plating density. Gleba (1978) reported that droplets larger than 2 μl did not support the division of individual cells. The microdroplet method has been successfully used to culture hybrid cells of *Nicotiana glauca* + *Glycine max* (Kao, 1977) and *Arabidopsis thaliana* + *Brassica campestris* (Gleba and Hoffmann, 1978).

Koop and Schweiger (1985a) described a microculture system based on a computer controlled set-up for the efficient selection, transfer and culture of isolated single protoplasts of tobacco in microdroplets (ca. 50 nl) of fully defined medium. Each microdroplet is contained within a separate drop (1 μl) of mineral oil and 50 such droplets are placed on a coverglass (see Fig. 12.6). For culture, the coverglass is kept in a moist chamber. Subsequently, this technique was successfully applied for plant regeneration from hypocotyl protoplasts of *Brassica napus* (Spangenberg

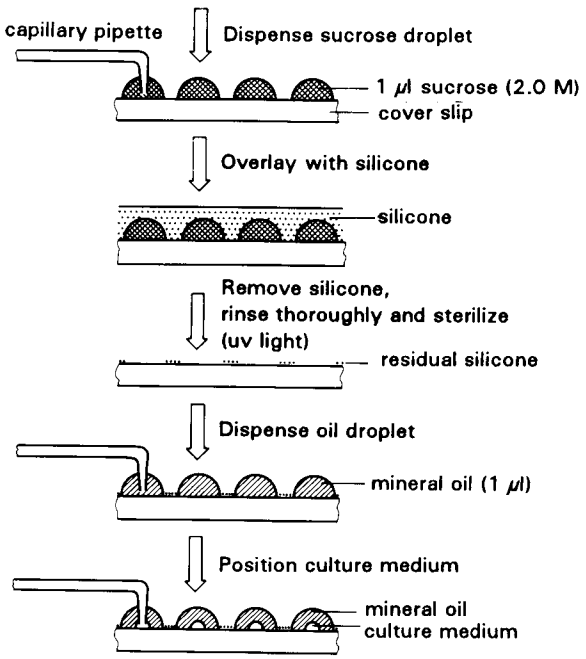

Fig. 12.6. Diagrammatic summary of the procedure followed to prepare a microchamber to culture individual protoplasts, using a 24 × 40 mm coverglass. Fifty 1.0 μl droplets of 2 M sucrose are applied onto the coverglass in an array of 5 rows of 10 drops each. The coverglass carrying the drops is covered with 'Repel silance (solution of dimethyldichlorosilance in 1,1,1-trichloroethane; LKB), drained, washed with water, rinsed with ethanol, dried and UV sterilized. One microlitre of mineral oil droplet is pipetted onto the spot that was occupied by sucrose and injected with 15–100 nl culture medium. The coverglass so prepared is kept in a two-compartment petri plate, with the outer compartment filled with 2 ml of 0.2 M mannitol solution (reprinted by permission from: H.U. Koop and H.G. Schweiger, 1985, J. Plant Physiol., 121: 245–257; © Gustav Fischer Verlag).

et al., 1985, 1986). A summarized description of the experimental set-up and protocol to be followed has been described by Schweiger et al. (1987).

Culturing the protoplasts at a high density for 4–5 days and then transferring to a fresh medium at very low densities (1–15 cells ml^{-1}) has been reported by Caboche (1980) and White and Bhojwani (1981). This two-step procedure would be useful in somatic hybridization if the hybrid cell lines can be distinguished from the parent types at the time of dilution.

(iv) *Physical treatments*. Electroporation treatment of isolated protoplasts has been shown to trigger early onset of cell divisions and bring

about a significant increase of protoplast plating efficiency in herbaceous and woody species (Rech et al., 1987; Chand et al., 1988; Ochatt and Power, 1992; Gupta et al., 1988). The protoplasts suspended in a buffer solution at four times their final density required for culture are exposed to three successive high voltage (250–2000 V) DC pulses, each of 10–50 μs, at intervals of 10 s. The effect of electric treatment of protoplasts seems to be lasting. The calli recovered from electroporated protoplasts of colt cherry showed increased proliferation ability and higher frequency shoot bud differentiation (Chand et al., 1988; Ochatt et al., 1988a; Ochatt, 1990) and such shoots produced a more prolific root system (9–11 roots per shoot) than the untreated controls (Ochatt et al., 1988b). Electroporation of parent protoplasts markedly increased the throughput of heterokaryons following fusion of protoplasts of *Prunus avium* × *pseudocerasus* and *Pyrus communis* (Ochatt et al., 1989). Ochatt and Power (1992) have suggested that the promotory effect of electroportion could be due to enhancement of DNA synthesis which could in turn bring about an earlier expression of genes controlling the early stages of differentiation. This may be coupled with permanent membrane modifications leading to sustained capacity for a longer/more efficient uptake of the requisite media components.

In contrast to the above examples of stimulation of cell division and regeneration in protoplast cultures by electroportion, involving high voltage DC currents, in *Medicago sativa* (Dijak and Simmonds, 1988) and *Trifolium subterraneum* (Zhongyi et al., 1990) low voltage electric treatment of protoplasts is reported to enhance division of protoplasts. Promotion of embryogenic differentiation by low voltage electric treatment is described in Section 6.3.7.

In rice, heat-shock prior to plating the protoplasts doubled the number of protoplasts entering division as well as increased the plating efficiency (Thompson et al., 1987). Gupta et al. (1988) observed stimulation of division and colony formation in protoplast cultures of *Pennisetum squamulatum* by electroporation or heat shock treatment (45°C for 5 min, followed by 10 s on ice). Chilling freshly isolated mesophyll protoplasts of tomato enhanced the plating efficiency by more than twofold (Muhlbach and Thiele, 1981).

(v) *Storage conditions.* Freshly isolated protoplasts should be stored in diffuse light or dark. In some species protoplasts are very sensitive to light and require storage in complete darkness for the first 4–7 days (Krishnamurthi, 1976; Landgren, 1976; Scott et al., 1978). Pea-root protoplasts exposed to 5 min of green filter incandescent light on the stage of the microscope resulted in incomplete inhibition of mitotic activity

(Landgren, 1976). With the regeneration of a proper wall after 5–7 days the cells may become light tolerant and at this stage the cultures may be transferred to light. It has been suggested that in cases where protoplasts are light sensitive observations should be kept to a minimum and those observed should be discarded from subsequent accountability (Torrey and Landgren, 1977). Protoplast cultures are generally maintained at 25–30°C. Very little attention has been paid to the effect of temperature on wall regeneration and subsequent division in protoplast cultures. Mesophyll protoplasts of *Lycopersicon esculentum* and *L. peruvianum* (Zapata et al., 1977) and cultured cell protoplasts of *Gossypium hirsutum* (Bhojwani et al., 1977b) either fail to divide or do so at a very low frequency when stored at 25°C but at 27–29°C they divide with a high plating efficiency. It has been suggested that elevated temperatures may not only influence the rate of division but may also be a pre-requisite for the initiation and maintenance of division in hitherto non-dividing protoplast systems.

(vi) *Plant material.* For reproducibly high plating efficiency the physiological state of the source tissue and the quality of the protoplasts are as critical as, if not more important than, the culture conditions. Therefore, when using tissues from intact plant organs the source plants should be grown under controlled conditions of light, temperature, and humidity. Leaves from field-grown plants often give erratic results. In *Lycopersicon* spp., reduction of the day length from 16 to 9 h and a cold treatment (at 4°C) of the donor plants significantly increased the plating efficiency (Tabaeizadeh et al., 1984).

To overcome the problem of low reproducibility of protocols for protoplast isolation, culture and regeneration, in many species axenic cultures of shoots, maintained under highly controlled environmental conditions are now widely used as the donor tissue. Schenck and Hoffmann (1979) reported that mesophyll protoplasts of *Brassica campestris* and *B. oleracea* isolated from plants grown in a glasshouse or growth chamber failed to divide but those from aseptically growing shoots formed calli. Axenic shoot cultures are also valuable in providing juvenile material of woody species (McCown and Russell, 1987; Revilla et al., 1987; Ochatt, 1990).

The culture response of protoplasts is also affected by the part of aseptic seedlings from which they are derived. In *Helianthus annuus* the hypocotyl-derived protoplasts divided, formed microcalli and, subsequently, somatic embryos but those from cotyledons or leaflets did not even divide (Dupuis et al., 1990). The plating efficiency and the frequency of somatic embryogenesis varied with the portion of hypocotyl from where the pro-

toplasts were obtained. Those from the basal part were most regenerative. Protoplast source can also influence the type of regeneration. Whereas the root protoplasts of *Medicago sativa* showed direct embryogenesis those from leaves or hypocotyl-derived suspension cultures initially formed callus on which somatic embryos could be induced (Pezzotti et al., 1984).

Sometimes when protoplasts from freshly harvested leaves did not divide it has been possible to obtain protoplasts capable of dividing by preculturing the leaves in a suitable medium for 3–7 days (Gatenby and Cocking, 1977; Donn, 1978; Kao and Michayluk, 1980).

In spite of intensive attempts during the past three decades it is still not possible to induce sustained cell divisions in mesophyll protoplasts of graminaceous species (Cutler et al., 1991; Vasil and Vasil, 1991); the recent report of regeneration of plants from mesophyll protoplasts of rice being an exception (Gupta and Pattnayak, 1993). For these plants embryogenic suspension cultures have proved to be the most suitable source of dividing and totipotent protoplasts, and plants have been obtained from protoplasts of almost all graminaceous species for which regenerable suspension cultures have been established.

12.3.3. Plant regeneration

The first report of plant regeneration from isolated protoplasts, in *Nicotiana tabacum* was published in 1971 by Takebe et al. Until 1983 this was achieved with only 44 species, which included 31 solanaceous species (Bhojwani and Razdan, 1983). During 1985–1993 protoplast technology gained considerable momentum and a large number of taxonomically diverse species, including most of the cereals and grasses, several legumes, cotton and tree species, have been shown to regenerate full plants from isolated protoplasts (Fig. 12.4). According to Roest and Gilissen (1993) this has been achieved in 330 species of higher plants (10 species of gymnosperms, 32 monocotyledons and 288 dicotyledons; see Table 12.4).

The main factors responsible for this grand progress are the selection of genotype and choice of source tissue. Plant regeneration in cereals is largely restricted to the protoplasts from embryogenic suspension cultures. Maltose (1.5%) in combination with sucrose (1.5%) substantially improved the regeneration rate from protoplast-derived calli of two indica rice varieties (Jain et al., 1995). In many cases protoplast regeneration appears to be strongly dependent on the genotype. In *Trifolium repens* inter (Yamada, 1989) and intra-varietal (Bhojwani et al., 1984a) variation for regeneration from protoplasts has been reported. Kyozuka et al.

TABLE 12.4

Some examples where whole plants, shoots or embryos have been regenerated from isolated protoplasts[a]

Taxon	Donor tissue	Mode of regeneration	References
Gymnosperms			
Pinaceae			
Abies alba	EC	E	Lang and Kohlenbach (1989)
Larix decidua	EC	E	Von Aderkas (1992)
L. xeurolepis	EC; ES	EP	Klimaszewska (1989)
Picea abies	ES	EP	Gupta et al. (1990)
P. mariana	ES	E	Tautorus et al. (1990)
P. glauca	ES	E	Attree et al. (1987)
Pinus caribaea	ES	E	Laine and David (1990)
P. kesiya	EC	E	Kumar and Tandon (1991)
P. taeda	ES	EP	Gupta and Durzan (1987)
Pseudotsuga menziensii	ES	E	Gupta et al. (1988)
Angiosperms			
MONOCOTYLEDONS			
Amaryllidaceae			
Hemerocallis fulva	P	E	Zhou (1989)
Hemerocallis cv.	SC	P	Fitter and Krikorian (1981)
Araceae			
Caladium bicolor	L	E, S-P	Jing and Wang (1991)
Iridaceae			
Crocus sativus	SC	SP	Isa et al. (1990)
Liliaceae			
Allium cepa	L	B, P	Wang et al. (1986)
Asparagus officinalis	St	Sh	Bui-Dang-Ha and Mackenzie (1973)
Lilium formolongi	EC	EP	Mii et al. (1991a)
Orchidaceae			
Phalaenopsis	C	ShP	Kobayashi et al. (1993)
Poaceae			
Agrostis alba	ES	EP	Asano and Sugiura (1990)
A. palustris	ES	SP	Terakawa et al. (1992)
Festuca rubra	ES	EP	Zaghmout and Terello (1990)
F. arundinacea	ES	EP	Dalton (1988a,b)
Hordeum vulgare	ES	P	Luhrs and Lorz (1988)
Lolium perenne	ES	P	Creemers-Molenaar et al. (1988),
	SC	EP	Dalton (1988a,b)
L. multiflorum	ES	SP	Dalton (1988b)
Oryza refipogon	ES	EP	Baset et al. (1993)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
O. sativa	SC	EP	Abdhullah et al. (1986)
	C	SP	Coulibaly and Demarly (1986),
	SC	P	Fujimura et al. (1985)
	C	P	Hayashi et al. (1986),
	C, SC	EP	Kyozuka et al. (1987),
	SC	EP	Thompson et al. (1986b),
	SC	P	Toriyama et al. (1986),
	SC	SP	Yamada et al. (1986),
Panicum maximum	ES	EP	Lu et al. (1981)
P. miliaceum	SC	EP	Heyser (1984)
Paspalum dilatatum	ES	EP	Akashi and Adachi (1992)
P. scrobiculatum	ES	EP	Nayak and Sen (1991)
Pennisetum americanum	ES	EP	Vasil and Vasil (1980)
P. purpureum	ES	EP	Vasil et al. (1983)
Poa pratensis	SC	Stp	Van der Valk and Zaal (1988)
Saccharum officinarum	SC	SP	Chen et al. (1988)
	ES	EP	Srinivassan and Vasil (1985)
Setaria italica	EC	EP	Dong and Xia (1989)
Sorghum vulgare	ES	SP	Wei and Xu (1990)
Triticum aestivum	ES	EP	Harris et al. (1988)
Zea mays	EC	EP	Cai et al. (1988),
	ES	EP	Kamo et al. (1987),
	ES	EP	Rhodes et al. (1988),
	ES	E	Vasil and Vasil (1987)
DICOTYLEDONS			
Actinidiaceae			
Actinidia chinensis	C	SP	Cai (1988)
A. deliciosa	L, St	P	Cai et al. (1991)
	C, L	E, SP	Oliviera and Pais (1991, 1992)
Apiaceae			
Daucus carota	SC	P	Grambow et al. (1972)
	R	EP	Kameya and Uchimiya (1972)
Foeniculum vulgare	SC	P	Miura and Tabata (1986)
Apocynaceae			
Rauvolfia vomitoria	L	E, ShP	Tremouillaux-Guiller and Chenieux (1991)
Araliaceae			
Panax ginseng	E	EP	Arya et al. (1991)
Asteraceae			
Brachycome dichromosomatica	C	ShP	Hahne and Hoffmann (1986)
Chrysanthemum hortorum	L	SP	Sauvadet et al. (1990)

C. morifolium	L	P	Otsuka (1986), Otsuka et al. (1985)
Cichorium intybus × *endivia*	L	EP	Sidikou-Seyni et al. (1992)
Helianthus annuus	L	ShP	Binding et al. (1980, 1981)
Lactuca saligna	L	ShP	Brown et al. (1987)
L. sativa	L, Co, R	ShP	Berry et al. (1982)
Scenecio fuchsii	Sh	ShP	Binding et al. (1992)
Brassicaceae			
Arabidopsis thaliana	L	SP	Binding et al. (1981)
	SC	ShP	Xuan and Menczel (1980)
Brassica alboglabra	L, Co, R, St	ShP	Pua (1987)
B. campestris	Co	ShP	Glimelius (1984)
B. carinata	Co	ShP	Chuong et al. (1987b)
B. juncea	L	EP	Chatterjee et al. (1985)
B. napus	L	ShP	Kartha et al. (1974),
	R	ShP	Xu et al. (1982b, 1985)
B. nigra	St	ShP	Chuong et al. (1987a),
	SC	E	Klimaszewska and Keller (1986)
B. oleracea	Co	ShP	Vatsya and Bhaskaran (1982)
	R	ShP	Xu et al. (1982b, 1985)
B. rapa	Co	Sh	Hegazi and Matsubara (1992)
Capsella bursa-pastoris	SC	EP	Bonfils et al. (1992)
Diplotaxis muralis	L	E, ShP	Sikdar et al. (1990)
Eruca sativa	L	EP	Sikdar et al. (1987)
Moricandia arvensis	L	SP	Murata and Mathias (1992)
Raphanus sativus	Co	ShP	Hegazi and Matsubara (1992)
Caricaceae			
Carica papaya × *cauliflora*	SC	EP	Chen and Chen (1992)
Caprifoliaceae			
Lonicera nitida	L	SP	Ochatt (1991a)
Caryophyllaceae			
Dianthus barbatus	L	SP	Nakano and Mii (1992)
D. caryophyllus	L	SP	Nakano and Mii (1992)
D. chinensis	L	SP	Nakano and Mii (1992)
D. plumarius	L	SP	Nakano and Mii (1992)
Chenopodiaceae			
Spinacia oleracea	L	ShP	Goto and Miyazaki (1992)
Convolvulaceae			
Ipomoea batatas	L, St	ShP	Sihachakr and Ducreux (1987)
I. trifida	L	P	Suga et al. (1990)
I. triloba	L, St	ShP	Liu et al. (1991)
Cucurbitaceae			
Cucumus melo	L	E,ShP	Moreno et al. (1986)
C. sativus	Co	EP	Colign-Hooymans et al. (1988)
Ebenaceae			
Diospyros kaki	L	ShP	Tao et al. (1991)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
Euphorbiaceae			
Manihot esculenta	L	ShP	Shahin and Shepard (1980)
Fabaceae			
Crotalaria juncea	Co	E,ShP	Rao et al. (1982, 1985)
Dolichos biflorus	SC	E	Sinha and Das (1986)
Glycine argyrea	Co, L	ShP	Hammatt et al. (1989)
G. canescens	Co	P	Davey and Power (1988)
G. clandestina	Co	P	Davey and Power (1988)
	Co	ShP	Hammatt et al. (1987)
G. max	Co	ShP	Wei and Xu (1988)
G. soja	SC	E	Gamborg et al. (1983)
G. tabacina	SC	E	Gamborg et al. (1983)
Lotus corniculatus	Co, R	ShP	Ahuja et al. (1983a)
L. pedunculatus	Co	ShP	Pupilli et al. (1990)
L. tenuis	R	ShP	Piccirilli et al. (1988)
Lupinus mutabilis × *hartwegii*	L	Sh	Schafer-Menuhr (1989)
Medicago arborea	L	ShP	Arcioni et al. (1985a)
	L,R	ShP	Mariotti et al. (1984)
M. coerulea	L, SC	EP	Arcioni et al. (1982)
M. difalcata	Co	EP	Gilmour et al. (1987)
M. falcata	Co	EP	Gilmour et al. (1987)
M. glutinosa	L, SC	EP	Arcioni et al. (1982)
	Co	EP	Gilmour et al. (1987)
M. hemicycla	Co	EP	Gilmour et al. (1987)
M. sativa	L	EP	Johnson et al. (1981),
	L	P	Kao and Michayluk (1980)
	Co, R	EP	Lu et al. (1982b)
	C, SC	EP	Mezentsev (1981)
	L	E,ShP	Dos Santos et al. (1980)
	R	EP	Xu et al. (1982a)
Onobrychis viciifolia	L	ShP	Ahuja et al. (1983b)
Phaseolus angularis	L	E, ShP	Ge et al. (1989)
Pisum sativum	L, Co	Sh	Puonti-Kaerlas and Eriksson (1988)
Psophocarpus tetragonolobus	C	ShP	Wilson et al. (1985)
Stylosanthes macrocephala	Co	ShP	Vieira et al. (1990)
S. scabra	Co	ShP	Vieira et al. (1990)
Trifolium hybridum	L, R	ShP	Webb et al. (1984, 1986)
T. lupinaster	SC	ShP	Zhao et al. (1991)
T. pratense	Co, L	EP	Davey and Power (1988)

T. repens	L	ShP	Ahuja et al. (1983b)
	SC	ShP	Gresshoff (1980)
T. rubens	L, SC	EP	Grosser and Collins (1984)
Trigonella corniculata	L	EP	Lu et al. (1982a)
T. foennum-graecum	L	Sh	Shekhawat and Galston (1983a)
Vicia narbonensis	Sh	EP	Tegender et al. (1991)
Vigna aconitifolia	L	EP	Shekhawat and Galston (1983b)
V. mungo	L	E	Sinha et al. (1983)
V. sinensis	L	ES	Davey et al. (1974)
Gentianaceae			
Eustoma grandiflorum	L	ShP	Kunitake et al. (1990)
Geraniaceae			
Pelargonium × *domesticum*	L	ShP	Dunbar and Stephens (1991)
P. crispum	C	ShP	Miyazaki et al. (1992)
P. odoratissimum	C	ShP	Miyazaki et al. (1992)
Labiatae			
Pogostemon cablin	SC	SP	Sakurai and Kawachi (1990)
Linaceae			
Linum usitatissimum	Co, R	ShP	Barakat and Cocking (1983)
L. catharticum	Sh	ShP	Binding et al. (1992)
Magnoliaceae			
Liriodendron sulipifera	ES	EP	Merkle and Sommer (1987)
Malvaceae			
Gossypium barbadense	Co, R	E, ShP	Elishihy and Evans (1986)
G. hirsutum	ES	EP	Chen et al. (1989), She et al. (1989)
Myrtaceae			
Eucalyptus sp.	Sh	SP	Ito et al. (1990)
Oxalidaceae			
Oxalis glaucifolia	C	SP	Ochatt et al. (1989)
Passifloraceae			
Passiflora edulis	L	ShP	Manders et al. (1991)
Platanaceae			
Platanus orientalis	L	ShP	Wei et al. (1991)
Plumbaginaceae			
Limonium perezii	SC	ShP	Kunitake and Mii (1990a)
Polygonaceae			
Fagopyrum esculentum	Co	E, ShP	Adachi et al. (1989)
Primulaceae			
Cyclamen persicum	EC	EP	Otani et al. (1989)
Primula malacoides	SC	ShP	Mii et al. (1990)
Rauunculaceae			
Nigella arvensis	L	ShP	Binding et al. (1980, 1981)
N. sativa	Sc	ShP	Jha and Roy (1982)
Ranunculus sceleratus	L	EP	Dorion and Bigot (1985)
Rosaceae			
Fragaria ananassa	L	ShP	Binding et al. (1982)
Malus × *domestica*	C, SC	E	Kouider et al. (1984)
Prunus avium	L	SP	Ochatt (1991b)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
P. avium × *P. pseudocerasus*	L, SC	R, P	Davey and Power (1988)
P. cerasifera	L	ShP	Ochatt (1992)
P. cerasus	L, SC	R, P	Davey and Power (1988)
	L	R, P	Ochatt and Power (1988)
P. spinosa	L	ShP	Ochatt (1992)
Pyrus communis	L, SC	R, P	Davey and Power (1988)
	L	ShP	Ochatt and Caso (1986)
Rosa persica × *xanthina*	ES	EP	Matthews et al. (1991)
R. rugosa	EC	E	Kunitake and Mii (1990b)
Rubiaceae			
Coffea arabica	EC	EP	Yasuda et al. (1986)
	ES	EP	Acuna and de Pena (1991)
C. canephora	E	EP	Schopke et al. (1987, 1988)
Rutaceae			
Citrus aurantium	C	EP	Vardi and Spiegel-Roy (1982)
C. limon	C	EP	Vardi and Spiegel-Roy (1982)
C. madurensis	EC	EP	Ling et al. (1989)
C. paradisi	C	EP	Vardi and Spiegel-Roy (1982)
C. reticulata	C	EP	Vardi and Spiegel-Roy (1982)
C. sinensis	C	EP	Kobayashi et al. (1985),
	C	EP	Vardi and Spiegel-Roy (1982)
C. unshiu	EC	EP	Ling et al. (1990), Kunitake et al. (1991a,b)
Salicaceae			
Populus alba	L	ShP	Sasamoto and Hosoi (1990)
P. alba × *P. glandulosa*	L	ShP	Park and Son (1988)
P. alba × *P. grandidentata*	L	ShP	Russell and McCown (1986, 1988)
P. glandulosa	L	ShP	Park et al. (1990)
P. nigra	C	ShP	Lee et al. (1987)
P. nigra × *P. maximowiczii*	L	ShP	Park and Son (1989, 1992)
P. nigra × *P. trichocarpa*	L	ShP	Russell and McCown (1988)
P. sieboldii	L	ShP	Sasamoto and Hosoi (1990)
Santalaceae			
Santalum album	C	EP	Bapat et al. (1985)
	SC	EP	Rao and Ozias-Akins (1985)
Scrophulariaceae			
Antirrhinum majus	L	E	Poirier-Hamon et al. (1974)
Digitalis lanata	L	ShP	Li (1981)

D. obscura	L	ShP	Brisa and Segura (1987)
Paulownia fortunei	L	ShP	Wei et al. (1991)
Simarubaceae			
Ailanthus altissima	C	ShP	Park and Lee (1990)
Solanaceae			
Atropa belladonna	SC	EP	Gosch et al. (1975)
Capsicum annuum	L	ShP	Saxena et al. (1981b)
Cyphomandra betacea	Sh	ShP	Binding et al. (1992)
Datura innoxia	L	ShP	Schieder (1975, 1977)
D. metel	L	ShP	Schieder (1977)
D. meteloides	L	ShP	Schieder (1977)
Duboisia myoporoides	SC	ShP	Kitamura et al. (1989)
Hyoscyamus muticus	L,SC	ShP	Lorz et al. (1979)
	L	SP	Wernicke and Thomas (1980) Wernicke et al. (1980)
Lycopersicon chilense	SC	ShP	Hassanpour-Estamhbanati and Demarly (1986)
L. esculentum	Co	ShP	Koblitz and Koblitz (1982ab, 1983)
	C	SP	Morgan and Cocking (1982)
L. hirsutum	L	SP	Montagno et al. (1991)
L. pennellii	L, SC	ShP	Tan et al. (1987)
L. peruvianum	L	ShP	Muhlbach (1980)
	L	ShP	Zapata and Sink (1981), Zapata et al. (1977)
L. pimpinellifolium	Co	SP	Imanishi and Suto (1987)
Nicotiana acuminata	L	ShP	Bourgin et al. (1979)
N. alata	L	SP	Bourgin and Missonier (1978), Bourgin et al. (1979),
	L	ShP	Passiatore and Sink (1981)
N. debney	L	ShP	Piven (1981),
	L	SP	Scowcroft and Larkin (1980)
	L	ShP	Shakurov (1982)
N. glauca	L	ShP	Bourgin et al. (1979)
N. glutinosa	L	EP	Liu and Xu (1988)
N. langsdorfii	L	ShP	Bourgin et al. (1979),
	L	ShP	Evans (1979)
N. longiflora	L	ShP	Bourgin et al. (1979)
N. otophora	L	ShP	Banks and Evans (1976),
	L	ShP	Bourgin et al. (1979)
N. plumbaginifolia	L	ShP	Bourgin et al. (1979)
	L	ShP	Gill et al. (1978)
N. repanda	L	ShP	Evans (1979)
N. rustica	L	ShP	Gill et al. (1979)
N. sylvestris	L	ShP	Banks and Evans (1976)
	L	ShP	Bourgin et al. (1976, 1979),
	L	ShP	Nagy and Maliga (1976)
N. tabacum	L	ShP	Nagata and Takebe (1971), Takebe et al. (1971)
Petunia alpicola	C, SC	ShP	Ford-Logan and Sink (1988)

TABLE 12.4 (*continued*)

Taxon	Donor tissue	Mode of regeneration	References
P. axillaris	L	ShP	Power et al. (1976)
P. hybrida	L	ShP	Durand et al. (1973) Frearson et al. (1973)
P. inflata	L	ShP	Power et al. (1976)
P. parodi	L	ShP	Hayward and Power (1975)
P. parviflora	L	ShP	Sink and Power (1977)
P. violaceae	L	ShP	Power et al. (1976)
Salpiglossis sinuata	C	ShP	Boyes and Sink (1981), Boyes et al. (1980)
Solanum dulcamara	L	ShP	Binding and Mordhorst (1984), Binding and Nehls (1977), Binding et al. (1980, 1981)
S. etubersoum	L	ShP	Barsby and Shepard (1983)
S. melongena	L	ShP	Bhatt and Fassuliotis (1981),
	SC	ShP	Gleddie et al. (1982),
S. tuberosum	L	ShP	Binding et al. (1978), Bokdlmann and Roest (1983)
Sterculiaceae			
Theobroma cacao	SC	E	Kanchanapoom and Kanchanapoom (1991)
Tiliaceae			
Corchorus capsularis	Co, L	E	Saha and Sen (1992)
Ulmaceae			
Ulmus campestris	L	P	Dorion et al. (1991)
U. × *'Pioneer'*	C	ShP	Sticklen et al. (1986)

[a]Based on Roest and Gilissen (1989, 1993); for additional examples references are given in this book. C, callus, Co, cotyledon/hypocotyl; E, somatic embryos; EC, embryogenic callus; EP, plants regenerated via somatic embryogenesis; ES, embryogenic suspension; L, leaf; P, plantlet; R, root; SC, suspension culture; Sh, shoot; SP, plants regenerated via shoot bud differentiation; St, stem.

(1988) observed intervarietal differences for regeneration from protoplast-derived calli of indica rice. Of the 65 genotypes belonging to *Brassica oleracea*, *B. campestris*, *B. napus*, *B. juncea* and *Raphanus sativus* only 4 genotypes of *B. oleracea* and *B. napus* exhibited high plating efficiency (>35%), and regenerated shoots at variable frequencies. Other genotypes either failed to divide or divided with very low frequencies (<6%) and showed very poor regeneration if any (Jourdan and Earle, 1989). Genotypic variation also occurs for the pattern of regeneration (see Section 6.3.2).

12.4. CONCLUDING REMARKS

During the last 15 years considerable progress has been made in isolation and culture of protoplasts. With the available enzymes and protocols it is possible to isolate protoplasts from virtually any tissue of in vivo or in vitro growing plant materials. The progress during this period is particularly outstanding with regard to the number of species for which plant regeneration has been achieved from isolated protoplasts. This has been possible due to selection of right source tissue and plant genotype. A noteworthy achievement in this area has been isolation of viable protoplasts from egg and sperm cells of maize and their manipulation (see Section 10.4).

APPENDIX 12.I: PROTOCOLS FOR ISOLATION AND CULTURE OF PROTOPLASTS OF FOUR SPECIES

12.I.1. *Nicotiana tabacum*

(a) Select fully expanded leaves from 7–8-week-old plants growing in a glasshouse.
(b) Surface-sterilize the leaves by first immersing in 70% ethanol for 30 s followed by rinsing in 0.4–0.5% sodium hypochlorite solution for about 30 min.
(c) Wash the leaves thoroughly with sterile distilled water to remove every trace of hypochlorite.
(d) Peel the lower epidermis with fine forceps and cut out the peeled areas with a fine scalpel.
(e) Place the peeled leaf pieces on a thin layer of 600 mmol l^{-1} mannitol-CPW solution[3] in such a way that the peeled surface is in contact with the solution.
(f) After about 30 min replace the mannitol/CPW solution by filter-sterilized enzyme solution containing 4% cellulase SS, 0.4% macerozyme SS, 600 mmol l^{-1} mannitol and CPW salts.
(g) Seal the petri plate with parafilm and incubate it in the dark at 24–26°C for 16–18 h.
(h) Gently squeeze the leaf pieces with a Pasteur pipette to liberate the protoplasts.

[3] Cell-protoplast washing medium (CPW) contains (mg l^{-1}); KH_2PO_4 (27.2), KNO_3 (101), $CaCl_2 \cdot 2H_2O$ (1480), $MgSO_4 \cdot 7H_2O$ (246), KI (0.16), $CuSO_4 \cdot 5H_2O$ (0.025), pH 5.8 (Cocking and Peberdy, 1974).

(i) Remove the large debris by filtering through a 60–80 μm nylon mesh.
(j) Transfer the filtrate to a screw-cap centrifuge tube and spin at 100 × *g* for 3 min.
(k) Remove the supernatant and transfer the sediment, on the top of 860 mmol l^{-1} sucrose solution (prepared in CPW) in a screw-cap centrifuge tube and spin it at 100 × *g* for 10 min.
(l) Collect the green protoplast band from the top of the sucrose pad and transfer it to another centrifuge tube.
(m) Add the protoplast culture medium (e.g. NT medium; for composition see Table 3.1) to suspend the protoplasts and centrifuge at 100 × *g* for 3 min. Repeat such washings at least three times.
(n) After the final washing add enough culture medium to achieve a protoplast density of 0.5×10^5 to 1×10^5 ml^{-1}.
(o) Plate the protoplasts as small (100–150 μl) droplets or a thin layer in petri plates.

12.I.2. *Arabidopsis thaliana* genotype C24 (after Damm and Willmitzer 1988, 1991)

(a) Take 1–2 g of leaf material from 3 to 4-week-old aseptically growing plants and place them in a 94 × 16 mm petri plate so that the lower side of the leaves is towards the bottom of the plate, and wet them with 4–7 ml of 0.5 M mannitol solution, in dark, at 25°C.
(b) Cut the leaves with a razor blade so that the leaf is cut once.
(c) Transfer the leaf material into two plates, each containing 10 ml of 0.5 M mannitol and plasmolyse them for 1–2 h in the dark at room temperature.
(d) Remove the mannitol solution and replace it with 12 ml of enzyme solution containing 1% cellulase 'Onozuka' R-10, 0.25% Macerozyme R-10, 8 mmol l^{-1} $CaCl_2$, 0.4 M mannitol (pH 5.5). Incubate the plates in dark at 25°C.
(e) After 16–20 h agitate the mixture and wait for another 30 min to complete the digestion.
(f) Separate the protoplasts from the undigested tissue by consecutive filtration of the mixture through a 125 μm stainless steel sieve on top of a 63 μm sieve. Wash the petri plate with 6 ml of 0.2 M $CaCl_2$ in order to recover the remaining protoplasts and add this solution to the filtrate through the sieve.
(g) Distribute the filtrate into four 12 ml centrifuge tubes and spin for 5 min at 60 × *g* in a swinging bucket rotor.

(h) Carefully remove the supernatant with a pipette and resuspend the pellet in a solution containing 3 ml of 0.5 M mannitol and 6 ml of 0.2 M $CaCl_2$ and centrifuge for 5 min at 40 × *g*.
(i) Repeat the washing of protoplasts as in step (h).
(j) Suspend the protoplasts of each centrifuge tube in a solution composed of 2 vols. of 0.5 M mannitol and 1 vol. of 0.2 M $CaCl_2$ and recentrifuge at 40 × *g*.
(k) Finally, suspend the protoplasts in 0.5 M mannitol at a density of 4–6 × 10^5 ml^{-1} and put on ice in dark for at least 30 min to stabilize the protoplasts.
(l) Embed the protoplasts in sodium alginate as follows (all solutions used are to be cooled in ice): mix equal volumes of 0.4 M mannitol solution containing 2.8% sodium alginate and the protoplast suspension. Add 1 ml of this mixture dropwise to 3 ml of solution 1 (50 mmol l^{-1} $CaCl_2$ in 0.4 M mannitol) in small (60–15 mm) petri dishes. Due to the presence of $CaCl_2$ Ca-alginate beads will be formed. After 1–2 h at room temperature replace the solution 1 by 3 ml of solution 2 (10 mM $CaCl_2$ in 0.4 M mannitol).
(m) After keeping the embedded protoplasts for 1–2 days at 4°C in the dark replace solution 2 by 3 ml of B_5 medium containing 0.4 M glucose, 1 mg l^{-1} 2,4-D and 0.15 or 0.5 mg l^{-1} BAP and incubate the plates at 26°C in the dark for 3 weeks. Renew the medium every 10 days.
(n) After 3 weeks add fresh medium and transfer the plants to light (700 lx) at 26°C. Renew medium every 2 weeks.
(o) After 5–7 weeks free the protoplast derived colonies by incubating the beads in a solution containing 0.3 M mannitol and 20 mM sodium citrate.
(p) Transfer the larger colonies to MS medium containing 2% sucrose, 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, and incubate in the light (16/8 h photoperiod) at 25°C, for shoot differentiation.
(q) After 2–5 weeks transfer the shoots to shoot elongation medium (MS + 0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 0.1 mg l^{-1} GA_3) and incubate at 21°C under photoperiod as in step (p).
(r) After another 8–10 weeks, root the shoots on a medium containing MS salts and vitamins at half strength, 1% sucrose and 1 mg l^{-1} IBA.

12.I.3. *Brassica napus* cv *isuzu* (after Chuong et al., 1985)

(a) Raise aseptic seedlings on 0.8% agar supplemented with 0.2% sucrose, in the dark, at 25°C.

(b) Excise 150–200 hypocotyl hooks (2–3 cm long) from 2-day-old seedlings and plasmolyse them for 1 h in CPW solution (for composition see Appendix 12.I.1) supplemented with (in mg l^{-1}) ampicillin (400), gentamycin (10), tetracyclin (10) and 13% mannitol ('CPW 13 M'), with pH set at 5.7.

(c) Transfer the hypocotyl pieces to the enzyme solution containing 2% Rhozyme HP-150 (Genecor, N.Y., USA), 4% meicelase (Meiji Seika Co., Tokyo, Japan) and 0.3% macerozyme R-10 (Yakult Pharmaceutical Co., Nishinomya, Japan) in CPW 13 M and incubate at 25°C in the dark on a shaker at 60 rev. min^{-1}.

(d) After 12 h of enzyme treatment gently agitate the mixture by taking up into and expelling from a pipette several times to enhance the release of the protoplasts.

(e) Filter the enzyme mixture through two layers a nylon mesh (60 μm pore size on top of 44 μm).

(f) Transfer the filtrate to centrifuge tubes and spin at 100 × g for 3 min.

(g) Suspend the pellet in CPW 13 M and spin again. Repeat this washing process three times.

(i) Finally, suspend the protoplast pellet, at a density of 2×10^5 protoplasts ml l^{-1}, in a modified Lichters medium[4], supplemented with 13% w/v sucrose, 5 g l^{-1} Ficoll 400 (Pharmacia Fine Chemicals, Sweden), 0.5 mg l^{-1} BAP, 1 mg l^{-1} NAA and 0.5 mg l^{-1} 2,4-D, with pH set at 5.7 and plate them in petri plates (2.5 ml of protoplast suspension per 15 × 60 mm petri plate. Incubate the plates at 25°C in dark.

(j) After 4–6 weeks transfer the floating microcalli to MS medium containing 200 mg l^{-1} casein hydrolysate, 5 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.6% agarose, with pH set at 5.7.

(k) After 3–4 weeks shoot buds differentiate.

12.I.4. *Oryza sativa* (*Indica* cvs *Nipponbare* and *Iwaimochi*) (after Kyozuka et al., 1987)

(a) Surface sterilize the mature seeds and plant them on MS medium supplemented with 2 mg l^{-1} 2,4-D.

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[4] Composition of Lichter's medium (mg l^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (500), KNO_3 (125), $MgSO_4 \cdot 7H_2O$ (125), KH_2PO_4 (125), $MnSO_4 \cdot 4H_2O$ (25), H_3BO_3 (10), $ZnSO_4 \cdot 4H_2O$ (10), $Na_2MoO_4 \cdot 2H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.025), $CoCl_2 \cdot 6H_2O$ (0.025), EDTA (0.037), $FeSO_4 \cdot 7H_2O$ (0.028), glycine (2), myo-inositol (100), nicotinic acid (5), pyridoxin·HCl (0.5), thiamin·HCl (0.5), folic acid (0.5), biotin (0.05), glutathione (30), L-glutamine (800) and L-serine (100).

(b) After 3–4 weeks transfer the scutellar callus to 125 ml flask containing 25 ml of liquid medium containing inorganic salts of R-2 medium[5], 5.6 mg l^{-1} $FeSO_4$, 7.5 mg l^{-1} Na_2EDTA, vitamins of MS, 1 mg l^{-1} 2,4-D and 3% sucrose, and place the flask on a shaker at low speed (50 rev. min^{-1}). Suspension can be maintained by weekly subcultures.

(c) Filter the cell suspension through a sieve of 500 μm pore size into a pre-weighed 10 cm plate. Remove the medium with a sterile pipette, leaving the cells in the plate and add (10 ml^{-1} g^{-1} fresh weight of cells), the enzyme mixture consisting of 4% cellulase RS (Kinki Yakult, Japan), 1% macerozyme R-10 (Kinki Yakult, Japan) and 0.4 M mannitol. Seal the plate with parafilm or nescofilm and incubate it in the dark at 30°C without shaking.

(d) After 3–4 h filter the enzyme mixture through a 20 μm nylon mesh and add four times the volume of KMC solution, consisting of equal volumes of 0.35 M KCl, 0.245 M $MgCl_2$ and 0.254 M $CaCl_2$, and pH set at 6.

(e) Centrifuge for 10 min at 800 rev. min^{-1}.

(f) Wash the pelleted protoplasts twice in KMC solution by centrifugation.

(g) Mix 1 ml of the protoplast suspension (1×10^6 protoplasts ml^{-1}) in culture medium (basal medium used for suspension cultures containing 2 mg l^{-1} 2,4-D and 0.4 M sucrose (pH 5.0) with an equal volume of the molten agarose medium (2.5% Sea Plaque agarose) in 6 cm petri plate.

(h) Cut the solidified agarose, containing the protoplasts, into 8 × 8 mm blocks and transfer them to 6 cm plate containing 5 ml of protoplast culture medium.

(i) Add rice Oc nurse cells (100 mg/plate) to the liquid part of the culture (mixed nurse method).

(j) After 10 days transfer the agarose blocks to new plates with nurse cells-free medium. Completely remove the nurse cells from agarose blocks by washing with the culture medium.

(k) After 4 weeks transfer the agarose blocks containing visible colonies to soft agarose medium containing N_6 basal medium (for composition see Table 7.2) supplemented with 2 mg l^{-1} 2,4-D, 6% sucrose and 0.25% agarose, and culture under light (3000 lx).

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[5] Composition of R-2 medium (mg l^{-1}): $NaH_2PO_4.2H_2O$ (307.78), KNO_3 (4040), $(NH_4)_2SO_4$ (330), $MgSO_4.7H_2O$ (256.32), $CaCl_2$ (110.9), Fe-EDTA (2.5), $MnSO_4.H_2O$ (0.5), $ZnSO_4.7H_2O$ (0.5), H_3BO_3 (0.5), $CuSO_4$ (0.05), $Na_2MoO_4.2H_2O$ (0.05), $CoCl_2.6H_2O$ (0.05), thiamine-HCl (1), sucrose (2%), 2,4-D (2), pH 6.

(l) After 2–3 weeks transfer individual colonies (Ca 1 mm in diameter) to the medium as in step (k) but with higher agarose concentration (0.5%).

(m) When the colonies attain a size of about 2 mm transfer them to the N_6 basal medium containing 6% sucrose, 1% agarose and 2 mg l^{-1} kinetin or 5 mg l^{-1} BAP.

(n) After 3–8 weeks transfer the regenerated shoots to hormone-free N_6 medium for rooting.

Chapter 13

Somatic Hybridization and Cybridization

13.1. INTRODUCTION

Plant protoplasts represent the finest single cell system and offer exciting possibilities in the fields of somatic cell genetics and crop improvement. In culture, isolated protoplasts often perform better than single, whole cells (Nagata and Takebe, 1971; Kao and Michayluk, 1975) and should, therefore, serve as an excellent starting material for cell cloning and development of mutant lines. They also provide experimental material for many other fundamental and applied studies. Freshly isolated protoplasts have been employed in studies related to cell wall synthesis, membrane properties and virus infection. However, the feature of isolated protoplasts that has brought them into the limelight is the ability of these naked cells to fuse with each other irrespective of their origin.

Protoplast fusion has opened up a novel approach to raising new hybrids. This technique of hybrid production through the fusion of body cells, bypassing sex altogether, is called somatic hybridization.

Unlike sexual reproduction in which organelle genomes are generally contributed by the maternal parent, somatic hybridization also combines cytoplasmic organelles from both the parents. In somatic hybrids recombination of mitochondrial genome occurs frequently. Chloroplast genome recombination is rare but segregation of chloroplasts of the two sources in hybrids causes selective elimination of chloroplasts of one or the other parent, forming novel nuclear-cytoplasmic combinations. Fusion products with the nucleus of one parent and extra-nuclear genome/s of the other parent are referred to as cybrid and the process to obtain cells or plants with such genetic combination/s is called cybridization. Somatic cell fusion, thus, offers new ground to achieve novel genetic changes in plants.

This chapter deals with the techniques of somatic hybridization and cybridization which involve a series of interdependent steps, shown in Fig. 13.1. Isolation and culture of protoplasts have been dealt with in Chapter 12. Regeneration of plants from hybrid cells does not warrant further discussion after having dealt with plant regeneration from unfused protoplasts in Section 12.3.3. Other aspects of the technique are discussed in the following pages. Application of the protoplast system in genetic transformation is dealt with in Chapter 14.

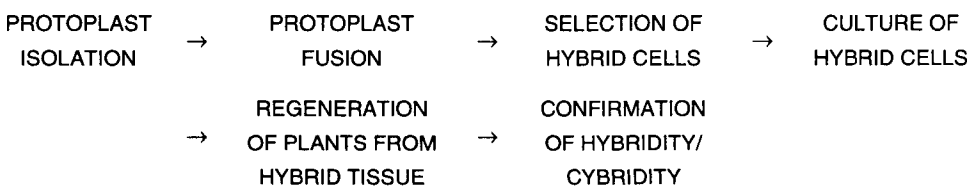

Fig. 13.1. Steps involved in somatic hybridization/cybridization.

13.2. PROTOPLAST FUSION

During enzymatic degradation of cell walls some of the adjacent protoplasts fuse together forming homokaryons (also referred to as homokaryocytes, each with two to several nuclei (Miller et al., 1971; Motoyoshi, 1971; Woodcock, 1973). This type of protoplast fusion, called 'spontaneous fusion', has been ascribed to the expansion and subsequent coalescence of the plasmodesmatal connections between the cells (Withers and Cocking, 1972). The occurrence of multinucleate fusion bodies is more frequent when protoplasts are prepared from actively dividing cultured cells. About 50% of the protoplasts prepared from callus cells of maize endosperm (Motoyoshi, 1971) and suspension cultures of maize embryos (Brar et al., 1979) were multinucleate. A sequential method of protoplast isolation, or exposing the cells to strong plasmolyticum solution before treating them with mixed enzyme solution would sever the plasmodesmatal connection and, consequently, reduce the frequency of spontaneous fusion.

So far as somatic hybridization and cybridization are concerned spontaneous fusion is of no value; these require the fusion of protoplasts of different origin. To achieve induced fusion a suitable chemical agent (fusogen) or electric stimulus is generally necessary. Since 1970 a variety of fusogens have been tried to fuse plant protoplasts of which $NaNO_3$, high pH and high Ca^{2+}, and polyethylene glycol treatments have been successfully used to produce somatic hybrid/cybrid plants. During the last decade fusion of protoplasts by electric stimulus (electrofusion) has gained increasing popularity.

13.2.1. Chemical fusion

(i) *$NaNO_3$ treatment.* As early as 1909, Kuster demonstrated that a hypotonic solution of $NaNO_3$ induces the fusion of sub-protoplasts within a plasmolysed epidermal cell. However, a reproducible, and controlled fusion of isolated protoplasts by $NaNO_3$ was reported by Power et al.

(1970). The technique is fully described by Evans and Cocking (1975). Although this fusogen was used by Carlson et al. (1972) to produce the first somatic hybrid in plants, the technique suffers from a low frequency of heterokaryon formation, especially when highly vacuolated mesophyll protoplasts are involved (Power and Cocking, 1971; Keller and Melchers, 1973; Burgess and Fleming, 1974; Melchers and Labib, 1974). This led to the search for more efficient fusion techniques.

(ii) *High pH and high Ca^{2+} treatment.* In 1973 Keller and Melchers reported that mesophyll protoplasts of two lines of tobacco could be readily fused by treating them in a highly alkaline (pH 10.5) solution of high Ca^{2+} ions (50 mM $CaCl_2 \cdot 2H_2O$) at 37°C for about 30 min. Using this technique Melchers and Labib (1974) and Melchers (1977) produced intraspecific and interspecific somatic hybrids, respectively, in the genus *Nicotiana*. For somatic hybridization in petunias this method of protoplast fusion was regarded as superior to the other two common chemical methods in terms of the throughput of hybrids (Power et al., 1980). However, for some protoplast systems such a high pH may be toxic (Kao and Wetter, 1977). For practical details of the technique, see Appendix 13.I.1.

(iii) *Polyethylene glycol (PEG) treatment.* Since 1974 (Kao and Michayluk, 1974; Wallin et al., 1974) PEG has achieved widespread acceptance as a fusogen of plant protoplasts because of the reproducible high frequency heterokaryon formation and comparatively low cytotoxicity to most cell types. Another merit of PEG-induced fusion, over the other two methods of chemical fusion of protoplasts, is the formation of a high proportion of binucleate heterokaryons (Wallin et al., 1974; Kao, 1977). Burgess and Fleming (1974) reported that treatment with a highly alkaline solution containing Ca^{2+} ions, at 37°C, produced large clumps comprising many protoplasts whereas with PEG the aggregation occurred mostly between two to three protoplasts. PEG-induced fusion is non-specific. In addition to fusing soybean-maize and soybean-barley (Kao et al., 1974), PEG brings about effective fusion between animal cells (Ahkong et al., 1975a; Pontecorvo, 1975), animal cells with yeast protoplasts (Ahkong et al., 1975b), and animal cells with higher plant protoplasts (Dudits et al., 1976b).

The steps involved in fusing protoplasts by PEG are given in Appendix 13.I.2. Briefly, the freshly isolated protoplasts from the two selected parents are mixed in appropriate proportions and treated with 15–45% PEG (1500–6000 MW) solution for 15–30 min followed by gradual washing of the protoplasts with the culture medium. Kao et al. (1974) observed that eluting PEG with a highly alkaline solution (pH 9–10) containing a high

Ca^{2+} ion concentration (50 mM $CaCl_2 \cdot 2H_2O$) led to a higher frequency of fusion than washing with the culture medium (see also Kao and Wetter, 1977). This method, which is essentially a combination of the original PEG method described by Kao and Michayluk (1974) and the high pH high Ca^{2+} ions method of Keller and Melchers (1973), is currently the most widely used method for plant protoplast fusion.

Several factors affect protoplast fusion by PEG:

(a) PEG of molecular weight (MW) higher than 1000 induces tight adhesion and high frequency fusion of protoplasts. Generally, PEG of MW 1500–6000 has been used at concentrations ranging from 15 to 45%.

(b) PEG-induced fusion is enhanced by enriching the PEG solution with Ca^{2+} ions.

(c) The dilution of PEG should be gradual. Drastic elution would result in the formation of very few heterokaryons.

(d) Prolonged incubation in PEG solution reduces heterokaryon formation.

(e) Protoplasts from young leaves and fast growing calli give better fusion.

(f) Whereas the protoplasts from cultured cells can tolerate enzyme, PEG, and high pH high Ca^{2+} treatments fairly well, those from the mesophyll cells have proved sensitive to these conditions. Preculturing the leaves for a few days improves the tolerance of mesophyll protoplasts to these treatments.

(g) Excessive dilution of the enzyme solution leads to poor fusion, probably because of rapid wall synthesis by the protoplasts.

(h) The types of enzymes and their concentrations used for protoplast isolation is another factor influencing protoplast fusion. Driselase yields highly fusable protoplasts but it may also adversely affect the viability of the protoplasts (Kao, 1978).

(i) Protoplast density also influences the fusion frequency. A 4–5% protoplast suspension (protoplast volume/liquid volume) usually gives the highest frequency of heterokaryon formation.

(j) High temperature (35–37°C) promotes fusion frequency while low temperature (15°C) promotes protoplast adhesion. According to Burgess and Fleming (1974) high temperature is especially promotive for the fusion of highly vacuolated protoplasts. In practice, however, the entire fusion experiment is performed at around 24°C.

(k) Repeated centrifugation after the fusion treatments, as is necessary when the fusion experiment is performed in centrifuge tubes, adversely affects the yield and viability of fused protoplasts.

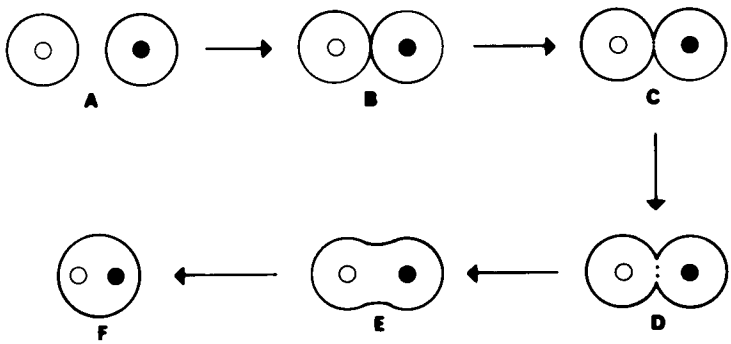

Fig. 13.2. Diagrams showing the sequential stages in protoplast fusion. (A) Two separate protoplasts. (B) Agglutination of two protoplasts. (C,D) Membrane fusion at localized sites. (E,F) Formation of a spherical heterokaryon.

Therefore, the technique of fusing the protoplasts on a coverslip, in 150 μl droplets, as described by Kao and Michayluk (1974), is preferred (for details see Appendix 13.I.2).

(iv) *Mechanism of fusion.* Protoplast fusion consists of three main phases: (a) agglutination, during which the plasma membrane of two or more protoplasts are brought into close proximity (see Fig. 13.2A,B), (b) membrane fusion at small localized regions of close adhesion resulting in the formation of cytoplasmic continuities or bridges between protoplasts (see Fig. 13.2C,D) and, (c) rounding-off of the fused protoplast due to the expansion of the cytoplasmic bridges forming spherical hetero- or homokaryons (see Fig. 13.2E,F).

Protoplast adhesion, which is temperature independent, can be induced by a variety of treatments but this does not necessarily lead to membrane fusion. Plant protoplasts carry a negative surface charge (Grout et al., 1972; Nagata and Melchers, 1978). Depending on the species this charge may vary from –10 to –30 mV. Due to the common charge the plasma membranes of agglutinated protoplasts do not come close enough to fuse. Membrane fusion requires that the membranes must be first brought into apposition at molecular distances of 10 Å or less (Cocking, 1976). The high pH-high Ca^{2+} ions treatment has been shown to neutralize the normal surface charges thus allowing the membranes of agglutinated protoplasts to come in intimate contact (Melchers, 1977). Ten millimolar $CaCl_2 \cdot 2H_2O$ completely removes the charge from tobacco protoplasts. High temperature, which promotes membrane fusion in plants as well as in animals, has been shown to cause perturbance of the lipid molecules in the plasma membranes, and the fusion occurs due to

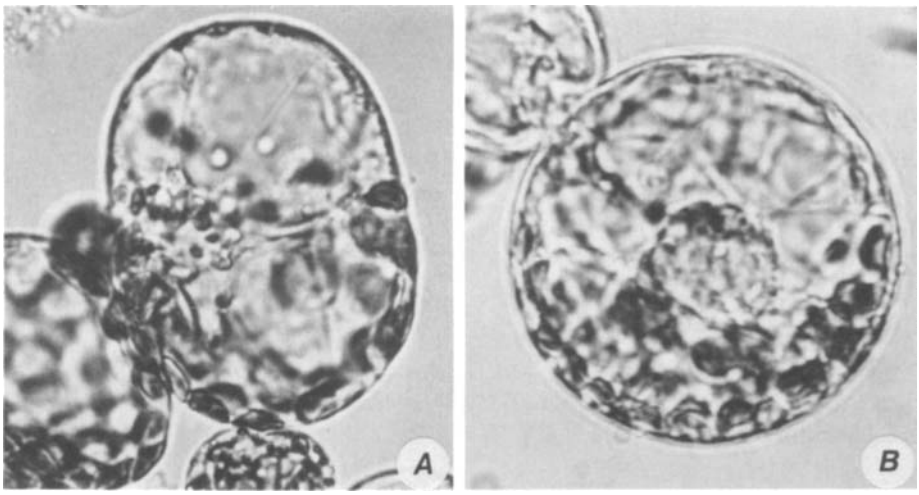

Fig. 13.3. Two stages in the fusion of a non-chlorophyllous protoplast from a suspension culture of *Petunia hybrida* with a green mesophyll protoplast of *P. parodii*. The cytoplasms are becoming progressively mixed together and a heterokaryon has been formed (B) (courtesy of J.B. Power, UK).

the interaction and intermingling of the lipid molecules in the intimately adhering plasma membrane (Ahkong et al., 1975a).

The protoplasts treated with PEG instantaneously agglutinate to form clumps of two or more protoplasts. Tight adhesion of the plasma membranes may occur over a large surface area (Wallin et al., 1974) or it may be restricted to small localized sites in the region of agglutination (Fowke et al., 1975) or both (Burgess and Fleming, 1974). Localized fusion of closely apposed plasma membranes occurs in the regions of tight adhesion, and small cytoplasmic channels are established. These channels gradually expand and the fusing protoplasts, passing through the dumbell-shaped stage (Fig. 13.3A), becomes spherical (see Fig. 13.3B). As the PEG is eluted the fusion bodies are deplasmolyzed and active streaming of the cytoplasm is re-established. This facilitates the rounding of the fusion bodies and mixing up of the cytoplasm which is completed in 3–10 h (Fowke et al., 1975; Gosch and Reinert, 1978).

The actual mechanism of PEG-induced fusion is not clear. Kao and Wetter (1977) have suggested that the PEG molecule, which is slightly negative in polarity, can form hydrogen bonds with water, protein, carbohydrate, etc., which possess positively polarized groups. When the PEG molecule chain is large enough it acts as a molecular bridge between the surface of adjacent protoplasts and adhesion occurs. PEG can bind Ca^{2+}

as well as other cations. The Ca^{2+} may form a bridge between the negatively polarized groups of protein (or phospholipids) and PEG, thus, enhancing adhesion. During the washing process the PEG molecules bound to the membrane, either directly or through Ca^{2+}, are eluted, resulting in disturbance and redistribution of the electric charge. Such a redistribution of charge in the regions of intimate contact of the membranes can link some of the positively charged groups of one protoplast to the negatively charged groups of the other protoplast and vice versa, resulting in protoplast fusion.

In animal cells PEG is reported to cause alterations in membrane structure, such as aggregation of intramembranous protein/glycoprotein particles. As a result, protein-free lipid bilayer regions appear in the plasma membrane. It has been suggested that membrane fusion during PEG treatment occurs in these regions (Ahkong et al., 1975a).

13.2.2. Electrofusion

Chemical fusion of plant protoplasts has many disadvantages: (1) The fusogens are toxic to some cell systems. Benbadis and de Virville (1982) observed destruction of mitochondria following PEG treatment at fusogenic level. (2) It produces random, multiple cell aggregates. (3) The fusogen must be removed before culture. In contrast, electrofusion is rapid (usually complete within 15 min), simple, synchronous, and more easily controlled (Walton and Brown, 1988; Jones, 1991). For somatic hybridization of *Solanum tuberosum* and *S. brevidens*, without selection, electrical fusion (12.3% hybrid shoots) was more effective than PEG-induced fusion (2.6% hybrid shoots; Jones et al., 1990). The somatic hybrids produced by electrofusion of protoplasts often show much higher fertility than those produced by PEG-induced fusion (Han San et al., 1990; Hossain et al., 1994; Asao et al., 1994).

Electrofusion of protoplasts was first demonstrated by Senda et al. (1979). Zimmermann and his co-workers (see Zimmermann and Vienken, 1982) developed this method further, and their work led to the production of an automatic 'Zimmermann Electrofusion System', by GCA Corp., Precision Scientific Group, USA, which is claimed to be 10 000 times more effective than any other method for protoplast fusion.

Since then electrofusion of protoplasts has been applied with great success to a range of systems, and a number of different electrofusion systems with fixed or movable electrodes have been tested (Koop et al., 1983; Zhakrisson and Bornman, 1984; Watts and King, 1984; Gaynor, 1986; Buckley et al., 1990; Hidaka et al., 1995). During the last 5 years several hybrids have been produced by electrofusion of protoplasts

(Mattheij et al., 1992; Hossain et al., 1994; Ling and Iwamasa, 1994; Asao et al., 1994; Motomura et al., 1995). Electrically fused egg and sperm protoplasts of maize also regenerated full plants (see Section 10.4).

The fusion systems generally consist of a DC pulse generator and a sine wave generator connected in parallel to a fusion chamber fitted with two electrodes about 200 μm apart. The fusion chamber, mounted on a glass microscope slide, is connected to two syringe pumps, one containing the protoplasts to be fused and the other fresh sterile osmoticum for washing and flushing the chamber. The chamber, connecting tubing and associated valving can be sterilized by first pumping through 70% ethanol for ca. 10 min followed by a large volume of sterile water. Before fusion the sterilized chamber is flushed with a large volume of fusion mixture. The protoplasts, suspended in the fusion medium of low conductivity (e.g. mannitol solution of appropriate osmolarity) are introduced into the chamber and placed between the two electrodes. A non-uniform high frequency (0.5–1.5 MHz) AC field (10–200 V cm^{-1}) is applied across the protoplasts. As the surface charge on the protoplasts becomes polarized they act as dipoles and migrate along the electric field lines to a region of highest field intensity (Fig. 13.4). If the field intensity is high (ca. 200 V cm^{-1}) the protoplasts may migrate to the electrode with higher electric field but with lower field intensity the aggregation occurs between the electrodes. As the protoplasts have been aligned in chain, one or two short (10–20 μs) DC pulses of high voltage (0.125–1 kV cm^{-1}) are applied which causes reversible membrane breakdown (pore formation) in the contact area of the adjacent protoplasts (Fig. 13.5). The AC field is briefly reapplied to maintain close protoplast contact as fusion begins and then reduced to zero. The fusion process (Fig. 13.6) takes about 10 min. Pretreatment of protoplasts with spermine and the presence of 1 mM $CaCl_2$ in the fusion mixture increases the fusion frequency. With both the treatments up to 60% fusion can be achieved (Lindsey and Jones, 1990). To achieve high frequency one-to-one fusion the protoplast density should be low (ca. 1×10^4 protoplasts ml^{-1}). Fusion in pairs of two protoplasts is also enhanced if the divergence of the electric field and the field strength are not too large (Zimmermann and Greyson, 1983).

In the microdroplet method of electrofusion (Fig. 13.7), developed by Koop et al. (1983), the desired pair of protoplasts is transferred, as described in the microdroplet method of protoplast culture (Section 12.3.2), to a droplet containing low ionic strength fusion medium. In one experiment several fusions are performed using several microdroplets on one coverglass overlaid with a common layer of mineral oil. Fusion is performed by introducing into the droplet a pair of platinum wire electrodes (0.5 μm diameter, 10 mm long). The distance between the electrodes,

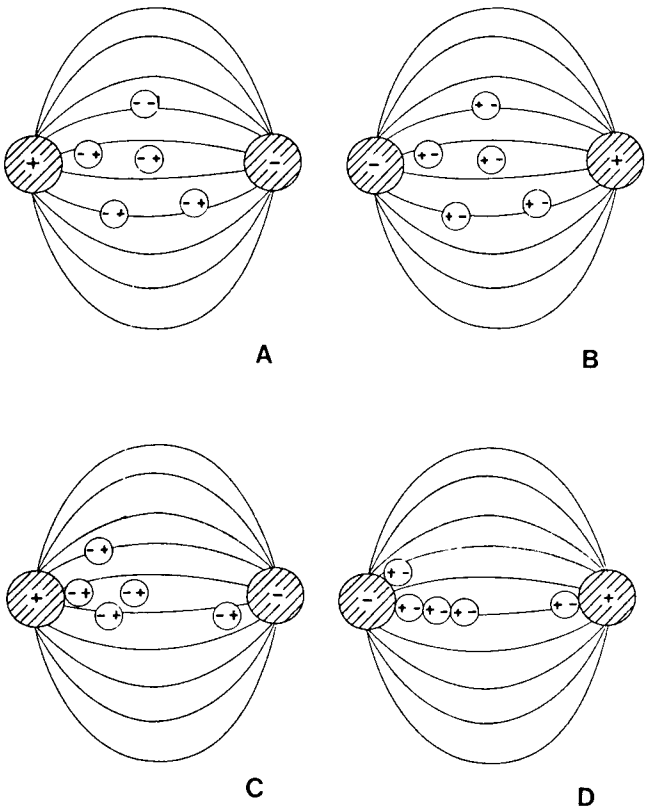

Fig. 13.4. Dielectropole collection of protoplasts (blank circles) in a non-homogeneous AC field. (A,B) The electric field-induced transient dipole in the protoplast membrane (represented by + and −). (C,D) Due to non-homogeneous electric field, the protoplasts move in the direction of higher field strength and attach to the nearest surface of the electrode (hatched circles). Since the bathing medium is of relatively low conductivity (in comparison to the cells) the protoplasts attached to the electrode surface act as local high field-strength region and attract other protoplasts. This leads to the formation of pearl-chain of protoplasts (after J.J. Gaynor, 1986, Handbook of Plant Cell Culture, Vol. 4, with permission of McGraw-Hill Co.).

mounted under the condenser, can be adjusted. After alignment of the protoplasts on one of the electrodes by AC current (1 MHz, 66 V cm^{-1}) fusion is induced by a single negative DC pulse (0.9 kV cm^{-1} for 50 μs). By this method, 50 one-to-one fusions could be performed in 1 h. Full plants have been regenerated after electrofusion of defined pairs of leaf protoplasts (Koop and Schweiger, 1985b).

Electrofusion is more suitable for the fusion of mesophyll protoplasts than root or callus protoplasts (Pelletier, 1993). The presence of large vacuole or amyloplasts is detrimental for the protoplasts during the fu-

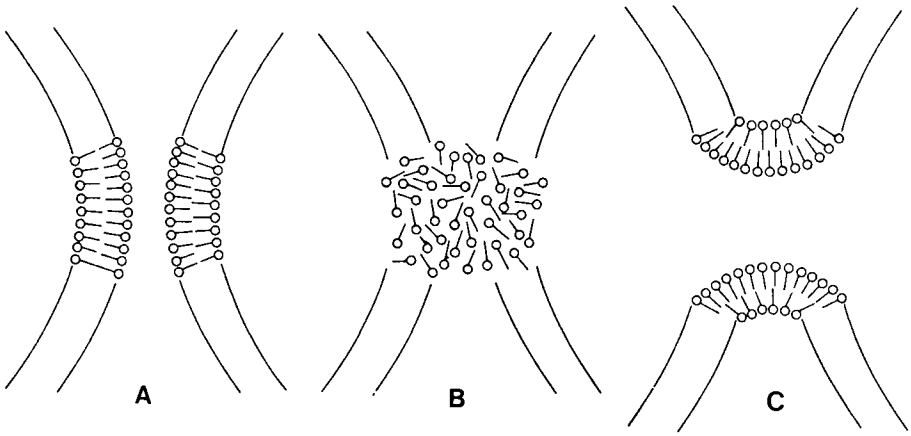

Fig. 13.5. Model of the molecular events occurring during electrofusion. (A) Membranes of neighbouring protoplasts are brought together and held in intimate contact by the process of dielectrophoresis. (B) Application of a single, high strength DC pulse leads to a breakdown of the plasma membrane at the poles of the cells. (C) Reannealing of membranes following fusion pulse. If the two neighbouring protoplasts were in close proximity at the time of local membrane disruption, then lipids reassemble into a single bilayer, fusing the protoplasts ((after J.J. Gaynor, 1986, Handbook of Plant Cell Culture, Vol. 4, with permission of McGraw-Hill Co.).

sion process. Despite the several advantages of the electrofusion method over PEG-induced fusion, the latter continues to be more popular probably because of high technical accuracy and, to some extent, high initial investment associated with the former.

13.3. SELECTION OF FUSION PRODUCTS

In somatic hybridization by electrofusion of protoplasts it may not be difficult to follow the fate of the fusion products because the fusion frequency is very high and sometimes it is possible to achieve one-to-one fusion of desired pairs of protoplasts. However, a chemical fusion treatment results in a heterogeneous mixture of the parental type protoplasts, heterokaryons and a variety of other nuclear-cytoplasmic combinations. The heterokaryons which are the potential source of future hybrids constitute a very small (0.5–10%) proportion of the mixture. Only a fraction of these heterokaryons show nuclear fusion (Pelletier, 1993). Moreover, being novel genetic combinations, several things may happen following fusion treatment which further reduce the number of potential hybrid cell lines to a very low level. It is, therefore, of key importance in somatic hybridization to be able to select the hybrid cells or their products. Numerous different ways of selecting hybrids have been proposed and practised, including morphological basis, complementation of biochemical and

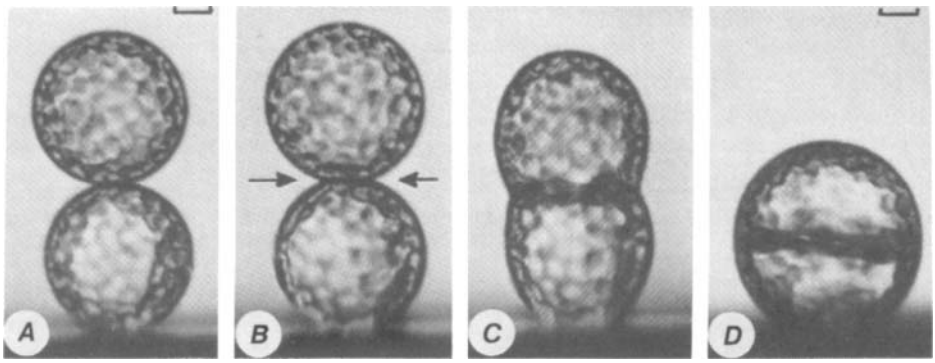

Fig. 13.6. Stages in the electrofusion of *Avena sativa* protoplasts. (A) Point-to-point contact between adjacent protoplasts suspended in 0.5 M mannitol solution and exposed to electric field (frequency 500 kHz and strength 200 V cm^{-1}). (B) Flattening of the protoplasts in the area of membrane contact. (C,D) Fusion of the protoplasts 10 and 30 s after a 15 μs high voltage (600 V cm^{-1}) DC pulse was applied. Bar = 10 μM (reprinted by permission from: U. Zimmermann and J. Vienken, 1982, J. Membr. Biol., 67: 165–182; © Springer-Verlag).

genetic traits of the fusing partners, and manual or electronic sorting of heterokaryons/hybrid cells. The last method is by far the most reliable and of wide application for somatic hybrid production.

13.3.1. Morpho-physiological basis

Some workers have cultured the whole mixture of protoplasts after fusion treatment and screened the calli or regenerated plants for their hybrid characteristics. However, it is a labour intensive method and may require considerable glasshouse space. Occasionally, the hybrid calli may exhibit heterosis and outgrow the parental cell colonies. Selection of putative hybrids based on callus morphology has been used in intra- (Deimling et al., 1988) and interspecific (Guri and Sink, 1988; Mattheij et al., 1992) somatic hybridization in the genus *Solanum*. In the cross *S. tuberosum* + *S. ceraceifolium* one of the parents produces bright green callus (*S. tuberosum*) and the other forms brown-yellow callus with purple coloured cells (*S. ciraceifolium*). The putative hybrid calli were identified by their intermediate morphology, i.e. green with purple coloured cells (Mattheij et al., 1992).

Nakano and Mii (1993a) obtained interspecific somatic hybrids between *Dianthus chinensis* and *D. barbatus* without an artificial selection system. PEG-fused protoplasts were cultured in the absence of a selection pressure and 30 calli exhibiting vigorous growth were selected. Two of the calli differentiated shoots, one more profusely than the other. The

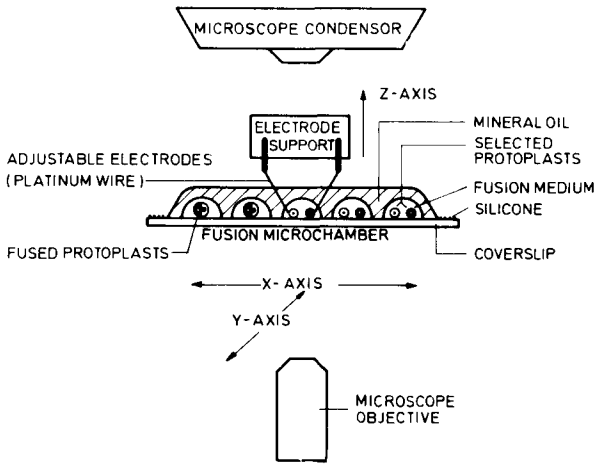

Fig. 13.7. Set-up for the microdroplet method of electrofusion of individually selected protoplasts (reprinted by permission from: H.G. Schweiger et al., 1987, Theor. Appl. Genet., 73: 769–783; © Springer-Verlag)

shoots were grown to the flowering stage in vitro. The flower colour, chromosome number and esterase isozyme pattern confirmed the hybrid nature of the plants. Similar hybrid vigour for callus growth was reported in an intraspecific combination involving *S. tuberosum* (Austin et al., 1985; Waara et al., 1989), interspecific combinations in *Datura* (Schieder, 1978), and *Brassica* (Taguchi and Kameya, 1986) as well as the intergeneric combination *Lycopersicon esculentum* + *Solanum muricatum* (Sakamoto and Taguchi, 1991).

In the interspecific cross *Dianthus chinensis* + *D. barbatus*, mentioned above, the two parents differed in their morphogenic potential which could have acted as a selection force at the final stage of plant regeneration. Whereas the protoplast-derived callus of *D. chinensis* showed 30% regeneration the other parent proved non-regenerable under the prevailing culture conditions. Plant regeneration in many cases has proved to be a dominant trait.

Recently, several reports have demonstrated that somatic hybrids can be efficiently selected by combining the dominant uniparental regeneration potentiality with iodoacetamide induced inactivation of the protoplasts of regenerable parent (Terada et al., 1987; Wright et al., 1987; Takamizo et al., 1991; Nakano and Mii, 1993b; Krasnyanski and Menczel, 1995). In such crosses the protoplasts of the regenerable parent are incapable of forming a callus. Since the calli derived from the other parent lacks regeneration potential, only the hybrid calli differentiate plants. This system seems to be quite promising as it requires neither the

production of mutants or transformants with genetic markers nor special equipment and technique.

The scope of this selection method is limited to certain combinations showing differences in their regeneration potential under specific culture conditions.

13.3.2. Complementation

In this method complementation of genetic or metabolic deficiencies of the two fusion partners are utilized to select the hybrid component. When protoplasts of two parents, each carrying a non-allelic genetic or metabolic defect are fused it reconstitutes a viable hybrid cell of wild type in which both defects are mutually abolished by complementation, and the hybrid cells are able to grow on minimal medium non-permissive to the growth of the parental cells. For such a complementation it is necessary that the defects are recessive and expressed in cultures (Harms, 1983).

Melchers and Labib (1974) demonstrated complementation to wild type green calli and plants after fusion of protoplasts from two chlorophyll deficient varieties of tobacco. Cocking et al. (1977) and Power et al. (1979, 1980) produced three interspecific hybrids of *Petunia* by applying a selection scheme involving complementation of cytoplasmic albino trait of one parent (*P. hybrida, P. inflata, P. parviflora*) and sensitivity to culture medium of the other parent (*P. parodii*). In all these combinations (see Fig. 13.8) when the protoplasts were plated in MS medium after the fusion treatment *parodii* protoplasts were eliminated at the small colony stage. Only the protoplasts of the other parent and the hybrid component developed into full callus. The calli of the hybrid nature could be clearly distinguished from the parental-type tissue by their green colour. The selection procedures used by Schieder (1978a), Dudits et al. (1977) and Krumbiegel and Schieder (1979) also rely partly on the use of nuclear albinos as one of the parents.

Glimelius et al. (1978) obtained tobacco colonies capable of growing on nitrate as the sole source of nitrogen when they fused protoplasts from a *cnx*-type and a *nia*-type mutant cell lines deficient in nitrate reductase activity.

Complementation selection can also be applied to dominant characters, such as dominant resistance to antibiotics, herbicides or amino acid analogues (Harms et al., 1981). Dominant expression of resistance in one parental line is supplemented, on the other hand, with sensitivity to a second drug for which the other fusion partner is resistant. Drug sensitivity behaves as a recessive trait. On a medium containing toxic levels of both drugs the double resistance of hybrid cells enables them to grow but ei-

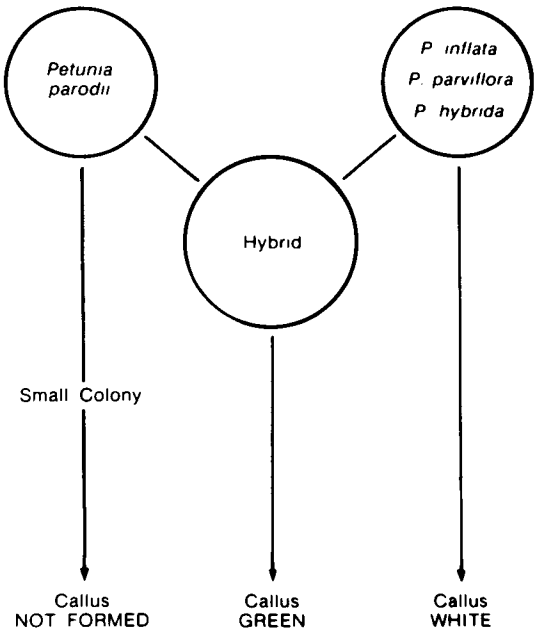

Fig. 13.8. Diagrammatized summary of the selection system used in interspecific somatic hybridization of *Petunia parodii* (wild type) with *P. hybrida*, *P. inflata* and *P. parviflora* (albinos). For details see text (after Cocking et al., 1977; Power et al., 1979, 1980).

ther parent is killed. Marker traits can be introduced into the parents either by mutagenesis or genetic engineering. Resistance to antibiotics (Hamill et al., 1984), amino acid analogues (Harms et al., 1981) and herbicides (Evola et al., 1983) have been employed in some cases using induced or spontaneously occurring mutations. Masson et al. (1989) and Thomas et al. (1990) utilized the transformation of both fusion parents, one with hygromycine and the other with kanamycin resistance genes and selected hybrids by adding both antibiotics to the culture medium.

The combination in the same parent of an auxotrophic mutation (such as nitrate reductase deficiency or albinism) and a dominant trait (such as antibiotic or herbicide resistance) result in so-called universal hybridizer. Hybrids between this and any wild type genotype belonging to the same or another species can be directly selected after fusion by culturing the fusion products in a minimal medium supplemented with the antibiotic or herbicide where both parents are unable to grow. Toriyama et al. (1987b) isolated a double mutant of *Sinapis turgida* which was deficient in nitrate reductase and was resistant to 5-methyltryptophane (5MT).

This line was used to produce intergeneric hybrids with *Brassica oleracea* and *B. nigra* by first culturing the fusion products on a medium containing NO_3^- as the sole source of nitrogen, which eliminated *S. turgida* protoplasts, and later transferred to NO_3^- medium containing 5MT to eliminate the other parent. All the plants finally regenerated were hybrids.

13.3.3. Isolation of heterokaryons or hybrid cells

The most reliable and widely applicable selection system is one which involves isolation of the heterokaryons or hybrid cells and their culture individually or at low density. This approach has gained strength from the success achieved with low density culture of protoplasts using media highly enriched with organic components, conditioned medium, feeder cell-layer technique, and microdrop technique (see Section 12.3.2). This approach not only allows definitive picking up of the hybrid components but also in purer forms. In other selection systems, especially where more than one type of cells is favoured to grow, the neighbouring cell clusters may fuse and make an adulterated tissue mass.

Manual isolation of heterokaryons requires that the two parental type protoplasts have distinct morphological markers and are easily distinguishable. Kao (1977) demonstrated that if green, vacuolated, mesophyll protoplasts of one parent were fused with richly cytoplasmic, non-green protoplasts from cultured cells of another parent the fusion products could be identified for some time in culture. Using this approach, Gleba and Hoffmann (1978, 1979) produced an intergeneric hybrid between *Arabidopsis thaliana* and *Brassica campestris*. Mesophyll protoplasts of *Brassica* were fused with cultured cell protoplasts of *Arabidopsis*. From 3 to 5-day-old cultures, the individual fusion products, mostly with four to eight cells, were mechanically isolated using a micropipette and cultured separately in Cuprak dishes. The inability of the parental protoplasts to grow well in the medium used initially served as the added selection pressure. A similar procedure was followed to obtain somatic hybrids between *Nicotiana tabacum* and *N. knightiana* (Menczel et al., 1981). A major advance in this field has been the development of a dual fluorescence labelling system for heterokaryons. Protoplasts labelled green by treatment with fluorescein diacetate (1–20 mg l^{-1}) are fused with protoplasts emitting a red fluorescence, either from chlorophyll autofluorescence or from exogenously applied rhodamine isothiocyanate (10–20 mg l^{-1}). The labelling can be achieved by adding the compound into the enzyme mixture. This system can be applied even for visual selection of heterokaryons formed by the fusion of morphologically indistinguishable protoplasts, such as mesophyll protoplasts of two parents.

Manual isolation of the heterokaryons by Pasteur pipette or an automatic pipette connected to a gentle suction has the advantage that multiple fusion products can be rejected but it is tedious and time consuming. Therefore, for physical isolation of dual-labelled heterokaryons some workers (Hammatt et al., 1990; Sundberg et al., 1991) have used fluorescence-activated cell sorter (FACS; Galbraith, 1991) which is accurate and exceptionally rapid (about 5×10^3 cells s^{-1}). After fusing the protoplasts labelled with different fluorochromes that fluoresce at different wavelengths, the mixture is fed to the FACS. In the machine the fluid stream carrying the protoplasts is passed between focused laser light and photocells that detect the fluorescence. The stream is dispersed into droplets and the droplets containing cells with single (parental) and double (heterokaryons) fluorescence are electrostatically deflected into different sterile containers.

13.4. VERIFICATION OF HYBRIDITY

Despite the numerous procedures that have been employed to select the desired hybrids/cybrids following protoplast fusion, it is evident from the available literature that no system is foolproof. Most of the selection systems have their characteristic disadvantages which preclude their widespread use. For example, the selection schemes relying on differential responses of the parental species towards the medium or culture regimes used, suffer from cross-feeding between the parental protoplasts when the fusion products are cultured. Therefore, successful passage through a selection system should be treated as first evidence for the hybridity of the selected materials. Further proof must be added from other, independent markers to finally prove or disprove the hybrid nature of the selected putative hybrids. This proof requires a demonstration of genetic contribution from both parents. Some of the commonly employed criteria for this purpose are listed below.

13.4.1. Morphology

Intermediate expression of numerous vegetative and floral characters, such as stalk height and diameter, leaf shape, type of trichomes formed, pigmentation, flower colour and morphology, have been screened for evaluation of presumed somatic hybrids to demonstrate their hybridity. This criterion is, however, not very reliable. Variations induced by tissue culture environment may alter some morphological characters or the hybrid may show entirely new traits not displayed by either of the parents.

13.4.2. Cytological analysis

One of the primary features to characterize somatic hybrids is the chromosome complement. Comparison of number and morphology of chromosomes would reveal if the putative hybrid possesses the expected chromosome complements from the two parents, it is an aneuploid or it involves intergenomic translocations. This is relatively easy when the parental species exhibit prominent differences in chromosome number and morphology. However, this approach will not be applicable to all species, particularly where fusion involves closely related species or where the chromosomes are very small (e.g. *Brassica* species).

A more generalized approach recently introduced to detect relative contributions of each parental genome and restructuring of chromosomes in somatic hybrids is in situ hybridization of 'species-specific' repetitive DNA probes to mitotic chromosomes (Piastuch and Bates, 1990; Itoh et al., 1991).

13.4.3. Isozyme analysis

Isozymes are defined as multiple molecular forms of an enzyme exhibiting similar or identical catalytic properties (Harms, 1983). If the two parents exhibit different band patterns for a specific isozyme the putative hybrid can be easily verified. The banding pattern displaying isozymes from both parents are usually sufficient proof of hybridity. The hybrid may also show isoenzyme bands derived from new combinations of enzymatic subunits. The isozymes commonly used for hybrid identification include acid phosphatase, esterase, peroxidase, phosphoglucoisomerase, phosphoglucomutase and glutamate oxaloacetate transaminase.

Isozyme banding pattern of a particular enzyme may vary considerably depending on the tissue source examined. Some enzymes, i.e. peroxidase isozymes, are particularly variable and do not provide reliable markers unless special care is taken. It is essential to use strictly comparable tissue samples when performing isozyme analysis.

13.4.4. DNA analysis

Recent developments of molecular biological techniques have greatly expanded our analytical tools which can serve to characterize somatic hybrids and cybrids. Demonstration of the presence of DNA from both parents provides the most direct proof of hybridity.

Restriction fragment length polymorphism (RFLP) analysis of nuclear (Williams et al., 1990) and organelle (Pehu, 1991) DNA has been widely

used to verify the hybrid and cybrid nature of the selected fusion products. Lately, southern blot analysis using species-specific repetitive DNA probes (Piastuch and Bates, 1990; Itoh et al., 1991; Fahleson et al., 1994a) or non-radioactive rDNA (ribosomal RNA genes) probes (Honda and Hirai, 1990; Nakano and Mii, 1993ac) have also been used to analyse nuclear genomes of somatic hybrids, and these methods are becoming increasingly popular in confirmation of hybridity of the putative somatic hybrids because of their efficiency and simplicity. These DNA-based methods of verifying hybrid nature are independent of the tissue source and can, therefore, be applied at a relatively early stage in somatic hybridization.

Randomly amplified polymorphic DNA (RAPD) is another recently introduced molecular method to screen hybrids (Xu et al., 1993; Nakano and Mii, 1993ac). Like rDNA analysis (Honda and Hirai, 1990) RAPD requires very small amount of tissue. However, it has been suggested that RAPD should be used only as a quick preliminary screening for putative somatic hybrids, and it should be followed by RFLP, species-specific DNA probes and/or chromosome counting to accurately confirm the nature of the hybrids (Xu et al., 1993).

13.5. GENETIC CONSEQUENCES OF PROTOPLAST FUSION

Fusion of protoplasts at the level of the plasmalemma is non-specific, and there is no barrier to interspecific, intergeneric, interfamily, or even interkingdom fusion of cells. Therefore, a range of wide crosses between sexually incompatible parents have been attempted through cell fusion to incorporate useful genes from wild species into present day cultivars of crop plants. Some examples of somatic hybrids with potential agronomic value are listed in Table 13.1.

Detailed cytological and biochemical analyses of somatic hybrid cell lines and the plants regenerated from them have revealed that hybrid cells can give rise to hybrid plants with full nuclear genomes from both the fusion partners (symmetric hybrids). More often, however, gradual elimination of chromosomes of one of the partners occurs during successive cell cycles, resulting in hybrids with full nuclear genome of one of the parents and only a part of the nuclear genome of the other parent (asymmetric hybrids). The third category of plants obtained from fused protoplasts are those which retain the nuclear genome of only one of the partners but with at least some alien extra-nuclear genes (Cybrids).

The fate of the nuclear genome in the course of somatic hybridization largely depends on three factors: (1) the number and type of parental cells participating in fusion; (2) genomic segregation during the first di-

TABLE 13.1

Examples of somatic hybrids with potential agronomic value

Combination	Resistance/ trait[a]	Reference
Nicotiana tabacum + *N. repanda*	TMV	Bates (1990b)
Solanum tuberosum + *S. ciraceifolium*	*Phytophthora*, *Nematode*	Mattheij et al. (1992)
S. tuberosum + *S. brevidens*	PLRV *Phytophthora* *Erwinia*	Austin et al. (1985), Helgeson et al. (1986), Austin et al. (1988)
S. tuberosum + *S. phureja*	Higher yield	Matheij and Puite (see Puite, 1992)
S. melongena + *S. integrifolium*	*Pseudomonas*	Kameya et al. (1990)
S. melongena + *S. saintwongsei*	*Pseudomonas*	Asao et al. (1994)
Oryza sativa + *O. officinalis*	Blast	Hayashi et al. (1989)
Brassica napus + *B. carinata*	*Phoma*	Sjodin and Glimelius (1989b)
B. napus + *B. juncea*	*Phoma*	Sjodin and Glimelius (1989b)
B. napus + *B. nigra*	*Phoma*	Sjodin and Glimelius (1989a,b)
B. napus + *B. tournefortii*	*Phoma*	Liu et al. (1995)

[a]TMV, tobacco mosaic virus; PLRV, potato leaf roll virus.

vision of the fusion product; and (3) chromosome segregation and/or rearrangement during colony formation and/or plant regeneration. Consequently, in a mass protoplast fusion experiment a wide variety of genetic recombinants may arise with different frequencies (Gleba and Shlumukov, 1990). Table 13.2 illustrates the degree of variation in chromosome number, and chloroplast segregation in somatic hybrids between different species in the tribe Brassiceae.

Somatic hybrid cells also show segregation of cytoplasmic inclusions. A heterokaryon formed after cell fusion contains two or more nuclei in a cytoplasm with plastids and mitochondria of the two parents. As a rule, the plastid population shows random segregation of the parental types in successive cell generations, so that the daughter cells, as early as small colony stage, are left with exclusively one or the other type of plastids (Kung et al., 1975; Chen et al., 1977; Melchers et al., 1978; Aviv et al., 1980; Akada and Hirai, 1986). Interparental recombination of plastid genomes (plastome) after cell fusion occurs only rarely (Medgyesy et al., 1985; Thanh and Medgyesy, 1989). In contrast, mitochondrial genomes (chondriome) very often undergo interparental recombination (Belliard et al., 1978, 1979; Rothenberg et al., 1985; Vedel et al., 1986; Menczel et al., 1987; Akagi et al., 1989; Kyozuka et al., 1989; Yang et al., 1989; Derks et al., 1991), and after segregation one of the many recombined genomes is

TABLE 13.2

Chromosome number, chloroplast segregation and fertility in somatic hybrids produced between different species within the tribe Brassiceae (after Glimelius et al., 1990)

Hybrid combination (no. of hybrid plants)	Chromosome number	% of hybrid plants	% of plants with chloroplasts from parent		Self fertility (% of rape)
A + B			A	B	
B. oleracea +	38	30			17
B. campestris	<38	9			–
(23)	>38	52	54	46	0.2
	Chimeric	9			–
B. napus +	54	67			4.6
B. nigra	<54	23			6.4
(30)	>54	7	88	12	0.4
	Chimeric	3			–
B. napus +	56	44			50
B. oleracea	<56	0			–
(18)	>56	50	68	32	2.0
	Chimeric	6			–
B. napus +	74	88			10
B. juncea	<74	0			–
(8)	>74	12	100	0	10
	Chimeric	0			–
B. napus +	60	8			2
Eruca sativa	<60	59			7
(24)	>60	29	85	15	1
	Chimeric	4			–

retained in a regenerated plant or its progeny (Pelletier, 1991). Independent assortment of chloroplasts and mitochondria results in a very large number of mitochondria-plastid combinations (Fig. 13.9). However, the most frequent cytoplasmic constitution of the cells derived after cell fusion are those where mitochondria with recombinant genomes are associated with one or the other parental plastid genomes (cases 7 and 8 in Fig. 13.9). The observed behaviour of nuclear genomes and cytoplasmic organelles in the course of somatic hybridization generates plants with novel nuclear-chloroplast-mitochondrial combinations.

Since asymmetric hybrids and cybrids are, generally, more valuable than full hybrids between completely unrelated plants, methods have been developed to promote asymmetric hybridization or cybridization.

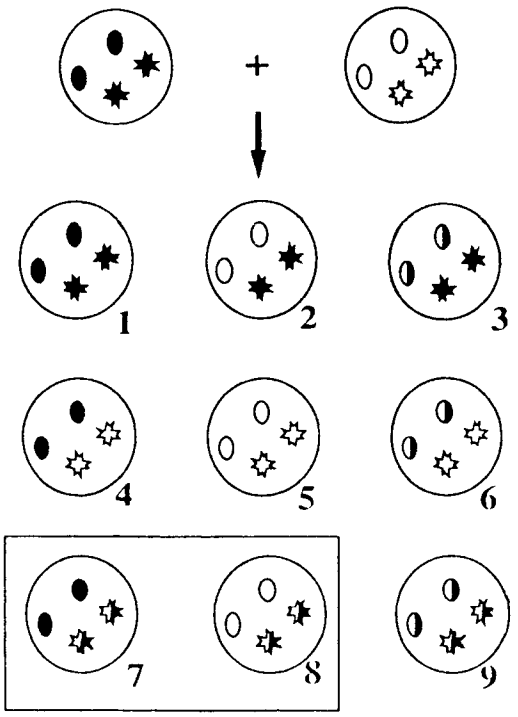

Fig. 13.9. The nine theoretical products of protoplast fusion between parents differing in plastid (○ , ●) and mitochondria (✡ , ✸), considering random elimination of one or the other parental genome and interparental recombination (◑ , ✡). Combinations No. 7 and No. 8 are most frequently observed (after Pelletier, 1993).

13.5.1. Symmetric hybridization

Despite a high incidence of chromosome elimination following fusion of protoplasts of distantly related parents, several interspecific, intergeneric, inter-tribal or even interfamily somatic hybrids have been produced, some of which are also fertile.

In the Brassicaceae several interspecific and intergeneric somatic hybrids have been produced (see Table 13.3). Sundberg and Glimelius (1986) and Sundberg et al. (1987) resynthesized *B. napus* by fusing the protoplasts of *B. oleracea* ($2n = 18$) and *B. campestris* ($2n = 20$). The heterokaryons were isolated 24 h after culture of PEG-treated protoplasts, either manually with a micropipette or by fluorescein activated cell sorter FACS-III (Glimelius et al., 1986) and cultured at low density. About 14% of the isolated fusion products formed calli of which 2% differentiated shoots. All the shoots regenerated from manually isolated heterokaryons were hybrids as against 87% shoots from flow sorted heterokaryons. As

TABLE 13.3

Examples of interspecific and intergeneric somatic hybrids produced during the past decade

Combination	Reference
Interspecific	
Brassica juncea + B. spinescens	Kirti et al. (1991)
B. napus + B. carinata	Sjodin and Glimelius (1988b)
B. napus + B. juncea	Sjodin and Glimelius (1988b)
B. napus + B. nigra	Sjodin and Glimelius (1988a,b)
B. napus + B. oleracea[a]	Jourdan et al. (1989a)
B. oleracea + B. campestris[a]	Sundberg and Glimelius (1986), Sundberg et al. (1987), Yamagishi et al. (1994)
Citrus sinensis + C. limon	Tusa et al. (1990)
C. sinensis + C. paradisi	Ohgawara et al. (1989)
C. sinensis + C. unshiu	Kobayashi et al. (1988)
Dianthus chinensis + D. barbatus	Nakano and Mii (1993a)
D. caryophyllus + D. chinensis	Nakano and Mii (1993b)
Helianthus annuus + H. giganteus[a]	Krasnyanski and Menczel (1995)
Lycopersicon peruvianum + L. pennellii	Adams and Quiros (1985)
L. esculentum + L. peruvianum[a]	Kinsara et al. (1986)
Oryza sativa + O. brachyantha	Hayashi et al. (1988a)
O. sativa + O. eichingeri[a]	Hayashi et al. (1988a)
O. sativa + O. officinalis[a]	Hayashi et al. (1988a)
O. sativa + O. perrieri[a]	Hayashi et al. (1988a)
Solanum melongena + S. integrifolium	Kameya et al. (1990)
S. melongena + S. sisymbriifolium	Gleddie et al. (1986)
S. melongena + S. khasianum[a]	Sihachakr et al. (1988)
S. melongena + S. torvum	Guri and Sink (1988a), Sihachakr et al. (1989)
S. melongena + S. nigrum	Guri and Sink (1988b)
S. melongena + S. ethiopicum	Daunay et al. (1993)
S. melongena + S. integrifolium	Kameya et al. (1990)
S. melongena + S. saintwongsei[a]	Asao et al. (1994)
S. tuberosum + S. brevidens[a]	Austin et al. (1985, 1986), Helgeson et al. (1986)
S. tuberosum + S. circaeifolium	Mattheij et al. (1992)
Intergeneric	
Brassica campestris + Barbarea vulgaris	Oikarinen and Ryoppy (1992)
B. carinata + Camelina sativa[a]	Narasimhulu et al. (1994)
B. juncea + Diplotaxis muralis[a]	Chatterjee et al. (1988)
B. juncea + Moricandia arvensis[a]	Kirti et al. (1992b)
B. juncea + Trachystoma ballii[a]	Kirti et al. (1992a)
B. napus + Arabidopsis thaliana	Forsberg et al. (1994)
B. napus + Barbarea vulgaris[a]	Fahleson et al. (1994)
B. napus + B. tournefortii[a]	Liu et al. (1995)

B. napus + *Eruca sativa*	Fahleson et al. (1988)
B. napus + *Thlaspi perfoliatum*[a]	Fahleson et al. (1994b)
B. oleracea + *Moricandia arvensis*	Toriyama et al. (1987a)
B. oleracea + *Sinapis turgida*[a]	Toriyama et al. (1987b)
Citrus aurantifolia + *Feroniella lucida*[a]	Takayanagi et al. (1992)
C. aurantifolia + *Swinglea glutinosa*	Takayanagi et al. (1992)
C. sinensis + *Atalantia ceylanica*[a]	Louzada et al. (1993)
C. reticulata + *Severinia buxifolia*[a]	Grosser et al. (1992)
C. sinensis + *Citropsis gilletiana*[a]	Grosser et al. (1990)
C. sinensis + *Murraya paniculata*[a]	Shinozaki et al. (1992)
C. sinensis + *Poncirus trifoliata*	Ohgawara et al. (1985), Grosser et al. (1988a)
C. sinensis + *Severinia disticha*[a]	Grosser et al. (1988b)
C. reticulata × *C. paradisi* + *Atalantia monophylla*[a]	Motomura et al. (1995)
C. reticulata + *Citropsis gabunensis*[a]	Ling and Iwamasa (1994)
C. reticulata × *C. paradisi* + *Severinia buxifolia*[a]	Motomura et al. (1995)
Oryza sativa + *Echinochola oryzicola*[a]	Terada et al. (1987)
Solanum lycopersicoides + *Lycopersicon esculentum*	Hossain et al. (1994)
Solanum tuberosum + *Lycopersicon pimpinellifolium*	Okamura (1988)

[a]Sexually incompatible combination, as indicated by the authors.

determined by chromosome counting and DNA content analyses, 30% of the hybrids showed 38 chromosomes. The 6 amphidiploid somatic hybrids with normal morphology showed 38–70% pollen viability (75% of the pollen viability of normal *B. napus*), and seed-set varied from 1 to 40%.

Some new species produced in the Brassicaceae by protoplast fusion are '*Brassicomoricandia* (*Brassica oleracea* + *Moricandia arvensis*; Toriyama et al., 1987a) and *B. naponigra* (*B. napus* + *B. nigra*; Sjodin and Glimelius, 1989a; Yamagishi et al., 1989). Fahleson et al. (1994a) made intertribal crosses between *B. napus* and *Barbarea vulgaris* but the somatic hybrids suffered from poor growth and differentiation of vital organs, such as roots. Even the rooted hybrids could not be established as full plants in the glasshouse. In contrast, fertile, intertribal symmetric somatic hybrids were obtained following the crosses *B. napus* + *Arabidopsis thaliana* (Forsberg et al., 1994) and *B. napus* + *Thlaspi perfoliatum* (Fahleson et al., 1994b).

A major objective of traditional plant breeding is the transfer of genes conferring disease resistance or stress tolerance from closely or distantly related wild species into modern high yielding crop cultivars, which is often thwarted because of sexual incompatibility barriers. Somatic hybridization can bypass these barriers. This is well illustrated by several

successful attempts to produce somatic hybrids involving *Solanum tuberosum* (potato) as one of the parents. Austin et al. (1985, 1986) produced somatic hybrids between *S. tuberosum* (tetraploid) and *S. brevidens* (diploid); the latter is a non-tuber producing wild species with genes for resistance against some common viral and fungal diseases of potato. The resulting tuber-bearing amphiploid (hexaploid) hybrids, with full chromosome compliments of the two parents were fertile and cross compatible with *S. tuberosum* (Ehlenfeldt and Helgeson, 1987). The wild species genes conferring resistance to potato leaf roll virus (PLRV) and *Phytophthora infestans* and tuber forming trait of *S. tuberosum* were present in the hybrids (Austin et al., 1985; Helgeson et al., 1986). Moreover, the hybrid tuber had resistance to 'tuber soft rot', a disease caused by the bacteria *Erwinia cartovora*, which was not predictable because *S. brevidens* does not form tubers.

Mattheij et al. (1992) reported another successful attempt to transfer disease resistance into potato from a wild *Solanum* species by somatic hybridization. Three out of four somatic hybrids obtained after fusing the protoplasts of diploid *S. circaeifolium* and dihaploid *S. tuberosum* were fully resistant to the pathogen *Phytophthora infestans*, and all four hybrids were highly resistant to the nematode, *Globodera pallida*. Sexual crosses between the somatic hybrids (as a female parent) and tetraploid *S. tuberosum* yielded viable seeds, demonstrating the potential of the hybrids in potato breeding. In a field trial of six tetraploid somatic hybrids between *S. tuberosum* and *S. phureja*, one hybrid gave three times higher tuber yield than the potato cultivar (Matheij and Puite; cited in Mattheij et al., 1992).

Somatic hybrids between *S. melongena* and *S. integrifolium*, possessing total chromosome number of the two parents ($2n = 48$) and showing high resistance to *Pseudomonas solanacearum* than either of the parents, were produced by Kameya et al. (1990). Fertile somatic hybrids showing resistance to *P. solanacearum* were obtained by PEG induced protoplast fusion of *S. melongena* and *S. saintwongsei* (Asao et al., 1994).

Melchers et al. (1978) had produced 'pomato' by fusing the protoplasts of potato and tomato but all the hybrids were sterile, probably because the potato protoplasts involved in the fusion were derived from aneuploid callus cells. Fertile 'pomato' plants could be obtained by fusing mesophyll protoplasts of potato and *Lycopersicon pimpinellifolium* (Okamura, 1988). Somatic and sexual hybridization in the genus *Lycopersicon* has been reviewed by Lefrancois et al. (1993).

Recently, Krasnyanski and Menczel (1995) have produced fertile somatic hybrids between *Helianthus annuus* and *H. giganteus*. Seeds from two of such hybrids produced normal fertile F_2 plants. Several inter-

specific (Grosser et al., 1989; Ohgawara et al., 1989; Tusa et al., 1990) and intergeneric (Grosser et al., 1988, 1990) somatic hybrids of *Citrus* with sexually compatible and incompatible partners have been developed during the past decade (see Table 13.3).

13.5.2. Asymmetric hybridization

Interspecific somatic hybrids, as also sexual hybrids, between a wild species and a cultivated species contain many undesirable traits of the wild species besides the desired ones. Backcrossing of the hybrid with the cultivated species, required to remove the unwanted genes of the wild parent, is hampered because the more spectacular hybrids between remote species are generally sterile. Combining alien genomes may also interfere with normal development of the hybrid, so that the hybrid callus is incapable of regenerating plants (Gupta et al., 1984), the hybrid plants are necrotic and die before attaining maturity (Terada et al., 1987) or produce abnormal flowers. Gleba and Hoffmann (1979) had suggested that the formation of asymmetric hybrids through the induction of unilateral chromosome elimination might improve hybrid morphology and fertility.

It is evident from the analyses of somatic hybrids that even if barriers preventing sexual hybridization between two remote species are bypassed by protoplast fusion, barriers may still exist at the genomic level, resulting in spontaneous elimination of chromosomes during culture of the hybrid cells. Consequently, some of the hybrid plants regenerated from the fusion products lack some or most of the chromosomes of one of the fusion partners. The nucleolar chromosomes of *Solanum phureja* were eliminated preferentially in the somatic hybrid *S. tuberosum* + *S. phureja* (Pijnacker et al., 1987). The somatic hybrids that have lost chromosomes of one of the parents and are, therefore, phenotypically closer to the other parent are called asymmetric hybrids. The mechanism that determines which lot of chromosomes is to be partially eliminated is not well understood. Generally, the chromosomes of the parent with shorter cell cycle are retained. Another factor which may cause genomic incompatibility is the difference in the state of differentiation of the two cells involved in fusion (Warren, 1991). For example, when cells from actively growing suspension cultures and non-dividing mesophyll cells are fused there are higher chances of loss of chromosomes of the latter parent.

The observation of spontaneous occurrence of asymmetric hybrids led to a search for methods to achieve directed elimination of chromosomes of the donor parent, to allow the synthesis of asymmetric but fertile hy-

brids. This is now possible by X- or γ-irradiation of the donor protoplasts before fusing them with normal protoplasts of the recipient parent, a method originally developed for the transfer of cytoplasmic genetic information (Zelcer et al., 1978; see Section 13.5.3). Irradiation causes fragmentation of the chromosomes rendering the protoplasts incapable of dividing. When such protoplasts are fused with normal protoplasts of the recipient parent, some of the fragments may get integrated into the hybrid genome. Such asymmetric hybrids can be recovered by selection for nuclear traits of the donor. Dudits et al. (1980) first described the restoration of chlorophyll synthesis in carrot albino mutants by fusing its protoplasts with irradiated parsley protoplasts. Similarly, Somers et al. (1986) restored nuclear-coded nitrate reductase (NR) activity in a NR deficient tobacco mutant by fusion with irradiated barley protoplasts. Following similar strategies several asymmetric hybrids, with one or a few traits (Dudits et al., 1980, 1987; Gupta et al., 1984; Bates et al., 1987; Wolters et al., 1991) to many chromosomes (Imamura et al., 1987; Muller-Gensert and Schieder, 1987; Gleba et al., 1988; Famelaer et al., 1989; Yamashita et al., 1989; Wijbrandi and Koornneef, 1990) of the donor genome have been obtained based on irradiation of the donor parent protoplasts.

Yamashita et al. (1989) produced asymmetric hybrids by fusing *B. oleracea* protoplasts with X-irradiated protoplasts of *B. campestris* and established their hybridity on the basis of morphology, isozymes and chromosome number. Itoh et al. (1991) confirmed the asymmetric hybrid nature of these plants by using in situ hybridization of *B. campestris*-specific, middle repetitive DNA sequences to their metaphase chromosomes. By backcrossing of the asymmetric hybrids with *B. oleracea* twice, Itoh et al. obtained fully fertile plants with all the morphological characters of *B. oleracea* but showing a disease resistance derived from *B. campestris*. Sjodin and Glimelius (1989b) achieved interspecific transfer of resistance to *Phoma lingam*, a fungus causing severe diseases in several cruciferous crops, by asymmetric hybridization. Complete resistance to the pathogen occurs in *B. nigra* and related species, *B. carinata* and *B. juncea*. By fusing *B. napus* protoplasts with irradiated protoplasts of *B. juncea* or *B. carinata* and by addition of sirodesmin (a toxin produced by *P. lingam*) in the culture medium, as a selection pressure, asymmetric hybrids were regenerated which proved fully resistant to infection by pycnospores of the fungus. Another interspecific asymmetric hybrid in the genus *Brassica* was produced by Schieder et al. (1991). Irradiated protoplasts of *B. nigra* ($2n = 16$) were fused with *B. napus* ($2n = 38$) protoplasts and hybrid colonies were regenerated on the basis of hygromycin resistance introduced in *B. nigra* by genetic engineering. Some of the hy-

brids were resistant to *Plasmodiophora brassicae*, a trait contributed by *B. nigra*. The hybrids showed a great inter- and intra-clonal variation in chromosome number. Cells with different chromosome numbers occurred in the same root, suggesting that chromosome elimination in somatic hybrids may continue even after plant regeneration.

Bates (1990a,b) recovered asymmetric hybrids following fusion of *Nicotiana tabacum* protoplasts with irradiated protoplasts of *N. repanda*. The hybrids, which appeared similar to *N. tabacum*, were obtained by selection for kanamycin resistance, introduced into *N. repanda* by *Agrobacterium*-mediated transformation. Two of the hybrids displayed *N. repanda's* hypersensitivity response when inoculated with TMV. Although largely male sterile, many of the hybrids produced viable seeds when backcrossed with *N. tabacum*. Bates (1990b) has also transferred hypersensitivity to TMV from *N. glutinosa* to *N. tabacum* through asymmetric hybridization.

In asymmetric hybridization the degree of elimination induced by irradiation varies considerably with the species involved. When normal protoplasts of *N. plumbaginifolia* were fused with *N. tabacum* protoplasts exposed to γ-irradiation as low as 50 Gy, cybrids containing *N. plumbaginifolia* nuclear DNA and *N. tabacum* chloroplasts were obtained (Menczel et al., 1982). In contrast, fusion of irradiated *Solanum tuberosum* protoplasts with the protoplasts of *Lycopersicon esculentum* did not yield even a single hybrid lacking potato DNA (Wolters et al., 1991). γ-Irradiation up to 500 Gy was insufficient for total elimination of potato chromosomes from the fusion products.

Partial donor genome transfer to raise asymmetric hybrids can also be achieved by using microprotoplasts containing one or a few chromosomes (Ramulu et al., 1992). Miniprotoplasts can be obtained after inducing micronuclei formation by treatment with antimicrotubule agents (Sree Ramulu et al., 1991).

13.5.3. Cybridization

In sexual hybridization the plastid and mitochondrial genomes are generally contributed by only the female parent whereas in somatic hybridization the extranuclear genomes from both the parents are combined. Consequently, the latter approach to crossing plants offers a unique opportunity to study the interaction of the cytoplasmic organelles. Interparental recombination of mitochondrial genomes and independent assortment of chloroplasts and mitochondria following cell fusion results in plants with novel combinations of nuclear/plastid/mitochondria genomes. A plant having nuclear genome mostly derived from one of the

fusion partners with at least some alien organelle genome, derived from the other fusion partner, is termed cybrid (Galun, 1993).

Some of the desirable traits, such as cytoplasmic male sterility (CMS), certain types of disease resistance and herbicide resistance are encoded in extranuclear genomes. Alloplasmic lines, with nucleus of one parent in the cytoplasm of another parent are conventionally obtained by crossing the two parents with the cytoplasm-donor (hereafter called 'donor') as the female parent, followed by a series of backcrossing with cytoplasm-recipient (hereafter, called 'recipient') parent as the recurrent pollinator. This process is time consuming and may require several years. Another drawback of this method is that alloplasmic transfer can only be performed between sexually compatible species. Moreover, this approach does not allow combining two cytoplasmically controlled traits occurring in different plants. By cell fusion, on the other hand, cybrids can be produced in a single manipulation, and it is an efficient method to transfer cytoplasmic characters from one parent to the other or combining cytoplasmic characters from two parents.

(i) *Methods to produce cybrids.* In an experiment involving fusion of full protoplasts of two parents, cybrids may arise through: (a) fusion of a normal protoplast with an enucleate protoplast; (b) fusion between a normal protoplast and a protoplast containing non-viable nucleus; (c) elimination of one of the nuclei after heterokaryon formation; or (d) selective elimination of chromosomes at a later stage. Although cybrids have been produced by this approach, lacking a nuclear fusion control, it is possible to improve the chances of recovery of desired cybrids by inactivating the nucleus of the donor parent by X- (Menczel et al., 1987) or γ- (Barsby et al., 1987a) irradiation (5–30 kR) of its protoplasts, before fusion (Zelcer et al., 1978; Aviv and Galun, 1980). These treatments apparently do not have any deleterious or mutagenic effect on organelle genomes, probably because these genomes are present at a high copy number in each plant cell. Consequently, when the irradiated protoplasts of the donor plant are fused with normal protoplasts of the recipient plant and fusion products are cultured, only the protoplasts of the recipient parent with cytoplasmic genome of either or both the parents are able to divide and regenerate plants. However, in cybrid production using irradiated donor protoplasts incorporation of fragmented nuclear DNA of the donor parent into the recipient genome may occur. After all this is also the most popular method to raise asymmetric hybrids (Section 13.5.2). Probably, the chances of cybrid formation by this method can be increased by additional selection pressure in favour of the nuclear genome of the recipient parent.

Contribution of nucleus by the donor parent can also be avoided by using their cytoplasts (enucleated sub-protoplasts) for fusion with normal protoplasts of the recipient parent. Highly purified preparations of cytoplasts can be obtained in a single step by high-speed centrifugation (20 000–40 000 × *g* for 45–90 min) of protoplasts in an iso-osmotic density gradient, with 5–50% Percoll (Lorz et al., 1981). A modification of this method of cytoplast production is described by Lesney et al. (1986). Maliga et al. (1982) were the first to demonstrate the transfer of streptomycin-resistant chloroplasts in *Nicotiana* by cytoplast-protoplast fusion. Sakai and Imamura (1990) produced CMS *B. napus* by fusion of cytoplasts isolated from CMS *Raphanus sativus* Kosena with iodoacetamide-inactivated protoplasts of male *B. napus*. A problem with using cytoplasts could be that often nucleate miniprotoplasts may occur with cytoplasts as contaminant (Pelletier, 1991).

When an irradiated protoplast or a cytoplast of the donor parent is fused with a normal protoplast of the donor parent, the fusion product receives the cytoplasm of both the parents, and interparental recombination of organelle genomes may occur before random segregation of the organelles. To retain only the cytoplasmic organelles of the donor parent, Medgyesy et al. (1980) recommended the treatment of the recipient protoplasts with metabolic inhibitors, such as iodoacetate (IOA) or iodoacetamide (IOAM). In an experiment involving fusion of irradiated donor protoplasts and metabolically inactivated recipient protoplasts the parental protoplasts are unable to divide but due to metabolic complementation the fusion products may divide and form tissues in which cells possess the nucleus of the recipient partner and cytoplasm of the donor parent. This donor-recipient method (Sidorov et al., 1981), which considerably enhances the chances of selecting the desired nucleus-cytoplasm combinations is now most widely used to produce cybrids. However, in this approach a careful analysis of the cybrids is required because some nuclear information of the irradiated donor may persist as individual or translocated chromosomes (Menczel et al., 1987; Sidorov et al., 1981).

(ii) *Some examples of useful cybrids produced through cell fusion.* Cybridization has been used successfully to make intergeneric and interspecific transfer of cytoplasm in tobacco, petunia, rice and *Brassica* species. In recent years most extensive work on cybrid production has been done in the genus *Brassica*.

Several CMS systems (encoded in mitochondrial genome) are available in the genus *Brassica*, including *Nap* (Thompson, 1972), *Ogu* (Bannerot et al., 1977) and *Polima* (Sernyk, 1983) types. *Ogu* type of CMS, discovered in *Raphanus sativus* by Ogura (1968), was introduced into *B. oler-*

acea and *B. napus* through intergeneric sexual crosses (Bannerot et al., 1974, 1977). However, the resulting plants, although male sterile, suffered from several deficiencies. In all the alloplasmic CMS lines produced by sexual crosses the plants exhibited yellowing at low temperatures (<15°C) and, although green at higher temperature they always maintained low level of chlorophyll. Screening among varieties of various *Brassica* species for genes to correct the chloroplast deficiency or restorer genes for CMS trait have been unsuccessful (Yarrow, 1992). Moreover, the flowers of CMS plants of *Brassica* species exhibited poor development of nectaries, resulting in reduced production of nectar (Pelletier et al., 1983). By fusing the protoplasts of chlorophyll deficient CMS lines of *B. napus* with those of male fertile *B. napus* (with normal cytoplasm) or *B. campestris* (with atrazine resistant chloroplasts), Pelletier et al. (1983) obtained CMS *B. napus* plants in which cold sensitive chloroplasts of *R. sativus* were replaced by those of *B. napus* or *B. campestris* as a result of random segregation of the plastids. Plants with a new combination of mitochondrial male sterility and fully functional plastids were identified by morphological traits after random regeneration from parental and fused protoplasts. In field tests some of the cybrids also showed correction for other deficiencies related to floral morphology (Pelletier et al., 1988). They produced enough nectar to be as attractive to bees as fertile plants and possessed female fertility. The findings of Pelletier et al. (1983) were confirmed by Menczel et al. (1987). However, these workers used X-irradiated (5.7 kR) protoplasts of the CMS donor parent which made selection of the desired genotypes easier because protoplasts of one of the parents did not divide.

A wild *B. campestris* showing resistance to the triazine herbicide atrazine, due to a single point mutation in the plastome genome, was isolated by Matais and Bouchard (1978). This cytoplasmically controlled trait had been transferred to cultivars of *B. napus* and *B. campestris* (Beversdorf et al., 1980). Yarrow et al. (1986) and Barsby et al. (1987b) combined, by cybridization, the two useful cytoplasmically controlled traits, viz. CMS (encoded in chondriome) and atrazin resistance (encoded in plastome), occurring in different plants. However, the two studies employed different selection strategies. Yarrow et al. manually picked up the heterokaryons and cultured them using *Nicotiana tabacum* nurse cell system, earlier used for the selection of novel cytoplasm-nuclear combinations in *N. tabacum* (Flick et al., 1985) and in the synthesis of *B. napus* through somatic hybridization (Sundberg and Glimelius, 1986). The atrazine resistant CMS (*nap*) *B. napus* plants produced by Yarrow et al. (1986) were morphologically normal and produced seeds on pollination with viable pollen. Barsby et al. (1987b) combined the *Polima* type CMS

and herbicide resistance of *B. campestris* in *B. napus* using the donor-recipient selection system.

Jourdan et al. (1989b) synthesized atrazine resistant CMS *B. napus* by cybridization between CMS (*Ogu*) *B. oleracea* and atrazine-resistant *B. campestris*. This group also transferred atrazine-resistant chloroplasts from *B. napus* to *B. oleracea* (Jourdan et al., 1989a).

Kameya et al. (1989) demonstrated that somatic hybridization between sexually incompatible species offers the possibility of producing male sterile plants. They fused IOAM-treated protoplasts of *B. oleracea* with normal protoplasts of *B. campestris* and cultured them on MS medium which does not favour division of *R. sativus* protoplasts. From the regenerants CMS plants with nucleus of *B. oleracea* and plastids of *R. sativus* were obtained.

13.6. CONCLUDING REMARKS

The current methods of isolated gene transfer (Chapter 14) have somewhat overshadowed the importance of somatic hybridization in crop improvement. However, the application of genetic engineering is limited to transfer of single gene traits. The characters, such as yield and stress resistance, which require transfer of cluster of genes are not amenable to improvement by the present methods of genetic engineering. In this regard somatic cell fusion acquires significance.

Somatic hybridization provides a new approach to widen the genetic base of our crop plants by facilitating gene flow between sexually incompatible species. Somatic cell fusion may not be so useful to produce spectacular hybrids such as 'pomato' as once expected but certainly has helped in producing several intergeneric and interspecific hybrids, which could not be obtained sexually and has generated some useful breeding material. The progress in asymmetric hybridization and cybridization by somatic cell fusion has considerably enhanced the importance of this asexual method of plant breeding.

Hybrid selection after protoplast fusion has been a serious bottle-neck in somatic hybridization. By electrofusion of protoplasts it has become possible to achieve very high frequency fusion. In some cases protoplasts can be fused in desired pairs and the fusion products cultured separately to obtain hybrids without an additional selection system.

APPENDIX 13.I: PROTOCOLS FOR FUSING PLANT PROTOPLASTS

13.I.1. High pH and high Ca^{2+}-induced fusion (after Keller and Melchers, 1973; Melchers and Labib, 1974)

(a) Mix freshly isolated protoplasts of the selected parents in a ratio of 1:1 with a final density of ca. 2.5×10^5 protoplasts ml^{-1}.
(b) Pellet the protoplasts by centrifuging at $50 \times g$ for 3–5 min.
(c) Remove the supernatant and add 2 ml of the fusion mixture, containing 50 mM glycine–NaOH buffer, 50 mM $CaCl_2 \cdot 2H_2O$, and 400 mM mannitol (pH 10.5).
(d) Pellet the protoplasts by centrifuging at $50 \times g$ for 3–5 min.
(e) Place the centrifuge tube in a water bath at 37°C for 10–30 min.
(f) Replace the fusion mixture by washing medium (600 mM mannitol, 50 mM $CaCl_2 \cdot 2H_2O$) and leave for 30 min.
(g) Wash twice with the washing medium.
(h) Suspend the protoplasts in culture medium and culture as small drops.

13.I.2. PEG-induced fusion (after Kao, 1976)[1]

(a) Mix freshly isolated protoplasts (while still in the enzyme solution) of the two desired parents in a ratio of 1:1. Pass the suspension through a 62-μm pore size filter and collect the filtrate in a centrifuge tube. Seal the mouth of the tube with a screw cap.
(b) Centrifuge the filtrate at $50 \times g$ for 6 min to sediment the protoplasts.
(c) Remove the supernatant with a Pasteur pipette.
(d) Wash the protoplasts with 10 ml of solution I (500 mM glucose, 0.7 mM $KH_2PO_4 \cdot H_2O$ and 3.5 mM $CaCl_2 \cdot 2H_2O$, pH 5.5).
(e) Resuspend the washed protoplasts in solution I to make a suspension with 4–5% (v/v) protoplasts ml^{-1}.
(f) Put a 2–3 ml drop of Silicon 200 fluid (100 cs) in a 60 × 15 mm petri dish.
(g) Place a 22 × 22 cm coverslip on the drop.
(h) Pipette ca. 150 μl of the protoplast suspension onto the coverslip with a Pasteur pipette.

[1] This method could be modified to fuse protoplasts in a centrifuge tube but in that case every washing would be followed by centrifugation which has been reported to adversely affect the fusion process and the viability of mesophyll protoplasts.

(i) Allow about 5 min for the protoplasts to settle on the coverslip forming a thin layer.

(j) Add drop-by-drop 450 μl of PEG solution (50% PEG 1540, 10.5 mM $CaCl_2 \cdot 2H_2O$, 0.7 mM $KH_2PO_4 \cdot H_2O$) to the protoplast suspension. Observe protoplast adhesion under an inverted microscope.

(k) Incubate the protoplasts in the PEG solution for 10–20 min at room temperature (24°C).

(l) Gently add two 0.5 ml aliquots of solution II (50 mM glycine, 50 mM $CaCl_2 \cdot 2H_2O$, 300 mM glucose, pH 9–10.5) at 10-min intervals. After another 10 min add 1 ml of protoplast culture medium.

(m) Wash the protoplasts five times at 5-min intervals with 10 ml of the fresh protoplast culture medium. At the end of each washing do not remove the entire medium from the coverslip. Leave behind a thin layer of the old medium over the protoplasts and add to it fresh medium. If the parent protoplasts are distinguishable visually it may be possible to assess the frequency of heterokaryon formation at this stage.

(n) Culture the fused products together with the unfused protoplasts on the same coverslip in a thin layer of 500 μl of culture medium. Put additional 500–1000 μl medium in the form of droplets around the coverslip to maintain the humidity inside the petri dish.

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Chapter 14

Genetic Engineering

14.1. INTRODUCTION

Deliberate alteration of the genome of an organism by introduction of one or a few specific foreign genes is referred to as 'genetic engineering' or 'genetic transformation', and the modified organism is described as a 'transformed' or 'transgenic' organism. In this highly sophisticated method of genetic modification of plants the genetic material or DNA sequence coding for a desirable trait is located in the donor organism by a variety of molecular techniques and then cut out from the parental DNA using the 'molecular scissors', restriction endonucleases. The relatively small piece of DNA, in a vector (a piece of DNA involved in the insertion of the foreign DNA into the chromosomal DNA) is then introduced into the recipient plant cells by one of several possible methods (see Section 14.2) and plants regenerated from them.

Genetic transformation of plants is becoming an indispensable aid to plant physiologists and biochemists in understanding the role of individual genes in the life of a plant. On the practical side, the recombinant DNA technology is ushering in the era of 'molecular breeding' of plants which offers many advantages over the established methods of foreign gene transfer into plants by sexual or, for that matter, somatic hybridization (Logemann and Schell, 1993): (1) traditional breeding allows movement of genes only between closely related plants but through molecular breeding the sources of new genetic material to be introduced is unlimited. For example, genes from viruses, bacteria, yeast, animals and completely unrelated plants can be added, in a functional form, to the genetic information of an established plant cultivar. (2) The addition of a useful trait by molecular breeding would not disrupt an elite phenotype which is often a problem with conventional breeding. In the latter case, whole genomes are transferred from the donor organism to the recipient organism and it is often difficult to separate beneficial genes from deleterious genes which are closely linked. Consequently, the process of plant breeding is slow, taking up to 15 years to produce a new variety. By the introduction of single well defined genes, that usually do not disrupt the elite genotype of the given cultivar, the breeding process can be consid-

erably accelerated. (3) Once a particular gene has been restructured it can be cloned and used in a wide variety of crop plants.

During the past 10 years considerable progress has been made to understand the regulation of gene expression and utilization of this knowledge in genetic modification of plants (Grierson, 1991a,b; Kung and Wu, 1993a,b). Techniques are now available to isolate genes and introduce them into living plants, so that they are stably integrated into the genetic system of the recipient plant. Provided that the foreign genes are appropriately restructured they will usually function in a predictable fashion after their introduction into plant cells. Since 1987 numerous potentially useful transgenic plants of cotton, maize, potato, rapeseed, raspberry, soybean, tomato, etc. have been generated (see Table 14.1) and at least one of these (tomato cv. Flavr Savr) has already been released as a new cultivar (Beachy, 1993; Christou, 1993). During 1988–1992 the Animal and Plant Health Inspection Service of USDA alone issued 284 permits for field trials of transgenic plants of 17 crops in the USA (Fisk and Dandekar, 1993). Recently, insect resistant maize, potato and cotton have been cleared for the first stage of commercialization in the United States (Hoyle, 1995). Full scale commercialization of these transgenic pesticidal plants is expected by early 1996.

The subject of plant genetic engineering involves hardcore molecular biology aspects, such as isolation and restructuring of genes, gene cloning, construction of vectors using suitable promoters and marker genes, molecular characterization of transformed plants, etc. These topics are discussed at length in some recent books and reviews (Schell and Vasil, 1989; Kung and Wu, 1990a,b; Grierson, 1991a,b). In this chapter the various techniques of gene transfer in plants and some examples of the production of stably transformed, non-chimeric plants for use in agriculture or horticulture are described.

14.2. METHODS OF GENE TRANSFER

A generalized strategy for plant transformation requires the construction of a genetic vehicle or vector composed of the desired gene flanked with the necessary regulatory sequences upstream (5′; correspond to the promoter region) and downstream (3′; correspond to the polyadenylation signal), replication sites and markers (Fig. 14.1) which allows for its cloning in a bacterial system and secure stable incorporation, multiplication and expression in the host cell. Among the various vectors used for genetic transformation of plants the Ti-plasmid of *Agrobacterium tumefaciens* has been studied in most detail. The transformation protocols based on *A. tumefaciens* are in general, more convenient and/or efficient than

the other methods described so far (Goodman et al., 1987). Therefore, *Agrobacterium* mediated transformation is dealt with in detail.

14.2.1. Agrobacterium tumefaciens mediated transformation

Induction of crown gall and hairy root diseases in several dicot plants by the common soil borne Gram-negative bacteria *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively, are examples of natural transformation of plants wherein the bacterial genes are stably introduced into the genome of higher plants (Chilton, 1983). Extensive studies made over the years to understand the process of crown gall formation has revealed that the virulent strains of the bacteria introduce a part of their genetic material into the infected cells where it gets integrated randomly with the genetic material of the host cell. The bacterial genes are able to replicate along with the plant genome and express themselves in terms of the synthesis of a special class of compounds, called opines, which are required for the growth of the bacteria but are useless to the host cells. The two most common opines are octopine and nopaline. A strain of *A. tumefaciens* can induce the synthesis of either octopine or nopaline and can metabolize only that particular opine and not the other one. The tumerous growth of the crown gall is caused by another set of bacterial genes introduced into the plant genome. These oncogenes are responsible for the synthesis of phytohormones (cytokinin and auxin) in the transformed plant cells, leading to uncontrolled proliferation of the cells.

The natural ability of *A. tumefaciens* to transform plant cells prompted scientists to investigate the molecular genetics of crown gall disease. The tumour inducing principle (Ti-principle) of the bacterium has been fairly well described and has been developed as an effective vector for introducing foreign genes into higher plants (Chilton, 1983; Klee and Rogers, 1989; White, 1993).

(i) *Ti plasmid.* The cells of virulent strains of *A. tumefaciens* contain, besides the chromosomal DNA, a small circular non-chromosomal DNA, called Ti-plasmid, which carries ancillary genes such as those affecting pathogenicity and metabolism of opines (Fig. 14.2).

The Ti-plasmid is made up of 200 000 nucleotide pairs of which only a 23 000 nucleotide piece is introduced into the host cell. The transferred DNA (or T-DNA) carries genes for the synthesis of opines and phytohormones. The T-DNA is bordered on both sides by 25 base pairs (bp) direct repeated sequences of nucleotides which define the limit of T-DNA. The T-DNA border sequences are the only elements of the plasmid required in

TABLE 14.1

Species that have been transformed and regenerated into complete transgenic plants (after Fisk and Dandekar, 1993)

Species	Common name	Method	Reference
Actinidia deliciosa	Kiwi fruit	At	Uematsu et al. (1991)
		Ar	Yazawa et al. (1995)
Allocasuarina verticillata		Ar	Phelep et al. (1991)
Anagallis arvensis	Pimpernel	Ar	Mugnier (1988)
Antirrhinum majus	Snapdragon	Ar	Handa (1992)
Apium graveolens	Celery	At	Catlin et al. (1988)
Arabidopsis thaliana	Mouse ear cress	At	Lloyd et al. (1986)
		Ar	Pavingerova and Ondrej (1986)
		PB	Seki et al. (1991)
Arachis hypogaea	Peanut	At	McKently et al. (1995)
Armoracia lapathifolia	Horseradish	Ar	Noda et al. (1987)
Asparagus officinalis	Asparagus	At	Bytebier et al. (1987)
Atropa belladonna		Ar	Saito et al. (1992)
Beta vulgaris	Sugarbeet	At	Lindsey and Gallois (1990)
Brassica campestris	Chinese cabbage	At	Jun et al. (1995)
Brassica carinata		At	Narasimhulu et al. (1992)
Brassica juncea	Indian mustard	At	Barfield and Pua (1991)
Brassica napus	Oilseed rape	At	Fry et al. (1987)
		At	Pua et al. (1987)
		Ar	Guerche et al. (1987a)
		EP	Guerche et al. (1987b)
		IR	Neuhaus et al. (1987)
Brassica oleracea	Cauliflower	At	Srivastava et al. (1988)
		Ar	David and Tempe (1988)
	Cabbage	Ar	Berthomieu and Jouanin (1992)
	Broccoli	Ar	Hosoki et al. (1991)
Carica papaya	Papaya	PB	Fitch et al. (1990)

Carya illinoensis	Pecan	At	McGranahan et al. (1993)
Catharanthus roseus	Periwinkle	Ar	Brillanceau et al. (1989)
Cichorium intybus	Chicory	At	Vermeulen et al. (1992)
Citrus jambhiri	Rough lemon	PG	Vardi et al. (1990)
Citrus sinensis	Orange	At	Hidaka et al. (1990)
Citrus sinensis × *Poncirus trifoliata*	Carrizo citrange	At	Moore et al. (1992)
Convolvulus arvensis	Morning glory	Ar	Tepfer (1984)
Cucumis melo	Muskmelon	At	Fang and Grumet (1990)
Cucumis sativus	Cucumber	At	Chee (1990)
		Ar	Trulson et al. (1986)
Dactylis glomerata	Orchardgrass	EP	Horn et al. (1988)
		PG	Horn et al. (1988)
Dandrathema grandiflorum	Chrysanthemum	At	Seiichi et al. (1995)
Datura innoxia		At	Sangwan et al. (1991)
Daucus carota	Carrot	At	Scott and Draper (1987)
		Ar	Chilton et al. (1982)
Dendranthema indicum	Chrysanthemum	At	Ledger et al. (1991)
Dianthus caryophyllus	Carnation	At	Lu et al. (1991)
Fagopyrum esculentum	Buckwheat	At	Miljus-Djikic et al. (1992)
Festuca arundinacea	Tall fescue	PG	Wang et al. (1992)
Fragaria × *anannassa*	Strawberry	At	James et al. (1990)
		At	Nehra et al. (1990)
		EP	Nyman and Wallin (1992)
Foeniculum vulgare	Fennel	Ar	Mugnier (1988)
Glycine max	Soybean	At	Hinchee et al. (1988)
		PB	McCabe et al. (1988)
Gossypium hirsutum	Cotton	At	Firoozabady et al. (1987)
		At	Umbeck et al. (1987)
		PB	Finer and McMullen (1990)
Helianthus annuus	Sunflower	At	Everett et al. (1987)
		PB+At	Nethalie et al. (1994)
Ipomoea batatas	Sweet potato	At	Al-Juboory and Skirvin (1991)

TABLE 14.1 (*continued*)

Species	Common name	Method	Reference
Juglans regia	Walnut	At	McGranahan et al. (1988)
Kalanchoe laciniata		At	Jia et al. (1989)
Lactuca sativa	Lettuce	At	Michelmore et al. (1987)
Larix decidua	European larch	Ar	Huang et al. (1991)
Linum usitatissimum	Flax	At	Basiran et al. (1987)
Lotus corniculatus	Bird's-foot trefoil	Ar	Otten and Schell (1986)
Lycopersicon esculentum	Tomato	At	McCormick et al. (1986)
		At	Shahin et al. (1986b)
		Ar	Shahin et al. (1986b)
Malus × *domestica*	Apple	At	Sriskandarajah et al. (1994)
Malus pumila	Apple	At	James et al. (1989)
Medicago arborea		Ar	Damiani and Arcioni (1991)
Medicago sativa	Alfalfa	At	Shahin et al. (1986a)
		Ar	Golds et al. (1991)
Medicago truculata	Barrel medic	At	Thomas et al. (1992)
Medicago varia	Alfalfa	At	Deak et al. (1986)
Musa sp.	Banana	PB	Sagi et al. (1995)
		At	May et al. (1995)
Nicotiana bigelovii		At	Schoelz et al. (1991)
Nicotiana clevelandii		At	Tavazza et al. (1988)
Nicotiana glauca		At	An et al. (1986)
		Ar	Taylor et al. (1985)
Nicotiana hesperis		Ar	Waltonn and Belshaw (1988)
Nicotiana plumbaginifolia		Ar	Horsch et al. (1984)
		At	De Block et al. (1984)
		Ar	Jouanin et al. (1987)
		PG	Horth et al. (1987)
Nicotiana rustica		At	An et al. (1986)

Nicotiana tabacum	Tobacco	At	An et al. (1986)
		Ar	Tepfer (1984)
		PB	Klein et al. (1988)
		EP	Riggs and Bates (1986)
		PG	Paszkowski et al. (1984)
		LP	Deshayes et al. (1985)
		US	Zhang et al. (1991)
Onobrychis vicifolia		Ar	Golds et al. (1991)
Oryza sativa	Rice	PB	Christou et al. (1991)
		EP	Toriyama et al. (1988)
		EP	Zhang et al. (1988)
		IR	Luo and Wu (1988)
		PG	Zhang and Wu (1988)
Panax ginseng	Ginseng	At	Lee et al. (1995)
Petunia hydrida	Petunia	At	Fraley et al. (1985)
		Ar	Ondrej and Biskova (1986)
Phaseolus vulgaris	Bean	PG	Russell et al. (1993)
Pisum sativum	Pea	At	De Kathen and Jacobsen (1990)
		At	Puonti-Kaerhus et al. (1990)
Populus alba × *P. gradidentata*	Aspen	At	Fillatti et al. (1987)
		PB	McCown et al. (1991)
Populus alba × *P. tremula*	Aspen	At	De Block (1990)
Populus tremuloides	Quaking aspen	At	Tsai et al. (1994)
Populus trichocarpa × *P. deltoides*	Poplar	At	De Block (1990)
		Ar	Pythoud et al. (1987)
Prunus amygdalus	Almond	At	Archiketi et al. (1995)
Prunus armeniaca	Apricot	At	Machado et al. (1992)
Prunus domestica	Plum	At	Mante et al. (1991)
Prunus persica	Peach	At	Smigocki et al. (1991)
Ribes nigrum	Blackcurrant	At	Graham and McNicol (1991)
Rubus spp.	Berry	At	Graham et al. (1990)

TABLE 14.1 (*continued*)

Species	Common name	Method	Reference
Saccharum officinarum	Sugar cane	PB	Bower and Birch (1992)
		EP	Arencibia et al. (1995)
Secale cereale	Rye	IR	De la Pena et al. (1987)
Solanum dulcamara		Ar	McInnes et al. (1991)
Solanum integrifolium		At	Rotino et al. (1992)
Solanum melongena	Eggplant	At	Guri and Sink (1988)
Solanum muricatum	Pepino	At	Atkinson and Gardner (1991)
Solanum nigrum	Nightshade	Ar	Wei et al. (1985)
Solanum tuberosum	Potato	At	An et al. (1986)
		At	Shahin and Simpson (1986)
		Ar	Ondrej et al. (1989)
		Ar	Visser et al. (1989)
Stylosanthes humilis	Towsville stylo	At	Manners (1988)
Torenia fournieri	Torenia	At	Aida and Shibata (1995)
Trifolium repens	White clover	At	White and Greenwood (1987)
Triticum aestivum	Wheat	PB	Vasil et al. (1992)
Vaccinium macrocarpon	Cranberry	PB	Serres et al. (1992)
Vicia narbonensis		At	Pickardt et al. (1991)
Vigna aconitifolia	Moth bean	At	Eapen et al. (1987)
		PG	Kohler et al. (1987)
Vinca minor	Lesser periwinkle	Ar	Tanaka et al. (1994)
Vitis rupestris		At	Mullins et al. (1990)
Zea mays	Maize	PB	Gordon-Kamm et al. (1990)
		EP	Rhodes et al. (1988)

At, *Agrobacterium tumefaciens*; Ar, *Agrobacterium rhizogenes*; PB, particle bombardment; EP, electroporation; PG, polyethylene glycol mediated; MI, microinjection; IR, injection of reproductive organs; LP, liposome mediated; US ultrasonication.

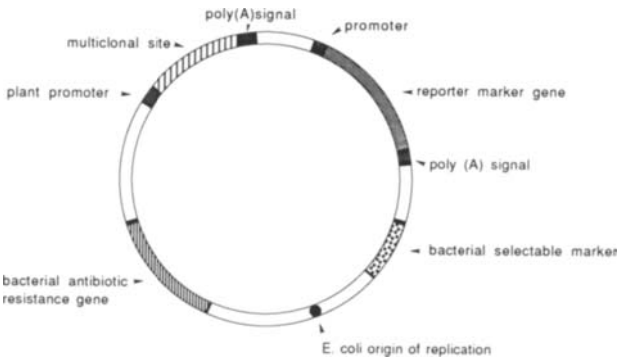


Fig. 14.1. A generalized vector for direct gene transfer into plant cells.

cis for T-DNA transfer to the plant genome. Any DNA piece inserted between the border sequences is introduced into the plant cells.

Just outside the right border segment of T-DNA is a 24 bp 'overdrive' (*ode*) region which contributes to the efficiency of T-DNA transfer (Peralta et al., 1986). A 35 000 base pair region of the plasmid, outside the T-DNA region, termed virulence region (*vir* region), is required to transfer T-DNA into plant cells. The *vir* region of the octopine Ti-plasmid consists of eight distinct loci, *vir* A to *vir* H; the nopaline Ti-plasmid lacks the *vir* F and *vir* H loci (Hooykaas and Beijersbergen, 1994). The *vir* genes regulate various aspects of T-DNA transfer (Table 14.2). However, the *vir* genes need not be physically linked to T-DNA to perform their function; they can function in *cis* and in *trans*. Induction of all *vir* genes is necessary for optimal T-DNA transfer. Only gene A is expressed constitutively. Other genes are induced by phenolic compounds produced by dicotyledonous plants. Certain phenolic compounds, such as acetosyringone, have been purified from plant exudates, which act as inducers of the *vir* genes.

The other genes present on the Ti-plasmid outside the T-DNA borders are opine catabolism genes, the origin of replication gene and plasmid conjugation genes.

(ii) *Ti-plasmid based vectors*. Initially wild type Ti-plasmid was used to transform plant cells. However, the tumorigenic properties of the transformed cells due to the introduction of oncogenes along with the T-DNA makes it difficult to regenerate morphologically normal transformed plants. To overcome this problem, *A. tumefaciens* strains were developed in which the oncogenes were deleted from the T-DNA without affecting its infectivity. Such 'disarmed' plasmids are now used as vectors in genetic transformation of plants.

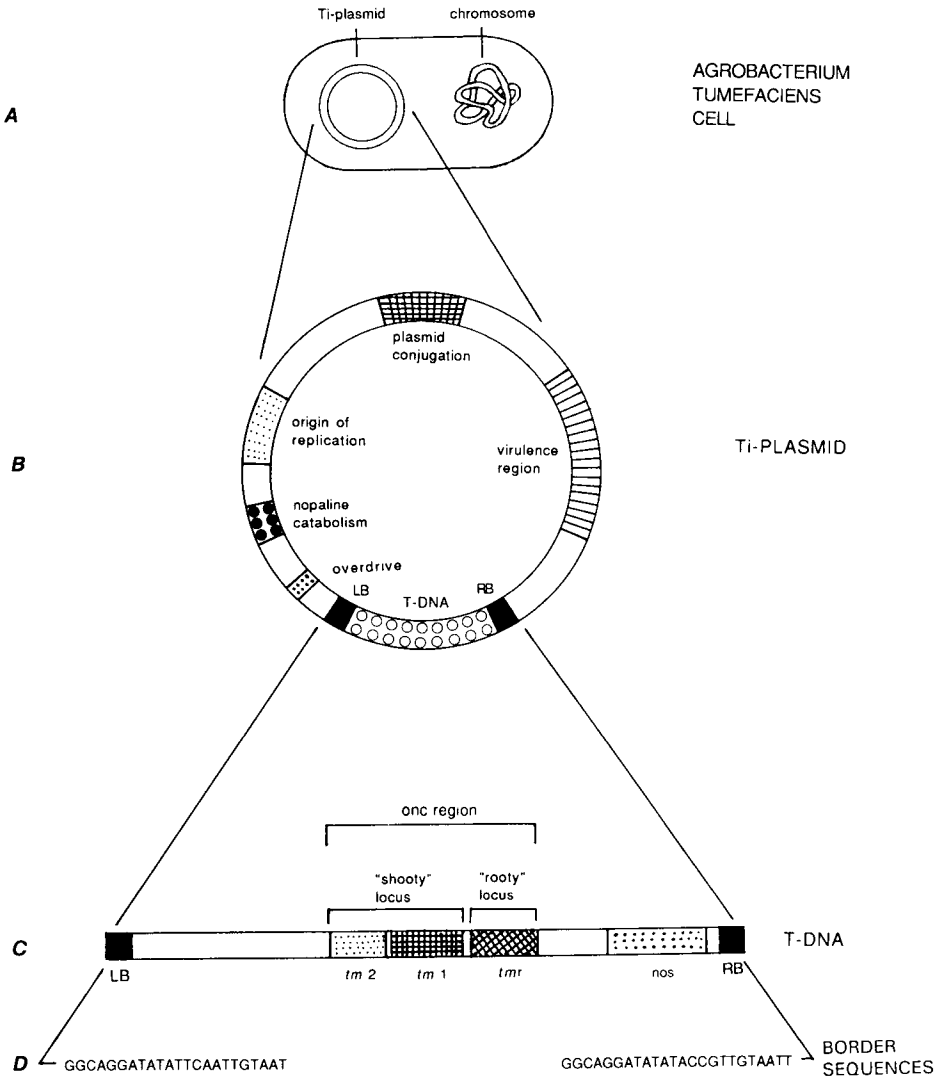


Fig. 14.2. Diagrammatic structure of wild type nopaline Ti-plasmid. (A) A cell of *Agrobacterium tumefaciens* harbouring the wild type Ti-plasmid; (B) structure of the Ti-plasmid; (C) structure of T-DNA; (D) border sequences.

The Ti-plasmid based transformation vectors are classified according to whether the DNA to be introduced is physically linked to the *vir* genes of the T-plasmid or is separate. The former type of vectors are known as 'co-integrating vectors' or '*cis* vectors' and the latter type are referred to as 'binary vectors' or '*trans* vectors'.

TABLE 14.2

Role played by six *vir* genes of Ti-plasmid in T-DNA transfer (based on Binns and Thomashow, 1988; Klee and Rogers, 1989; Zambryski, 1992)

Gene	Function
A	Phenolic sensor
B	Formation of the putative T-DNA transfer complex
C	Binding to 'overdrive', a sequence outside of the right border, necessary for optimal T-DNA transfer
D	Endonuclease to cut T-DNA at the border sequences to generate single stranded T-DNA
E	DNA binding protein, possibly involved in protection of single stranded T-DNA intermediate from degeneration
G	Transcriptional regulator

Co-integrating vectors. These vectors (Figs 14.3, 14.4) are based on a wild type Ti-plasmid from which a portion of the T-DNA, coding for phytohormone autonomy of plant cells, is replaced by a novel sequence of DNA. Such disarmed Ti-plasmids are stable within *Agrobacterium* and they retain *vir* genes and all other elements necessary for transfer of sequences located between the border sequences.

The foreign DNA to be inserted between the T-DNA borders is cloned on an 'intermediate vector' or 'shuttle vector' that can be manipulated in *E. coli* and which has a region of homology with the sequences between the border repeats of the co-integrative vector. The intermediate vector carrying the DNA to be introduced is transferred from *E. coli* to *Agrobacterium* containing co-integrative vector by conjugation in the presence of helper plasmid. Once inside the *Agrobacterium* the plasmids lacking suitable origin of replication are lost. However, recombination occurs between the intermediate plasmid and the disarmed Ti-plasmid so that the former can be rescued and retained in the bacteria. The *Agrobacterium* cells with recombined plasmids can be selected for by an appropriate genetic marker, such as antibiotic resistance (Section 14.3).

The two co-integrating plasmid systems have been used are pGV 3850:1103 and SEV. The former (Fig. 14.3) is based on disarmed Ti-plasmid pGV 3850 in which almost the complete T-DNA is replaced by the intermediate vector based on pBR 322 (Zambryski et al., 1983). This vector carries the entire intermediate vector, including its unwanted sequences which may limit its usefulness. Fraley et al. (1985) applied a modified approach which minimized the transfer of unwanted sequences of the intermediate vector. In this split-end vector (SEV; Fig. 14.4) system the right border and all of the phytohormone genes were removed

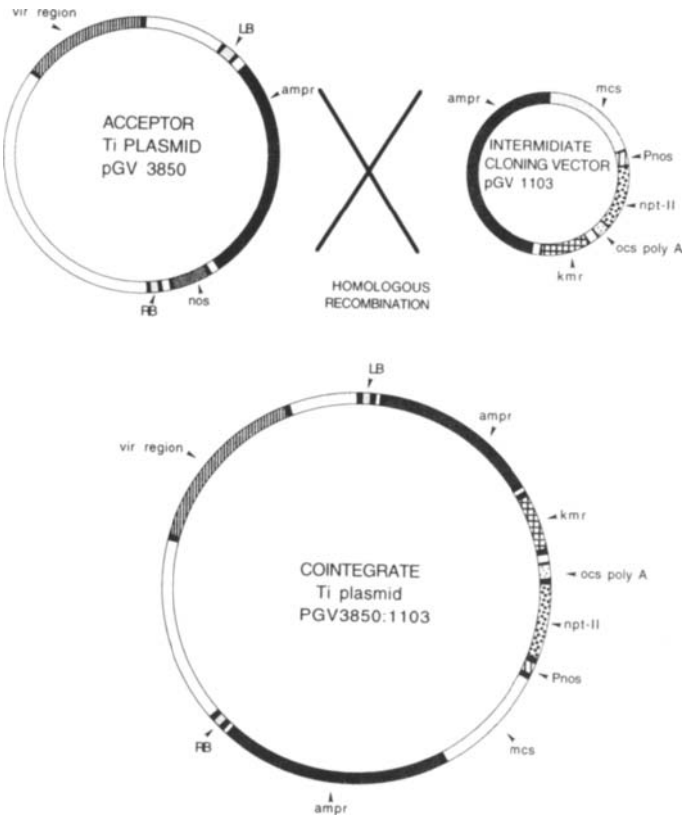


Fig. 14.3. Diagram showing the use of Ti acceptor plasmid pGV3850 (derived from a nopaline type Ti- plasmid) and *E. coli* intermediate cloning vector pGV1103 to synthesize co-integrated plasmid (pGV3850:1103) by homologous recombination. pGV3850 contains sequences from *E. coli* cloning vector 1103 in place of tumour inducing genes of T-DNA. The intermediate vector carries prokaryotic kanamycin resistance gene (kmr) and a plant selectable marker kanamycin resistance gene (npt-II) in addition to the multiclonal site (mcs) for the insertion of the gene to be transferred to plant cells. The intermediate vector is introduced into *Agrobacterium* containing the acceptor Ti-plasmid by conjugation transfer. A single crossover between the two plasmids in the region of homology (ampr) results in a larger, co-integrated plasmid pGV3850:1103 with all the genes of the intermediate vector inserted into the T-DNA region of the acceptor plasmid (after Zambryski et al., 1983).

from the Ti-plasmid pGV 3111. A small portion of the T-DNA containing the left border sequences, referred to as the left inside homology (LIH), remained in Ti-plasmid. The intermediate vector contains the LIH region as well as a right border sequence. When co-integration occurs, a functional T-DNA containing both a left and a right border results (Fig. 14.4). Several other improved co-integrating vectors have been developed. For example, in the vector pGV 2260 both the borders are removed from the

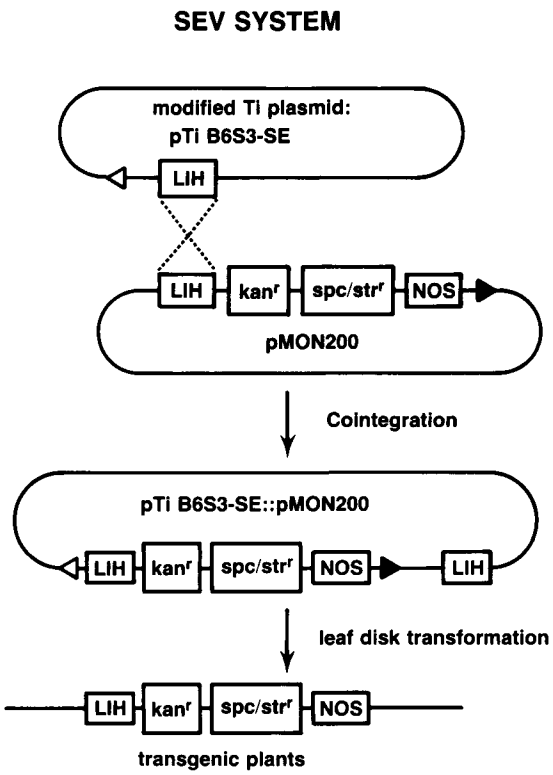


Fig. 14.4. Split end vector system (SEV) for plant transformation. The disarmed Ti-plasmid pTiB6S3-SE contains the left border sequence (hollow arrow) and a region of T-DNA (LIH) but none of the phytohormone biosynthesis genes. The pMON200 plasmid contains a region of T-DNA homology (LIH) for homologous recombination, a chimeric kanamycin-resistance gene (kan^r), a bacterial spectinomycin-streptomycin- resistance determinant (*spc/str*^{r}) for selection of co-integrates, a nopaline synthesis gene (NOS), and the right border sequence (solid arrow). Reciprocal recombination within LIH of the plasmids leads to a co-integrate plasmid, pTiB6S3-SE::pMON200, which contains complete T-DNA. During *Agrobacterium* mediated transformation the T-DNA located between the borders is transferred into the plant genome, resulting in selectable kanamycin resistance and production of nopaline, which is easily assayed. Reprinted by permission from: R.B. Horsch et al., 1985, Cold Spring Harbor Symp., 50: 433–437; © Cold Spring Harbor Laboratory Press.

disarmed Ti-plasmid and are present on the intermediate vector (Deblaere et al., 1985). pGV 2260 can be used as a co-integrating vector or as a part of the binary vector depending on the presence or absence of an origin of replication for *Agrobacterium* on the intermediate vector.

Binary vectors. Large size of the Ti-plasmid and consequently, absence of unique restriction enzyme sites makes it difficult to manipulate this

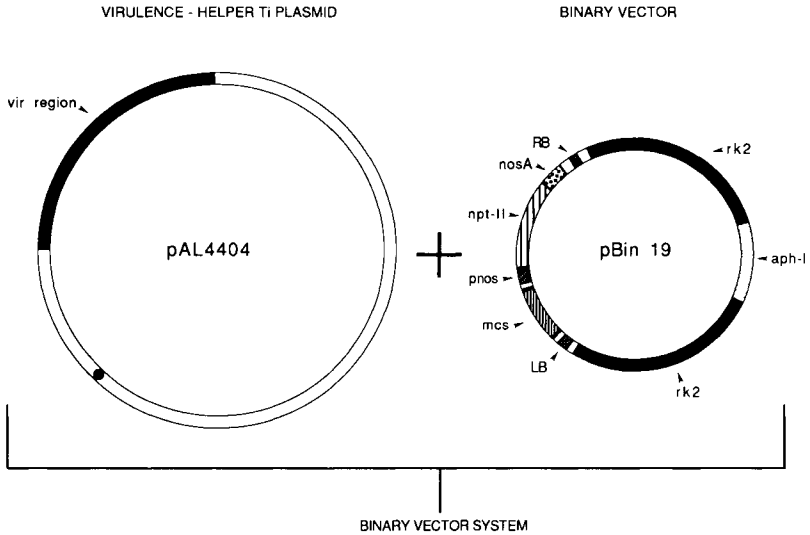


Fig. 14.5. Diagrammatic structure of a binary vector system, with pBin19 as the binary vector and pAL4404 as the helper plasmid. The vector, based on pRK252, contains wide host range rk_2 origin of replication and rk_2 conjugation function, prokaryotic kanamycin resistance gene (aph-I), and T-DNA borders derived from pTiT37. Within the borders, close to the right border (RB), is a plant selectable marker kanamycin resistance gene (npt-II) under the control of nopaline synthase promoter (Pnos) and polyadenylation signal (nosA). Close to the left border (LB) is the multiclonal site (mcs) for insertion of gene to be transferred into plant cells. The non-oncogenic helper plasmid carries the virulence genes (vir region) which help in the transfer of the genes present within the T-DNA borders on the binary vector (after Bevan, 1984).

plasmid. This problem was overcome by constructing binary vectors based on the knowledge that T-DNA and *vir* genes could be separated on different plasmids within the same bacterial cell without the loss of the T-DNA transfer capabilities (De Framond et al., 1983; Hoekema et al., 1983). The development of a binary vector strategy involves two plasmids (Fig. 14.5): (1) a disarmed Ti-plasmid containing *vir* genes and (2) a separate small binary vector plasmid containing T-DNA and broad host range plasmid replication origins (*oriv*) so that it can replicate both in *Agrobacterium* and *E. coli*. The desired foreign genes are inserted into the binary vector T-DNA between the left and right border sequences.

A selectable marker is also inserted into T-DNA. Since this vector can exist in *E. coli*, its cloning and manipulation are easier. The binary vector is introduced into *Agrobacterium* from *E. coli* by triparental mating procedure. Since it can replicate in *Agrobacterium* autonomously the frequencies with which transformation occurs is 10^3–10^4 times greater than that with co-integrating vectors. Binary vector pBin19 (Fig. 14.5), which became available in 1984 (Bevan, 1984) is still a popular general purpose binary vector.

(iii) *Method for Agrobacterium mediated transformation*: *Agrobacterium* containing the vector can be used in a number of ways to produce transgenic plants. The earlier studies involved co-cultivation of regenerating protoplasts with the bacteria. The bacteria attach to the plant cell wall and, presumably, introduce the T-DNA into the cells in a manner similar to plant infection. Despite the success of this method with systems such as *Petunia hybrida* (Fraley et al., 1983), *Nicotiana tabacum* (Herrera-Estrella et al., 1983), *N. plumbaginifola* (Horsch et al., 1984) and *Hyoscyamus muticus* (Hanold, 1983), the requirement for protoplast isolation and regeneration of plants from them limits its applicability with many crop plants.

A major technical advancement was made when Horsch et al. (1985b) demonstrated that transgenic plants could be obtained from leaf discs following their co-cultivation with *Agrobacterium*. The ease and reproducibility of this method of 'explant co-cultivation' allows for routine production of transformed plants with precise insertion of limited genes (De Block, 1993). Virtually every explant source (cotyledon, leaf, thin tissue layer, peduncle, hypocotyl, stem, microspores, proembryos) has been used to obtain transgenic plants by this method. Most transgenic plants produced so far were created through the co-cultivation method and they have shown stable integration and expression of foreign genes. The progeny from most of these transformed plants showed Mendelian segregation of the transgene (Fraley et al., 1986).

The explant co-cultivation method (Fig. 14.6) essentially involves incubation of surface sterilized explants or explants derived from aseptic plants with freshly grown bacteria for 24–48 h so that the cut surface of the explant is able to interact with the bacteria. The concentration of bacteria and composition of medium may vary with the bacterial strain and plant host. The explants are then transferred aseptically to a selection medium containing antibiotics such as cefotaxime, claforon, carbenicillin, triacillin and vancomycin, to kill the bacteria. Under proper selection pressure untransformed cells are unable to grow while the transformed cells grow and form callus. The cells are further manipulated using standard tissue culture techniques to regenerate transgenic plants from them.

Agrobacterium-mediated transformation is largely restricted to dicots which exhibit strong wound response. The monocots, particularly cereals, lack wound response and are, therefore, not susceptible to infection by the bacterium. However, recently two independent groups of scientists (Chan et al., 1993; Hiei et al., 1994) have reported heritable transformation of rice mediated by *A. tumefaciens*. Hiei et al. (1994) substituted the wound response by adding acetosyringone to the co-cultivation medium

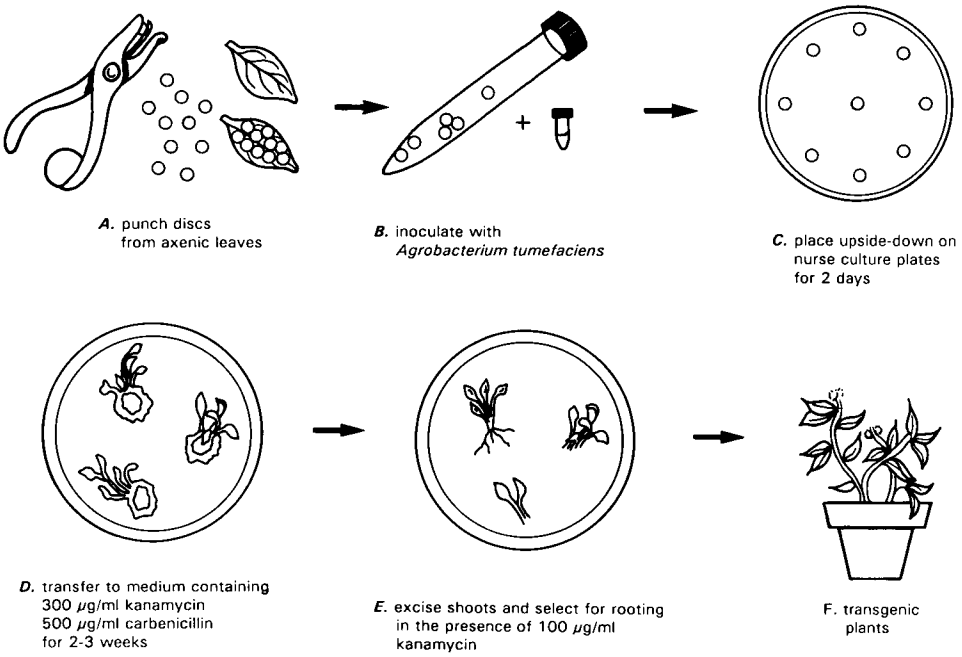


Fig. 14.6. Diagrammatic summary of the leaf disc transformation system. Reprinted by permission from: R.B. Horsch et al., 1985, Cold Spring Harbor Symp., 50: 433–437; © Cold Spring Harbor Laboratory Press.

and found that the temperature (22–28°C) during co-cultivation was critical. Of all the tissues screened scutellar callus was the best material for rice transformation.

14.2.2. Agrobacterium rhizogenes mediated transformation

The pathogenic function of *A. rhizogenes*, which is manifested in the form of hairy root disease, is also plasmid borne. This Ri plasmid is very similar to Ti plasmid except that their T-DNAs have homology only for auxin and opine synthesis sequences; the T-DNA of Ri plasmid lacks genes for cytokinin synthesis. It is possible to mobilize the T-DNA of Ti plasmid with Ri or Ti plasmid *vir* genes and vice versa.

Compared to the T-DNA of Ti plasmid very little is known about the functions encoded in T-DNA of Ri-plasmid. However, the mechanism of transfer of T-DNA of the two plasmids appears to be the same.

Hairy root cultures have been established for a wide range of species by *A. rhizogenes* mediated transformation (Hamill et al., 1987; Mugnier, 1988).

14.2.3. Virus mediated transformation

The ability of plant viruses to introduce their genome into plant cells prompted scientists to use them as plant transformation vectors in early 1980s. However, the progress in this direction has not been as expected because the plant viruses vary enormously in terms of their genomic make-up (RNA, single stranded DNA, double stranded DNA), mode of infection and replication, host range and basic life style. Moreover, the virus genome does not integrate with the plant genome and, therefore, can be used only for transient gene expression studies.

Of all the viruses cauliflower mosaic virus (CaMV) is considered a most potential vector for plant transformation as its genome is composed of double stranded DNA. Brisson et al. (1984) introduced methotrexate resistant dihydrofolate reductase (DHFR) gene of *E. coli* into turnip plants using CaMV as the vector. The passenger gene was introduced into CaMV genome replacing a part of the genome not required for virus propagation. The introduced gene spread systemically to various parts of the plants. However, it is not expected to bring about heritable transformation as neither the gene gets integrated into plant genome nor is the virus transmitted through seeds. Another problem in using CaMV as a plant transformation vector is that genes larger than a few hundred bp (DHFR is 234 bp) interfere with the packaging of the genome into the virion particle (Brisson et al., 1984).

CaMV may not be very suitable for stable transformation of plants but CaMV 35S RNA promoter from several strains of CaMV has proved to be a strong promoter, especially for dicots, and is widely used in plant transformation studies.

14.2.4. Direct gene transfer

(i) *Chemical transformation*: Many chemicals such as polyamines (poly-L-ornithine and poly-L-lysine) and dextran sulphate stimulate DNA uptake into protoplasts. However, they also highly reduce cell viability (Draper and Scott, 1991). Of the many chemicals tested for their ability to stimulate DNA uptake into protoplasts polyethylene glycol (PEG) has proved most effective.

The optimum chemical transformation under the influence of PEG is very simple. The protoplasts suspended in a medium containing biologically active DNA are treated with a relatively high concentration (28%) of high molecular weight (4000–6000, pH 8–9) PEG (Paszkowski et al., 1989). It is recommended that DNA is added before PEG (Shillito et al., 1985). Treatment of protoplasts with $MgCl_2$ (5–25 mM) significantly im-

proves transformation rates (Negrutiu et al., 1987). The DNA carrying the gene to be introduced should be offered to protoplasts in linear form.

(ii) *Electroporation*: A popular physical method for introducing new genes into protoplasts is the use of electric field which makes the protoplasts temporarily permeable to DNA (Langridge et al., 1985; Shillito et al., 1985; Fromm et al., 1987). In electroporation, plant cell protoplasts are suspended in an ionic solution containing the vector DNA in a small chamber that has electrodes at opposite ends. A pulse of high voltage is applied to the electrodes which produces transient pores (ca. 30 nm) in the plasma membrane, allowing the DNA to diffuse into the cell. After a short while, the membrane reseals. If properly treated, the cells can regenerate cell wall, divide to form callus and, finally, regenerate complete plants. The critical part of the procedure is to determine conditions which produce pores that are sufficiently large and remain open long enough to allow for DNA insertion. At the same time, the conditions should make pores that are temporary. With a 1 cm gap between the electrodes and protoplasts of 40–44 μm diameter, 1–1.5 kV cm^{-2} of field strength for 10 μs is required for efficient introduction of DNA. Shillito et al. (1985) found that the presence of 13% PEG (added after DNA) during electroporation significantly raised the transformation frequency.

The other factors which may improve the transformation frequency by electroporation are linearizing of plasmid, use of carrier DNA, heat shock (45°C for 5 min) prior to addition of vector, and placing on ice after pulsing. Under optimal conditions transformation frequencies of up to 2% have been reported. Stably transformed cell lines (Lorz et al., 1985; Fromm et al., 1986; Uchimiya et al., 1986) and full plants (Rhodes et al., 1988; Zhang et al., 1988) of a number of cereals have been produced through electroporation. Shimamoto et al. (1989) used this method of DNA delivery to raise fertile transgenic rice.

(iii) *Microinjection.* Direct injection of DNA into plant protoplasts or cells, using fine tipped (0.5–10 μm diameter) pipette has also been tried to transform plant cells (Crossway et al., 1986; Reich et al., 1986). The cells/protoplasts are immobilized on a solid support, such as agarose, or held with a macropipette under suction (Celis, 1984) under a Nomarski phase interference microscope and the DNA is injected into the nucleus or cytoplasm. Crossway et al. (1986) reported that the frequency of transformation was significantly higher (14%) when DNA was injected into the nucleus than in the cytoplasm (6%).

To avoid some of the problems associated with handling of protoplasts and regeneration of plants from isolated single cells, Neuhaus et al.

(1987) injected DNA into the cells of young pollen embryos of *Brassica napus* and regenerated transformed plants. This approach of transforming cells of multicellular systems is likely to yield chimeric transformed plants. However, the ability of pollen embryos of *B. napus* to differentiate secondary embryos enabled Neuhaus et al. (1987) to recover solid transformants. Optimum transformation response (10–30%) was obtained with 12-celled pollen embryos. Other potential target systems for transformation by microinjection are immature zygotic embryos, cell clumps from suspension cultures, isolated ovules, and cells in vegetative and floral meristems (Draper and Scott, 1991).

Besides the problems of plant regeneration from selected protoplasts, or single cells and the formation of chimeras, the major disadvantages of this approach to genetic transformation of plants are: (1) it is an extremely slow process, and (2) requires expensive set-up and highly skilled and experienced personnel.

A laser microinjection method of gene transfer into plants has been described by Hattori et al. (1991). The cells irradiated with a Nd-YAG laser beam (ca. 3 μm in diameter), using a 'laser cell processor', were able to take-up DNA from the surrounding liquid containing DNA through the fine aperture. By this method genes can be introduced into protoplasts or cells. However, more work is required to comment on the suitability of this method of gene delivery for genetic transformation of plants.

(iv) *Particle bombardment method*: Particle bombardment (or Biolistics), wherein microscopic tungsten or gold particles (1–3 μm) coated with genetically engineered DNA are explosively hurled into plant cells with high velocity, has become the second most widely used method for plant genetic transformation (Gray and Finer, 1993). The particles, penetrate the cell wall and lodge themselves within the cell and liberate the DNA, leading to the transformation of individual cells of the explant. This method can overcome many of the biological barriers associated with other methods of transformation, such as the host range specificity of the *Agrobacterium* and regeneration of complete plants from protoplasts. The introduction of DNA into organized, morphogenic tissues, such as seeds, embryos or meristems, by particle bombardment, has enabled the transformation of soybean (Christou et al., 1988), wheat (Vasil et al., 1992), rice (Christou et al., 1991) and maize (Gordan Kamm et al., 1990), thus showing the enormous potential of the method (Christou, 1992; Klein et al., 1992).

The first biolistic gun, designed by Sanford et al. (1987) employed a gunpowder charge to propel the DNA coated particles (microcarriers). Subsequently, several other types of guns have been described (Kikkert,

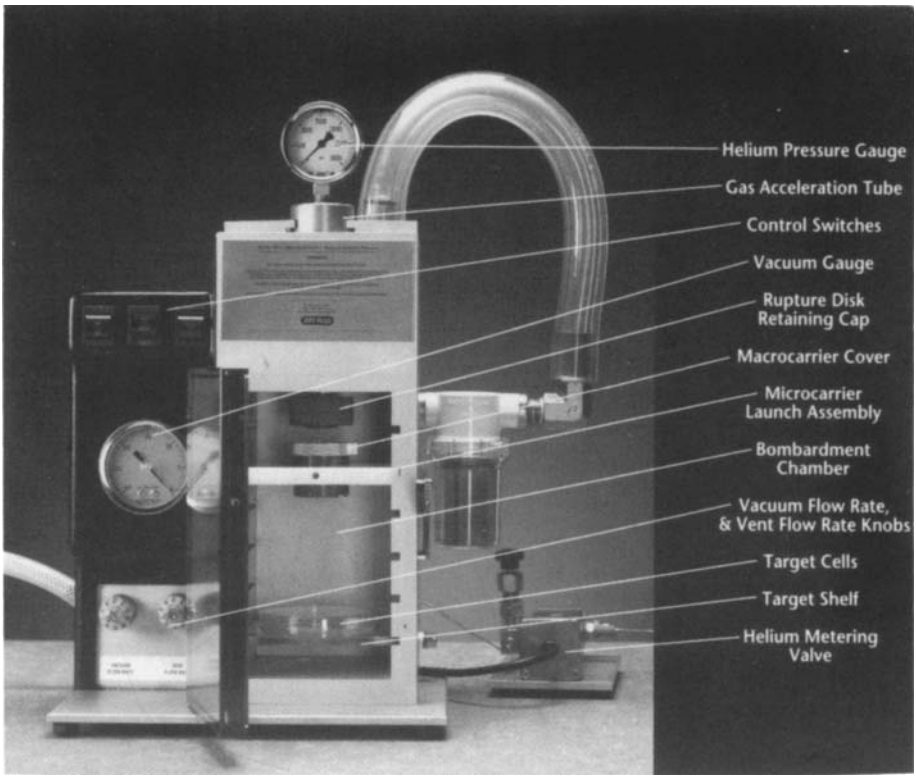


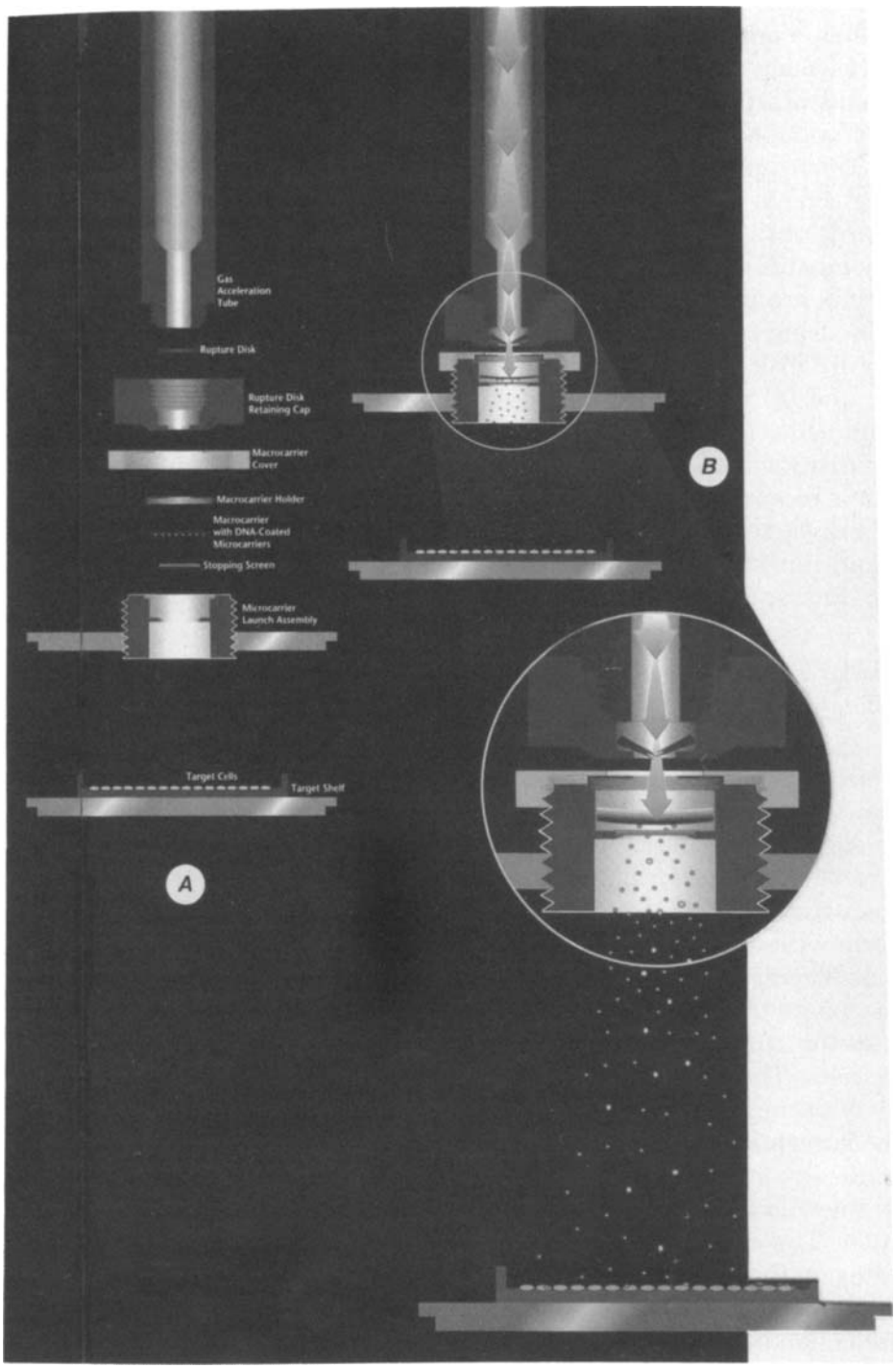
Fig. 14.7. PDS-1000/He Biolistic gun (courtesy of BIO-RAD Laboratories, USA).

1993; McCabe and Christou, 1993; Oard, 1993; Sautter, 1993; Vain et al., 1993) but the only one available commercially (PDS-1000/He), from BIO-RAD, is driven by helium gas.

A PDS-1000/He device (Fig. 14.7) comprises a bombardment chamber, the gas acceleration tube and microcarrier launch assembly (Fig. 14.8). The bombardment chamber is fitted at the bottom with a rupture disk retaining cap which holds the rupture disk in position. The microcarrier launch assembly houses the macrocarrier carrying the DNA coated microcarriers and its holder, the stopping screen and its support.

During operation, first the VAC switch is pressed to evacuate the small high pressure chamber within the gas acceleration tube. When the vacuum gauge registers the required vacuum level (160–760 mm), the switch is put on the hold position. The fire button is then pressed and

Fig. 14.8. Schematic representation of the PDS-1000/He biolistic gun before (A) and upon (B) activation. The arrows indicate the direction of helium flow. For details see text (courtesy of BIO-RAD Laboratories, USA).


Gas
Acceleration
Tube
Rupture Disk
Rupture Disk
Retaining Cap
Macrocarrier
Cover
Macrocarrier Holder
Macrocarrier
with DNA-Coated
Microcarriers
Stopping Screen
Microcarrier
Launch Assembly
Target Cells
Target Shelf
A
B

held, which allows helium pressure to build up in the gas acceleration tube. When a critical pressure is reached the rupture disk automatically bursts releasing a powerful gas shock. The ensuing helium shock drives the second plastic disk (macrocarrier) which separates from its holder and flies unimpeded downwards until it impacts against a rigid stopping screen. Upon impact the macrocarrier deforms and is retained by the stopping screen. However, the microcarriers are launched and continue downwards until they impact and penetrate the target cells at the bottom of the bombardment chamber. Microcarrier velocity and penetration into target cells are influenced by: (1) the helium pressure, which can be adjusted by using rupture disks of different thickness; (2) distance between the rupture disk and the macrocarrier; (3) the distance the macrocarrier travels; and (4) position of the target cells within the sample chamber. For plant cells, 1100 psi (7584 kPa) pressure, 1 cm distance between the rupture disk and the stopping screen, and a microcarrier flight distance of 12 cm is recommended (Kikkert, 1993). The use of gold particles is preferred because the tungsten particles are more heterogeneous in size and shape and tungsten can catalytically degrade DNA over time and it may be toxic (Russell et al. 1992).

14.3. SELECTION AND IDENTIFICATION OF TRANSFORMED CELLS

Genetic selection of transformed cells is an important component of any plant transformation system. In the absence of a proper selection system one would be faced with the option of screening every shoot that regenerates in a transformation experiment. In cases where transformation frequency is high this may be possible but for plant species that transform with low frequencies this would be a laborious, if not impossible, task. Therefore, a selectable marker gene (Table 14.3) is introduced into the plant transformation vectors and a suitable selecting agent is added to the culture medium which favours the growth of only transformed cells. The genes used as selectable markers are dominant and usually of bacterial origin. For successful selection, the target plant cells must be susceptible to relatively low concentration of the selecting agent in a non-leaky manner. The compound that inhibits the growth but does not kill the wild type cells is preferred as a selecting agent in plant transformation. The concentration of the selecting agent used varies widely depending on the sensitivity of the plant species and/or explant source. In some instances, such as potato (Grant et al., 1991), it may be desirable to delay selection until 6–8 days after gene insertion.

TABLE 14.3

Selectable marker genes used in plant transformation (after Draper and Scott, 1991)

Gene	Enzyme encoded	Selective agent(s)	Reference
Antibiotics			
ble	Enzymic activity not known	Bleomycin	Hille et al. (1986)
dhfr	Dihydrofolate reductase	Methotrexate Trimethoprim	Herrera-Estrella et al. (1983)
hpt	Hygromycin phosphotransferase	Hygromycin B	De Block et al. (1984)
npt II	Neomycin phosphotransferase	G418 Kanamycin	Bevan et al. (1983) Herrera-Estrella et al. (1983)
Herbicides			
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones	Haughn et al. (1988)
aro A	5-Enolpyruvylshikimate 3-phosphate synthase	Glyphosate (Roundup)	Comai et al. (1985) Shah et al. (1986)
bar	Phosphinothricin acetyltransferase	Phosphinothricin (Bialaphos)	De Block et al. (1987)

Generally, a screening (also called scorable or reporter) gene (Table 14.4) is also inserted into the transformation vectors which allows for the detection of transformed cells, tissues or plants. The screening markers presently used are mostly derived from bacterial genes coding for an enzyme that is readily detectable through the use of chromogenic, fluorigenic, photon emitting or radioactive substrates. A screening marker gene is useful only if an enzyme with comparable activity is not present in non-transformed cells.

The utility of any particular gene construct as a transformation marker varies depending on the plant species and the tissue involved. To date kanamycin resistance is probably the most widely used selectable marker phenotype and *Uid*A gene (also referred to as *gus*), which encodes β-glucuronidase, is the most versatile reporter gene.

The selected cells and the plants regenerated from them are further subjected to biochemical analyses, such as Southern hybridization (Southern, 1975), PCR and Northern hybridization (Thomas, 1980). The former determines the presence and the number of copies of the introduced gene while the latter demonstrates the presence of transcripts of the transgene.

TABLE 14.4

Screenable marker genes used in plant transformation (after Draper and Scott, 1991)

Gene	Enzyme encoded	Substrate(s) and assays	Reference
CAT	Chloramphenicol acetyl transferase	[^{14}C]chloramphenicol and acetyl CoA; TLC separation of acetylated [^{14}C]chloramphenicol-detection by autoradiography	Herrera-Estrella et al. (1983)
lac Z	β-Galactosidase	As β-glucuronidase; problems with background activity in some species	Helmer et al. (1984)
GUS	β-Glucuronidase	Range of substrates depending on assay; colourimetric, fluorometric, and histochemical techniques available	Jefferson et al. (1986)
lux	Luciferase: bacterial insect	Decanal and $FMNH_2$	Koncz et al. (1987)
		ATP and O_2 and luciferin	Ow et al. (1986)
		Bioluminescent assays: quantitative tests on extracts or in situ tissue assays with activity detected by exposure of X-ray film	
npt-II	Neomycin phosphoryltransferase	Kanamycin and [^{32}P]ATP	Reiss et al. (1984)
		In situ assay on enzyme fractionated by non-denaturing PAGE; enzyme detected by autoradiography	
		Quantitative dot-binding assay on reaction products	McDonnell et al. (1987)

14.4. RECOVERY OF TRANSFORMED PLANTS

In most circumstances the desired product of transformation is the regenerated plants expressing the introduced gene. It is, therefore, important that the cells, tissues and explants selected for transformation exhibit high regeneration frequencies after integration of the foreign gene. The regeneration frequency of the transformed cells is influenced by, besides the factors listed in Chapters 5 and 6, the method of transformation (infection by *Agrobacterium*, trauma of cells by particle bombardment) and the presence of a selecting agent (e.g. an antibiotic) and other antibiotics used for elimination of *Agrobacterium* in the culture medium. Sensitivity of the transformation system to different bacterial strains may also affect the transformation frequency. For example, octopine strains of *A. tumefaciens* are less effective than the nopaline strains of the bacterium for transformation of *Brassica napus* (Fry et al., 1987). Only 2.5% of nearly 1000 soybean cultivars were found to be susceptible to tumour formation by *Agrobacterium* (Wang et al., 1983; Hinchee et al., 1988).

Besides the frequency of regeneration, the type of regeneration is also critical in the recovery of transformed plants. Transformed plants are obtained only if regeneration occurs within the region of the explants where the cells can be in contact with the *Agrobacterium* or other instruments of gene transfer. Regeneration from single cells is desirable to obtain solid transformants.

14.5. GENE EXPRESSION

A promoter that confers high level expression of genes in most cell types, from virtually any plant species tested (monocot or dicot) and, therefore, most widely used for transformation is 35S promoter from cauliflower mosaic virus (Weissing et al., 1988; Benfey and Chua, 1989).

From the point of view of biotechnologists it is important to tailor the expression of introduced gene to the need of the experimental objective. For example, overexpression of a gene product throughout the growth season may unnecessarily deprive the plant resources required for optimum growth and production efficiency or somehow adversely affect the quality of the commodity in question. Therefore, promoters capable of directing gene transcription spacially, temporally or quantitatively in vegetative (Conkling et al., 1990) or floral organs (Koltunow et al., 1990) as well as in response to wound (Thornburg et al., 1987), fruit ripening, light, anaerobic stress, heat shock, cold stress (Hajela et al., 1990) and seed development have been selected (Goldberg et al., 1989; Matzke et al., 1990). Genetic engineering of male sterility, described in Section

14.7.5, is a good example of controlled expression of gene only in the targeted cells of transformed plants.

Promoters are complex multicomponent structures that contain specific *cis* acting sequences with a modular organization (Benfey et al., 1990a,b; Benfey and Chua, 1990; Ohta et al., 1991; Matzke et al., 1990; Cuozzo-Davis et al., 1990). The precise nature of these sequences and their relative position in the promoter are responsible for large scale diversity in plant gene expression (Fisk and Dandekar, 1993). Expression pattern induced by a promoter may also differ with the plant genome in which it is lodged (Yang and Christou, 1990; Terada and Shimamoto, 1990; Benfey et al., 1990a,b).

The present methods of gene insertion into plant genome result in random integration of foreign DNA throughout the genome of the recipient cells. Considerable variation observed between different transformants of the same plant with the same gene construct can result from differences in transgene position in the genome and transgene copy number (Weissing et al., 1988; De Almeida et al., 1989).

14.6. GENETIC STABILITY AND FIELD PERFORMANCE

For commercial applications, the ultimate test of a transgenic plant is its performance in the field. The field trials not only permit determination of the genetic stability and mode of inheritance of the introduced trait but also allows the evaluation of other agricultural characteristics pertinent to field performance and production, such as yield and quality. In most instances the transgenic plants have been produced by inserting the new gene into existing commercial cultivars. In such cases the emphasis of field trial would be not only to evaluate the introduced trait but also determine if any changes have occurred as a result of transformation.

In most countries a prior regulatory approval is required for field introduction of transgenic plants. In the US the Animal and Plant Health Inspection Service of USDA (USDA/APHIS) is responsible for approving field trails of transgenic plants. Of the 284 permits issued between 1988 and 1992 by APHIS for field trials of transgenic plants, 84% pertain to six crops, viz. tomato (57), potato (49), cotton (34), soybean (34), maize (33) and tobacco (32). The tomato cultivar 'Flavr Savr' produced by Calgene is the first genetically engineered plant product to reach US markets.

14.7. APPLICATIONS

One of the most important goals of plant breeding is to produce plants that would help stabilize yields and render their cultivation sustainable

from ecological perspectives. Therefore, significant research and development efforts have been made to produce plants with a high degree of tolerance or resistance to pests (insects, nematodes, etc.) and diseases. The knowledge of the molecular basis of diseases caused by various pathogens has allowed testing different strategies to produce disease resistant transgenic plants. Genetic engineering has also been successful in producing herbicide-tolerant plants. These and some other applications of genetic engineering are discussed in this section.

14.7.1. Virus resistance

Viruses are responsible for over 1400 plant diseases (Beachy, 1993), causing considerable losses in agricultural productivity. In the USA alone viruses destroy tomatoes worth about US$50 million, wheat worth about $95 million, and potatoes worth about $30 million in a single year (Bialy and Klausner, 1986). Unfortunately, there is no viracidal compound to control these diseases. Some diseases, such as rice tungro disease, are caused by two or more distinct viruses and attempts to incorporate genes for resistance against them have not met with success (Beachy, 1993).

Three approaches have been investigated to engineer virus resistance in plants, viz. (1) expression of the virus-coat protein (CP) gene, (2) expression of satellite RNAs and (3) use of antisense viral RNA. Of these the first approach has been most popular (Scholthof et al., 1993).

(i) *Expression of virus coat protein.* CP-mediated resistance (CP-MR) is, presumably, based on the well known phenomenon of cross protection (McKinney, 1929), according to which when a plant is infected by a mild virus strain that produces few or no symptoms, it is protected against superinfection by a severe strain of related virus. This technique has been widely used in agriculture to confer protection against severe virus infection (Costa and Muller, 1980). According to one of the molecular explanations of this phenomenon, first offered by Fulton (1986), CP of the pre-existing (protecting) virus encapsulate the RNA of the superinfecting virus strain, thereby rendering it non-infectious.

Although cross protection has played an important role in reducing crop losses by viral diseases there are several potential disadvantages to the widespread use of cross protection (Buck, 1991): (1) the protecting virus strain might mutate to a more severe form, leading to extensive crop losses, (2) infection of cross protected plants with a second unrelated virus may cause a severe disease by synergistic interaction, (3) protecting

virus strain may cause a low but significant reduction in yields, and (4) in cross protection the protecting virus must be applied each growing season. Most of these problems can be offset by genetic engineering of CP-MR in plants.

Abel et al. (1986) created the first genetically engineered plants showing CP-MR. They produced a c-DNA encoding the capsid protein (CP) sequences of TMV, ligated it to a strong transcriptional promoter (CaMV 35S promoter) and transport sequences to provide constitutive expression of the gene throughout the transgenic plant, and flanked on the 3′ end by poly A signal from the nopaline synthase gene. This chimeric gene was introduced into a disarmed plasmid of *A. tumefaciens* and the modified bacterium was used to infect leaf disc cells of tobacco plants. The plants regenerated from the transformed cells accumulated TMV RNA as well as TMV capsid protein. The selfed progeny of the transgenic plants expressed protection against TMV infection, so that the plants showed fewer sites of infection on the leaves inoculated with the virus. The amount of TMV particles produced by replication after infection was substantially less and the plants did not show systemic disease symptoms at the same rate as the control plants. Often the plants remained completely symptomless and were protected against a number of different TMV strains. The first report of field expression of CP-genes and the associated virus resistance in transgenic tomato plants and their sexual progeny was published by Nelson et al. (1988; Fig. 14.9). Most interestingly, the expression of CP-genes did not adversely affect the growth and yield of the plants, whereas TMV infection of the control plants reduced yield by 25–30% (Nelson et al., 1988).

During the last few years several reports have confirmed that genetically engineered CP-MR is fully or partially effective under field conditions. Selfed progeny of the tomato plants transformed with alfalfa mosaic virus (AlMV) CP-sequences exhibited high level of protection against infection by AlMV (Tumer et al., 1991). Lawson et al. (1990) developed transgenic plants of potato cv. Russet Burbank that expressed CP genes of PVX and PVY. The resistance to PVX was more common than to PVY. Very few lines were resistant to both viruses. Vegetatively propagated progeny of several of these resistant lines were also resistant to PVX under field conditions. A few lines which were resistant to both PVX and PVY were highly productive and gave better yields (Kaniewski el al., 1990). R_1 progeny of transgenic plants of a virus susceptible cultivar of cucumber expressing CP-genes of CMV exhibited 24% infection in the field compared to 70% of the parent cultivar. R-2 progeny of the most resistant of the transgenic R-1 plants showed only 0–5% disease symptoms (Gonsalves et al., 1991).

Fig. 14.9. (A) Greenhouse evaluation of R_1 progeny of the tomato plants transformed with TMV coat protein gene. The control plants (in the foreground) and genetically engineered plants (in the background) were inoculated with PV230 strain of TMV ($10\,\mu g\ ml^{-1}$) 2 weeks after planting and the photographs were taken 4 weeks later. The engineered plants have remained healthy whereas the control plants appear diseased. (B) Field test of tomato plants containing TMV coat protein. Control (right) and engineered (left) plants photographed 4 weeks after virus inoculation as in (A). The fruit yield on control plants was 19.6 kg per plot compared to 62.4 kg per plot on the engineered plants (courtesy of Dr R.T. Fraley, Monsanto Co., USA).

Whereas some virus resistant plants engineered by introducing CP sequences exhibit positive correlation between the degree of protection and the level of CP accumulation (Nejidat and Beachy, 1989; Abel et al., 1990) others do not (Stark and Beachy, 1989; Laimer da Camara Machado, 1992). Transgenic plants of potato showed field resistance to

PVY without showing detectable levels of PVY coat protein (Kaniewski et al., 1990). This also applies to a number of other plants transformed with CP genes (Pang et al., 1992; de Haan et al., 1992). In these cases the protection may be mediated through CP mRNA, independent of accumulation of CP expression per se (Lindbo and Dougherty, 1992; de Haan et al., 1992). Indeed, transcript-mediated virus protection may be preferable to CP-MR because this precluded the need for accumulation of foreign protein.

(ii) *Expression of satellite RNAs.* Some viruses contain, in addition to their genomic RNA, a small RNA molecule termed satellite RNA (S-RNA). The S-RNAs require the presence of a specific 'helper' virus (a closely related virus) for their replication. S-RNA do not encode CP. They are encapsulated in the coat protein of their helper virus or satellite viruses which encode their own coat protein (McGarvey and Kaper, 1993).

The S-RNAs are receiving increasing attention in genetic engineering because of their ability to modify disease symptoms. Most of the S-RNAs reduce the severity of viral infection, presumably through interference with viral replication. Field inoculation of plants with helper virus–S-RNA combinations, which produces symptomless infection, has been practised in China on a large scale (over 10 000 hectares in 16 localities) to protect tomato, a number of pepper varieties, cucumber, eggplant, cabbage and tobacco plants against CMV (Tien et al., 1987; Tien and Wu, 1991).

The first report of S-RNA induced attenuation of viral symptoms involved the introduction of cDNA copies of CMV S-RNA into the genome of tobacco plants (Baulcombe et al., 1986). In these transgenic plants S-RNA was transcribed and when they were challenged with CMV, symptoms in systemically infected leaves were highly attenuated and the growth of the plants was almost normal. The attenuation of symptoms was correlated with the formation and replication of monomeric S-RNA to high levels and suppression of virus multiplication to 5–20% of that in the control. Infection of these plants with closely related tomato aspermy virus (TAV) also induced the amplification of S-RNA, and reduction of symptoms occurred in systemically infected leaves but it was not associated with a reduction in the amount of infectivity, suggesting that here the protection effect of S-RNA may not be solely due to the inhibition of viral replication.

Protection from systemic infection has also been seen in the plants transformed with S-RNA of tobacco ringspot virus (S-TobRV). Gerlach et al. (1987) produced transgenic plants of tobacco containing S-TobRV, either in (+) strand or (–) strand orientation. Infection of these plants with

TobRV caused increase in S-RNA concentration, and disease symptoms appeared on the infected leaf. However, the amount of virus and symptoms were greatly reduced or completely absent in systemically infected leaves. The protection was stronger in plants expressing (+) strand than (–) strand RNA.

Tomato plants susceptible to CMV strain were imparted protection against this virus by introducing S-CARNA 5, a non-necrogenic, attenuating satellite RNA associated with CMV (McGarvey and Kaper, 1993).

The apparent advantage of satellite-mediated tolerance/protection is that it seems to be independent of both the strength of the challenge inoculum (Harrison et al., 1987) and the amount of RNA expressed from the inserted gene (Jacquemond et al., 1988), unlike the other forms of engineered viral resistance, such as coat protein, which can be overcome by high concentrations of virus in the inoculum.

A major drawback of this approach to inducing protection against virus infection is that the attenuating S-RNA may mutate into a harmful variant and cause more severe disease in the source plant; virulent and benign satellite may differ in only a few nucleotides. Secondly, even where the replication of genomic RNA of the helper virus is inhibited, a limited amount of replication of virus is necessary for the replication of S-RNA. In the process, the S-RNA can be encapsulated in the helper virus particle and get transmitted to other plant species where it may cause severe disease.

(iii) *Use of antisense viral RNA.* The use of antisense RNA, which is a RNA molecule complementary to the mRNA (sense RNA) transcribed by a given gene, is another approach suggested for introducing viral resistance in plants. The sense RNA carries codons to synthesize polypeptides with a specified sequence of amino acids. The antisense RNA, on the other hand, does not contain the sequences to produce functional protein. When both sense and antisense RNA are present in a common cytoplasm they anneal to form a duplex RNA molecule which cannot be translated. Using this strategy, transgenic plants expressing antisense RNA to the 3′ region, including CP gene of TMV (Abel et al., 1989), PVX (Hemenway et al., 1988) or CMV (Cuozzo et al., 1988) RNAs were produced which showed some protection against infection with respective viruses or viral RNA. However, the degree of protection was much lower than that obtained with CP-gene. Further investigations revealed that the resistance against TMV RNA was induced by the antisense RNA to the 3′ untranslated region of TMV RNA. The antisense RNA may be interacting with the replicase binding site thereby interfering with virus RNA replication (Buck, 1991).

14.7.2. Resistance to fungi and bacteria

Considering the ecological damage caused by seasonal application of fungicides and bactericides to prevent losses due to the diseases caused by these microorganisms, introduction of genes for resistance in these plants sounds an attractive alternative. Infection of plants with pathogenic microorganisms has been shown to result in the accumulation of novel class of proteins termed 'pathogenesis related proteins' (PRP; van Loon, 1985). Several of these proteins correspond to hydrolytic enzymes, such as chitinase and β-1,3-glucanase. These two enzymes are capable of catalysing hydrolysis of two main carbohydrate components of most fungal walls, viz. chitin and β-1,3-glucan. Various soil microorganisms (bacteria and fungi) also produce these enzymes for their self-defence.

Certain strains of *Serratia marcescens* act as an effective biocontrol for a number of pathogenic fungi (e.g. *Sclerotium rolfsii*) through production and secretion of chitinase enzyme, which is resistant to heat and inhibits fungal growth in vitro. The tobacco plants transformed by introducing bacterial (Jach et al., 1992) or bean (Broglie et al., 1991) chitinase gene exhibited marked tolerance towards attack by *Rhizoctonia solani*, a chitinous, soil-borne fungus that infects numerous plants. Constitutional expression of the chimeric bean endochitinase gene, under the control of 35S promoter, in transgenic tobacco and oilseed rape plants increased protection against *R. solani* (Broglie and Broglie, 1993; Broglie et al., 1991). In these plants the invading fungal hyphae was subjected to chitin breakdown, wall disruption and protoplasm leakage (Benhamou et al., 1993; cited in Broglie and Broglie, 1993).

In addition to the inducible defence proteins associated with vegetative tissues, seeds often contain varying levels of proteins that protect them from insect and fungal attacks. For instance, the seeds of barley contain a ribosome-inactivating protein (RIP) which inhibits protein synthesis in target cells by specific RNA *N*-glucosidase modification of 28S rRNA. RIP is not toxic to its own rRNA but inhibits the growth of a number of plant pathogenic fungi (Leah et al., 1991). By introducing the barley RIP gene, Logemann et al. (1992) produced tobacco plants with enhanced resistance to the fungal pathogen *R. solani*. The gene was expressed under the regulatory control of wound inducible promoter.

Another natural mechanism in plants for resistance against pathogenic organisms is detoxification of the toxin produced by the pathogen (Yoneyama and Anzai, 1993). In most plant diseases caused by microbial attack, development of disease symptoms may result from direct or indirect effect of toxins produced by the pathogen. Since the first report of the *Fusarium* wilt toxin, by Gaumann (1954), a number of host specific and

non-host specific toxins have been identified and isolated from pathogenic bacteria and fungi. The primary site of action of host-specific toxins is related to cell functions, such as cell membrane, mitochondria and chloroplasts. All the non-host specific toxins isolated so far happen to be of bacterial origin.

Tabtoxin, a non-host specific toxin secreted by wildfire bacteria, *Pseudomonas syringae* pv. *tabaci*, shows toxic activity on a wide range of living organisms, from higher plants to algae and animals. To protect itself from the toxin the bacterium produces the enzyme acetyltransferase which inactivates the tabtoxin molecule by acetylation of its amino group. Anzai et al. (1989) isolated the acetyltransferase gene, also called tabtoxin resistance gene (ttr), from *P. syringae* and produced tobacco plants highly resistant to infection by *P. syringae* pv. *tabaci* by inserting the ttr gene (Anzai et al., 1989).

Recently, Hain et al. (1993) have shown the possibility of engineering pathogen resistance by introducing a foreign gene coding for phytoalexin.

14.7.3. Insect resistance

Insects are serious pests of agricultural products in the field and during storage. Even with the widespread use of pesticides about 13% of the harvested crop products are lost to insect pests (Boulter et al., 1990). Insects belonging to the orders Coleoptera, Lepidoptera and Diptera are the most serious plant pests (Gatehouse et al., 1991). Due to the obvious harmful side effects of broad spectrum chemical insecticides, biopesticides are becoming increasingly popular. *Bacillus thuringiensis* (*Bt*), a free-living, Gram-positive soil bacterium, has been employed as an insecticide for over 20 years (Dulmage, 1981) and its demand is continuing to increase because of its specificity towards lepidopteran pests and being environmentally safe (Andrews et al., 1987). However, the major problems in using *Bt* sprays to control insect attack on plants is the high cost of production of *Bt* insecticide and the instability of the protoxin crystal proteins under field conditions, necessitating multiple applications. To contain the problem transgenic plants expressing *Bt* toxin genes have been engineered. Insect resistant transgenic plants have also been created by introducing trypsin inhibitor gene.

(i) *Bt toxin gene expression*. Sporulating *Bt* produces non-toxic proteinaceous endotoxin (protoxin) of 130–140 kDa which upon hydrolysis, under highly alkaline conditions (pH 9–12) of the insect gut, produces smaller insecticidally active peptides of 65–70 kDa (Whiteley and Schnepf, 1986). These polypeptides specifically attack the brush border

membrane of midgut epithelial cells paralysing the insect and eventually killing it (Sacchi et al., 1986; Barton et al., 1987). Different strains of *Bt* are effective against different insects because the endotoxin polypeptides produced by them vary in structure. In some cases more than one type of protoxin may be present in the spore. Each toxin is, however, a single polypeptide produced by a single gene located on the plasmid (Gatehouse et al., 1991). *Bt* toxin is fairly safe to humans and other mammals as they do not provide conditions suitable for the production of toxic polypeptide from non-toxic protoxin crystals.

The first *Bt* gene was cloned and characterized in 1985 (Schnepf and Whiteley, 1985) and since then several research groups have used different *Bt* genes to create insect resistant crop plants. Early studies revealed that *Bt* gene sequence encoding about 610 amino acids in the amino terminal position of the protoxin polypeptide shows the entire insecticidal activity of the intact gene encoding 1176 amino acids (Barton et al., 1987; Vaeck et al., 1987; Perlak et al., 1990), and it was not only unnecessary but deleterious to use the entire *Bt* gene to produce insect resistant transgenic plants. The presence of the entire gene showed very poor expression of toxin sequences and was toxic to plant cell viability (Barton et al., 1987; Barton and Miller, 1993). Therefore, the first transgenic plants showing field resistance to insects were produced by introducing the truncated *Bt* toxin gene coding only 610 amino acids in the amino-terminal portion of the protoxin molecule (Vaeck et al., 1987). Expression of truncated *Bt* toxin gene could be dramatically enhanced by replacing some DNA sequences of *Bt* Cry-I gene by synthetic sequences (Perlak et al., 1990).

The first group to report success in producing insect resistant plants by inserting *Bt* gene was the Belgian biotech company, Plant Genetic Systems (Vaeck et al., 1987). The truncated *Bt* gene inserted into tobacco plants produced enough of the endotoxin to kill the first-instar *Manduca sexta* larvae attempting to feed on their leaves. The insects placed on the leaves of these plants displayed the same response as insects placed on leaves sprayed with commercial *Bt* products, i.e. feeding suppression by 18 h and death within 3 days. The resistance was stably inherited. In the same year Agricetus Co. also produced transgenic insect resistant tobacco plants by introducing *Bt* gene (Barton et al., 1987).

Bt toxin expressing transgenic tomato plants showed reduction in damage to their fruits by *Heliothis zea* (Fischhoff et al., 1987). The efficiency of genetic engineering of these plants was most dramatically demonstrated by a field trial in Florida where they showed substantial protection against tomato pinworm (*Keiferia lycopersicella*) despite heavy infestation of the area with this pest. Delannay et al. (1989) described

Fig. 14.10. Field evaluation of cotton plants transformed with *Bt* gene. Note the striking difference in the yield of the control (A) and transformed (B) plants (courtesy of Monsanto Co., USA).

field resistance of transgenic tomato plants to both tomato pinworm (*Keiferia lycopersicella*) and fruitworm (*Heliothis zea*). Insect resistant transgenic cotton plants (Fig. 14.10) showing segregation of *Bt* gene as a single dominant trait were produced by Monsanto company (Perlak et al., 1990).

Koziel et al. (1993) transformed immature maize embryos with *Bt* gene sequence coding only 648 amino acids in the protoxin. Cry IA (b) gene with 35S promoter or a combination of promoter from maize phospho-enolpyruvate carboxylase (PEPC), a pollen specific promoter, induced a

high level of insecticidal protein. First progeny of the transformed plant showed excellent resistance to repeated heavy infestation with European maize borer under field conditions.

During 1987–1990 transgenic plants of at least four crops, viz. tobacco, tomato, potato and cotton, expressing the *Bt* toxin gene have undergone field trials for insect resistance (Barton and Miller, 1993). Some of these might be ready for release as new cultivars.

(ii) *Trypsin inhibitor gene expression.* The occurrence of certain proteins in plants which inhibit the common mammalian digestive protease (Read and Haas, 1938) have been implicated in natural mechanism of plant defence against insect attack. For example, the leaves of a number of plants, including tomato, synthesize protease inhibitors in response to insect attack. Similarly, cowpea (*Vigna unguiculata*) trypsin inhibitor (CpTI), a 80 amino acid polypeptide, has been identified as an insecticidal component of cowpea seeds, which is active against a range of economically important field and storage pests, including bruchid beetle (*Callosobruchus maculatus*) a major pest of stored cowpea (Gatehouse and Boulter, 1983; Gatehouse et al., 1979).

A research group at Durham University, UK, characterized the CpTI gene, cloned it and prepared a chimeric gene with the 35S promoter from CaMV. Insertion of this gene in tobacco created transgenic plants with enhanced resistance to *Heliothis virescens* (Hilder et al., 1987). Infestation with young larvae of *H. virescens*, which ate away the control plants to the stump, caused very little damage to the transgenic plants, expressing the CpTI gene; the leaf area eaten by the insects was less and the larvae survival was very much reduced. The insecticidal transgenic plants did not suffer in their yield, and the introduced resistance was heritable (Boulter et al., 1990). Johnson et al. (1989) have demonstrated that expression of proteinase inhibitor gene from tomato in transgenic tobacco plants resulted in enhanced resistance to *Manduca sexta*. However, proper field trials of the plants transformed with proteinase inhibitor genes are lacking.

14.7.4. Herbicide tolerance

Weeds compete with crop plants for nutrients, moisture and light and cause considerable decline in the yields and the quality of their end products. Many herbicidal compounds are now available for weed management. Selection of a herbicide depends on its effectiveness against the problem weeds, the crop sensitivity, herbicide treatment and, in many cases, the effect of the herbicide on the environment.

Several classes of herbicides (glyphosate, chlorsulfuron, imazapur, DL-phosphinothricin, bromoxynil, atrazine) are quite effective for broad spectrum weed control. However, as they act by inactivating target proteins (mostly enzymes) essential for vital functions in plants, such as photosynthesis, shared by both crop and weed plants, they are either non-selective and kill the crop plants or cause significant injury to them at the application rates required to eliminate the weeds. Production of herbicide resistant/tolerant crop plants can considerably reduce these losses. Since many possible herbicide resistance gene determinants are single dominant traits and the precise steps in the metabolic pathways affected by some of the herbicides have been determined it has become possible to produce herbicide resistant transgenic plants (Stalker, 1991). Genes coding for herbicide sensitive or insensitive proteins have been isolated from plants and microorganisms and used to produce several herbicide resistant transgenic crop plants.

Three approaches have been followed to obtain herbicide resistant transgenic plants: (1) modification of the plant enzyme or other sensitive biochemical target of herbicidal action to render it insensitive to the herbicide; (2) overproduction of the unmodified target protein permitting normal metabolism to occur even in the presence of the herbicide; and (3) introduction of an enzyme or enzyme system to degrade and/or detoxify the herbicide prior to its action.

The herbicide glyphosate or 'Roundup' (*N*-phosphonomethylglycine), applied as foliar sprays, is rapidly transported to the growing tips and causes cessation of growth in shoot and root tip meristems. At the molecular level glyphosate inhibits the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a shikimate pathway enzyme involved in the synthesis of aromatic amino acids and secondary metabolites.

Petunia cell lines with up to 40-fold enhanced activity of EPSPS enzyme were recovered by transforming the sensitive plants with a chimeric EPSPS gene driven by 35S promoter (Shah et al., 1986). Four plants regenerated from these transformed cell lines survived glyphosate sprays at a concentration of 0.8 lb $acre^{-1}$, which is 2–4 times the dose required to kill all the wild type plants. Glyphosate resistant plants of tobacco (Comai et al., 1985) and tomato (Fillatti et al., 1987) were also obtained by introducing a mutated bacterial gene *aro* A encoding glyphosate resistant EPSP synthase. The transgenic plants were three times more tolerant to glyphosate than the control. However, the tolerance was insufficient for agronomic use because application of 1 kg ha^{-1} of glyphosate caused significant stunting of the plants. The poor tolerance to the herbicide was probably because the bacterial gene lacked the sequence coding for the 72 amino acid extension of the pre-EPSPS which is re-

quired for the transport of this precursor of EPSPS into the chloroplasts where mature 444 amino acid EPSPS is released.

Improved Roundup tolerance in transgenic tobacco plants has been introduced by using other EPSPS genes along with chloroplast transit peptide sequence (Della-Cioppa et al., 1987). Through successive improvement in EPSPS, transgenic soybean (Hinchee et al, 1988, 1993) and *Brassica napus* (Hinchee et al., 1993) plants showing commercially desirable tolerance to glyphosate have been produced.

Phosphinothricin (PPT or 'Basta') is another broad spectrum herbicide which acts by inhibiting the synthesis of glutamine synthase (GS) enzyme involved in assimilation of ammonia produced by nitrate reduction, amino acid catabolism and photorespiration. In the absence of this enzyme, ammonia toxicity damages the plants.

Introduction of alfalfa GS gene regulated by 35S promoter was effective in producing PPT tolerant tobacco plants. These plants regenerated at 20 times the lethal concentration of PPT but showed severe damage when sprayed at four times the normal field application of the herbicide (Eckes et al., 1989). The tissue from the transgenic plants showed overproduction of GS enzyme and 7-fold lower level of free NH_3 relative to the control tissue but grew and reproduced normally.

Basta resistant plants of *Brassica napus*, *B. oleracea* (DeBlock et al., 1989), maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), tobacco, tomato (DeBlock et al., 1987), alfalfa (d'Halliun et al. 1990) and rice (Cao et al., 1992) have also been produced by transforming them with the bacterial gene *bar* which encodes the enzyme phosphinotricin acetyl transferase (PAT). This enzyme catalyses conversion of L-phosphinothricin to *N*-acetyl-L-phosphinothricin (Hinchee et al., 1993). Many of the PPT resistant plants have been field tested and shown to survive the normally used concentration of the herbicide (Hinchee et al., 1993).

14.7.5. Nuclear male sterile lines for hybrid seed production

Natural male sterility may be cytoplasmic (CMS) or nuclear. In hybrid seed production mostly CMS lines have been used which may be unstable under certain environmental conditions or pose other problems, such as linkage of CMS character with sensitivity to *Helminthosporium maydis* in maize.

Recently dominant chimeric genes have been constructed which express a lethal function only in the tapetal cells of anther wall, causing male sterility (Fig. 14.11). Goldberg (1988) described the identification of the gene *TA29* from tobacco which is characterized by its extreme cell specificity in the tapetal cells of young anthers. Introduction of a chimeric

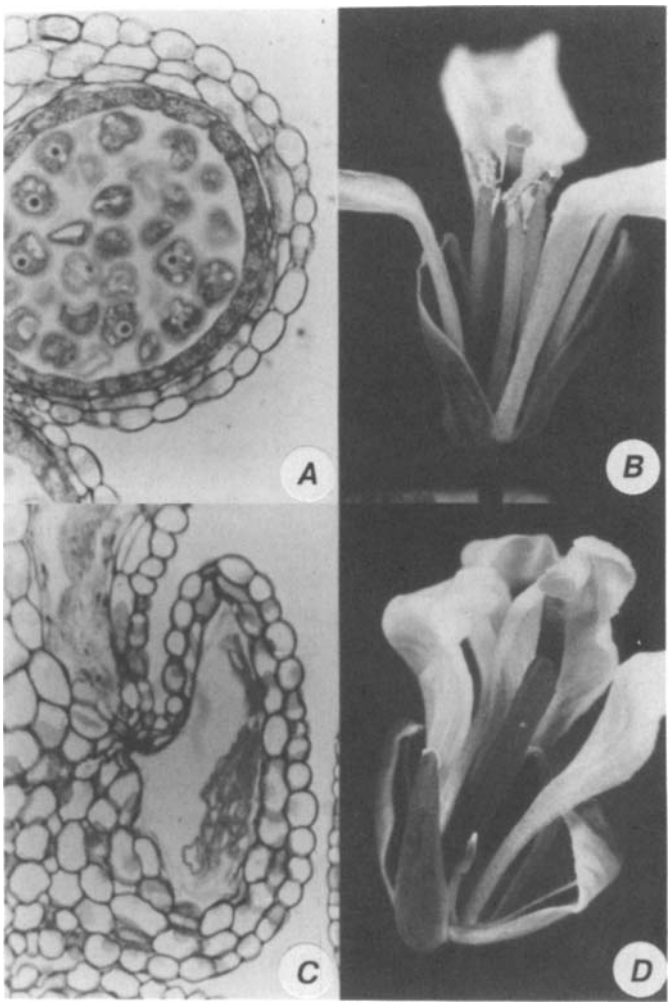


Fig. 14.11. Genetically engineered male sterility in cauliflower by introducing pTA *barnase* gene. (A) Cross-section of an anther from control plant, showing healthy tapetum and well developed pollen grains; (B) a flower from untransformed plant with well developed anthers; (C) cross-section of an anther from transformed plant, showing degenerated tapetum and empty locule; (D) a flower from transformed plant with poorly developed anthers (courtesy Dr A. Reynaerts, Plant Genetic Systems, Belgium).

gene comprising the 5′ regulatory region of TA29 (PTA 29) and a ribonuclease coding gene 'barnase' from *Bacillus amyloliquefaciens* caused precocious degeneration of tapetum cells and arrest of microspore development (Mariani et al., 1990). The promoter *PTA* 29, originally isolated

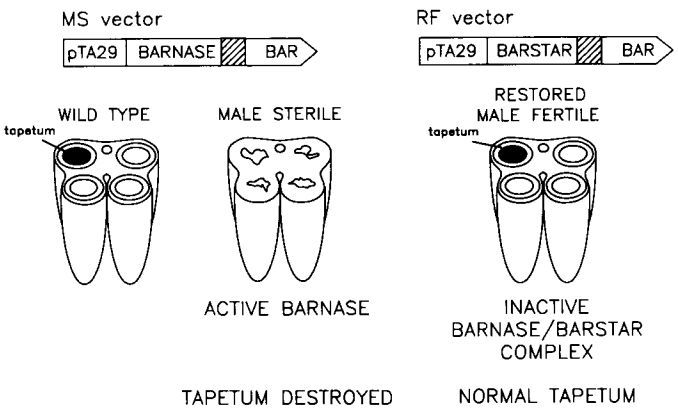


Fig. 14.12. Schematic representation of genetic engineering for fertility control using *barnase* and *barstar* genes (courtesy Dr A. Reynaerts, Plant Genetic Systems, Belgium).

from tobacco maintained its cell specific expression even in other plants and, therefore, could be used to engineer male sterility in other crops including oilseed rape, lettuce, tomato, cotton, maize, cauliflower (Fig. 14.12) and chicory (Mariani et al., 1992; Reynaerts et al., 1993).

Restorer lines for barnase male sterile lines could be engineered by introducing a chimeric gene consisting of *PTA29* promoter fused with the bacterial gene encoding barstar, the intracellular inhibitor of barnase. The progeny of crosses between male sterile lines which express barnase and male fertile lines that are homozygous for barstar produce fully normal pollen, because of simultaneous production of barnase and barstar. The two proteins form a highly stable one-to-one complex (Mariani et al., 1992; Reynaerts et al., 1993).

Figure 14.12 summarises the strategy used by Mariani et al. (1990, 1992) to engineer fertility control in tobacco and some other flowering plants.

As the engineered male sterility is a dominant nuclear trait, back crossing to the original fertile parent results in one to one segregation for fertile and sterile plants. Two approaches have been followed to maintain male sterile lines.

First, a gene coding resistance to the herbicide Basta is inserted along with the barnase gene. Consequently, when the segregating population, just after germination, is sprayed with the herbicide the sensitive male fertile lines are eliminated. Alternatively, homozygous male sterile lines are isolated using the restorer lines.

14.7.6. Control of senescence and fruit ripening

Genetic modification of genes involved in the senescence of cut flowers and the rate and degree of ripening of fruits would be of considerable importance to the horticulture industry. Controlling the metabolic processes which cause ripening and senescence has the potential to improve shelf-life and extend the market for the highly perishable flowers, fruits and vegetables.

Almost simultaneously, Smith et al. (1988) and Sheehy et al. (1988) reported that introduction of an antisense construct based on the genes for polygalacturonase (an enzyme which plays an important role in fruit softening by extensive degradation of the pectin fraction of the cell wall), inhibited softening of tomato fruits. However, the antisense tomatoes showed normal change of colour. Field trials have already been carried out with such engineered plants (Knight, 1989).

The conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, is catalyzed by the ACC synthase enzyme. Expression of antisense RNA of ACC synthase in transgenic tomato plants inhibited the synthesis of ethylene in the fruits. These fruits did not exhibit change of colour, aroma or softening (Oeller et al., 1991) even after 90–120 days after pollination on the plants or in storage. Under similar conditions, the control fruits ripened within 60 days after pollination. Application of 10 μl of ethylene for 15 days induced normal ripening of the engineered fruits.

14.7.7. Future possibilities

Improvement in the quality of plant products and yield of some important primary and secondary metabolites by manipulating the enzymes involved in their biosynthesis is an attractive area for future research. Meyer et al. (1990) produced a new variety of petunia by introducing A1-gene of *Zea mays* encoding dihydroflavanol reductase (DFR). This enzyme imparted brick red colour to the flower by catalysing the conversion of dihydrokampferol into leucopelargonidin-3-glycosides.

In one of the first attempts at genetic engineering of oil composition, seed specific antisense gene constructs of *Brassica rapa* stearoyl-ACP desaturase were used to reduce the protein concentration and activity of the desaturase enzyme in developing rapeseed embryos during storage lipid biosynthesis (Knutzon et al., 1992). This caused a dramatic increase in the level of seed stearate, a desirable trait for certain applications of edible oils, such as margarine formulations and the production of confectionery fats (Kishore and Somerville, 1993).

Most attempts to produce secondary metabolites in tissue cultures have failed either because the cells did not produce the compounds in sufficient quantity or the yields were unpredictable. In nature many secondary plant products (e.g. berberine, hyoscyamine, nicotine) are produced in roots (Chandler and Dodds, 1983; Flores et al., 1987). Secondary production by roots in vitro has been achieved but usually only with media containing growth regulators (Anderson et al., 1982). *A. rhizogenes*-induced hairy root cultures for the synthesis of secondary metabolites have been established for a large number of plant species (Flores et al., 1987; Hamill et al., 1987). The transformed roots, which are highly branched and ageotropic, are grown in flasks, stirred tank bioreactors or in immobilized systems where the culture medium is circulated over stationary roots held in column (Jung and Tepfer, 1987; Payne et al., 1987). Hairy roots can produce the same spectrum of compounds as the non-transformed roots, at a similar or increased concentration (Parr and Hamill, 1987). Secondary metabolite production by transformed roots is stably maintained over many generations (Hunter and Neill, 1990).

During the last 2 years some success has been achieved in engineering abiotic stress tolerance in plants. Transgenic tobacco plants with enhanced salt tolerance, as a result of production and accumulation of mannitol, have been produced by introduction of mannitol-1-phosphate dehydrogenase (*mtlD*) gene from *E. coli* (Tarczynski et al., 1992, 1993). Similarly, introduction of glycerol-3-phosphate acetyltransferase gene from chilling resistant plants into chilling sensitive plants has been shown to impart chilling resistance to the latter by enhancing unsaturated fatty acid content of their chloroplast membrane (Murata et al., 1992). Preliminary observations of Hightower et al. (1991) suggest that it may be possible to inhibit ice crystallization during storage of frozen fruits and vegetables by introducing genes encoding antifreeze proteins.

14.8. CONCLUDING REMARKS

Plant genetic engineering has made possible the transfer of genes across all taxonomic barriers. Genes from bacteria, viruses, insects and even mammals have been introduced into plants in functional form and, in several cases, the introduced genes have shown Mendelian inheritance. This feat is not only of academic interest, to understand the regulation of gene expression, but is revolutionizing the field of genetic improvement of crop plants. This molecular approach to plant breeding is expensive but this disadvantage is offset by the fact that it allows insertion of a specified gene without disrupting the desirable traits of an elite cultivar ('precision breeding'). Genetic engineering also cuts down the

breeding time to produce a new cultivar to almost one-third compared to plant breeding.

Genetic engineering requires diverse expertise. The remarkable progress made in this field during the last decade is the outcome of interdisciplinary efforts of plant cell and molecular biologists, biochemists, chemists, geneticists, plant tissue culturists, engineers, etc.

Commercialization and marketing of agricultural products derived from recombinant DNA technology is now becoming a reality. Genetically engineered tomatoes have already appeared in the American markets (Hamilton and Eliss, 1992). The other transgenic crop varieties to follow shortly are cotton, cucurbits, maize, potatoes, oilseed rape, soybean, etc. The list is expected to grow rapidly as new useful genes become available. With the availability of simple (*Agrobacterium*-mediated) or species non-specific (particle gun, electroporation) methods of gene delivery, which can transform intact cells of explants and organs, the recovery of transgenic plants should not be a serious limitation to crop improvement by genetic engineering.

There has been considerable debate regarding the possible environmental hazards by the introduction of transgenic cultivars in the field. A major concern has been the possibility of the introduced gene escaping with the pollen grains and making the weeds more weedy or the new cultivar itself becoming a super-weed. In this context Crawley et al. (1993) made a detailed study on the ecological performance of herbicide resistant transgenic *Brassica napus* versus the conventional oil seed rape in a variety of habitats and a range of climatic conditions and found no indication that genetic engineering of oilseed rape for kanamycin and herbicide tolerance increased its invasive potential.

Another serious problem associated with transgenic plants is the loss of the introduced trait by inactivation or suppression of transgenes in the first or subsequent generations. This phenomenon seems to be of wide occurrence but has been highlighted only recently (Finnegan and McElory, 1994). To realize the full potential of genetic engineering in agriculture it would be necessary to understand the causes of the instability of transgenes and examine means to stabilize their expression in transgenic plants.

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Chapter 15

Production of Pathogen-Free Plants

15.1. INTRODUCTION

Most of the crop plants, especially those propagated by vegetative means, are systemically infected with one or more pathogens. Strawberry plants, for example, are known to be attacked by 62 viruses and mycoplasmas which makes it essential to replace the mother plants every year (Boxus, 1976). Pathogen attack does not always lead to death of the plant. Many viruses may not even show visible symptoms. However, the presence of viruses in plants can reduce the yield and/or quality of crops (Wang and Hu, 1980). Yield increases up to 300% (averaging 30%) have been reported following replacement of virus-infected stock with specific pathogen-free plants (Murashige, 1980). Eradication of viruses and other pathogens is, thus, highly desirable to optimize the yield and also to facilitate the movement of living plant materials across international boundaries (Button, 1977). While plants infected with bacteria and fungi may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants.

A large number of viruses are not transmitted through seeds. In such cases it should be possible to obtain virus-free plants from infected individuals by using their seeds as the propagules. However, sexually reproduced plants often display genetic variability. Clonal multiplication of cultivars, which is so important in horticulture and silviculture, is generally achieved through vegetative propagation (see Chapter 16). If the complete stock of cultivar is not infected it should be possible to develop healthy nucleus stock by selecting out one or more healthy plants and propagating them vegetatively, but where the entire population of the clone is infected the only way to obtain pathogen-free stock is to eradicate the pathogen from vegetative parts of the plant and regenerate full plants from such tissues. Once a single pathogen-free plant has been obtained it can be multiplied vegetatively under conditions which would protect them from chance reinfection.

It is well known that the distribution of viruses in plants is uneven. In infected plants the apical meristems are generally either free or carry a very low concentration of the viruses (Quak, 1977; Wang and Hu, 1980).

In older tissues the virus titre increases with increasing distance from the meristem tips (Holmes, 1948; Kassanis, 1957). The reasons proposed for the escape of the meristem from virus invasion are (Mathews, 1970; Wang and Hu, 1980): (a) viruses readily move in a plant body through the vascular system which is absent in the meristem. The alternative method of cell-to-cell movement of the virus through plasmodesmata is rather too slow to keep pace with the actively growing tip; (b) high metabolic activity in the actively dividing meristem cells does not allow virus replication; (c) the 'virus inactivating systems' in the plant body, if any, has higher activity in the meristem than in any other region; thus, the meristem is protected from infection; (d) a high endogenous auxin level in shoot apices may inhibit virus multiplication.

The knowledge of the gradient of virus distribution in the shoot tip enabled Holmes (1948) to obtain virus-free plants from infected individuals of *Dahlia* through shoot-tip cuttings. On the same principle, Morel and Martin (1952) developed the technique of meristem-tip culture for in vivo virus eradication. They excised 100 μm long meristem tips of dahlias and cultured them in a nutrient medium. The meristem-tip derived shoots, however, failed to root. To develop full virus-free plants the shoots were grafted onto healthy rootstocks of *Dahlia*. Since then the advances in meristem-tip culture have been such that it has become the most efficient technique for obtaining completely virus-free plants (Belkengren and Miller, 1962; Mullin et al., 1974; Boxus et al., 1977) and it has been successfully applied to a wide range of crop plants (see Table 15.1). Although mainly used for virus elimination, meristem-tip culture has also enabled plants to be freed from other pathogens, including viroids, mycoplasmas, bacteria and fungi (Walkey, 1978; Murashige, 1980). It is now a popular horticultural practice. Before the meristem-tip culture technique was developed the in vivo eradication of viruses was achieved by heat treatment of whole plants (Hollings, 1965).

15.2. TERMINOLOGY

In the literature the term 'virus-free' plants has been loosely used. Plants are often infected with more than one type of virus and may also carry some unknown viruses. Thus, a plant can be claimed as free of only those viruses for which specific tests have given negative results. The use of term 'specific virus-free' or 'specific pathogen-free' plants has been recommended (Hollings, 1965; Langhans et al., 1977; Murashige, 1980). However, if the usage of the term 'virus-free' is still retained by commercial horticulturists for its magic appeal or any other reason it would be desirable that the label further specifies the viruses and/or other patho-

TABLE 15.1

Plant species for which virus-free plants have been obtained by tissue culture techniques[a]

Plant species	Virus eliminated[b]	Reference
Allium sativum (garlic)	GMV	Havranek (1973), Mori (1971a), Mori and Hamaya (1966), Quiot et al. (1972), Wang and Huang (1974)
	OYDV	Havranek (1973)
	GYSV	Bhojwani et al. (1982)
Ananas sativus (pineapple)	Unspecified	Walkey (1978)
Armoracia rusticana	TuMV	Mori and Hamaya (1966)
A. lapathifolia (horse radish)	CIMV, TuMV	Walkey (1978)
Asparagus officinalis	Unspecified	Yang and Clare (1976)
Brassica oleracea	CbBRSV	Paludan (1971)
(cauliflower)	TuMV, CIMV	Walkey et al. (1974)
Buddleia davidii	CMV	Duron and Morand (1978)
Caladium hortulanum (aroid)	Dasheen Mosaic	Hartman (1974)
Chrysanthemum sp.	Chlorotic Mottle	Paludan (1974)
	Complex of viruses	Mori (1971a,b), Mori and Hamaya (1966)
	Green flower	Hakkaart and Quak (1964)
	Stunt	Hollings and Stone (1970), Paludan (1974)
	Tomato Aspermy	Asatani (1972), Paludan (1973)
	Vein Mottle	Asatani (1972), Hakkaart and Quak (1964)
	Virus B	Asatani (1972), Hakkaart and Quak (1964), Hollings (1963), Paludan (1973, 1974), Quak and Hakkaart (1966)
Colocasia esculenta (taro)	Dasheen Mosaic	Hartman (1974)
Cymbidium sp.	Cymbidium Mosaic	Morel (1960), Nyland and Milbrath (1962)
Dahlia spp.	Complex of viruses	Mori (1971b)
	Dahlia Mosaic	Morel and Martin (1952), Mori (1971a), Mori and Hamaya (1966), Mullin and Schlegel (1978), Paludan (1971)
	Tomato Aspermy	Asatani (1972), Paludan (1973)

TABLE 15.1 (*continued*)

Plant species	Virus eliminated[b]	Reference
	Vein Mottle	Asatani (1972), Hakkaart and Quak (1964)
	Virus B	Asatani (1972), Hakkaart and Quak (1964), Hollings (1963), Paludan (1973, 1974), Quak and Hakkaart (1966)
Daphne sp.	AMV, CMV, RbRSV	Sweet et al. (1979)
D. odora	Daphne Virus S	Cohen (1977)
Dianthus barbatus	Latent, Mottle, Ringspot, Vein Mottle	Stone (1968)
D. caryophyllus (carnation)	Complex of viruses	Mori (1969, 1971a,b)
	Etched Ring	Paludan (1970, 1971)
	Latent	Stone (1968)
	Mottle	Hollings and Stone (1964), Maia et al.(1969), Paludan (1971), Stone (1963, 1968), Kowalska (1974)
	Streak	Paludan (1971), Phillips (1962)
	Ringspot	Hollings and Stone (1965), Stone (1968)
	Unidentified	Maia et al. (1969), Mori and Hamaya (1966), Mitrofanova et al. (1977)
	Vein Mottle	Paludan (1973), Paludan and Begtrup (1974), Stone (1968)
Forsythia × *intermedia*	Unspecified	Duron (1977)
Fragaria sp. (strawberry)	Complex of viruses	Mori (1971a,b), Mori and Hamaya (1966), Quak (1964), Wang and Huang (1975)
	Crinkle	Kacharmozov and Izvorska (1974), Miller and Belkengren (1963), Vine (1968)
	Edge	Kacharmozov and Izvorska (1974)
	Latent A	Ben-Jaacov and Langhans (1972), Smith et al. (1970), Vine (1968)
	Latent C	McGrew (1965)
	Mottle	Kacharmozov and Izvorska (1974), Mullin et al. (1974)

	Pallidosis	Mullin et al. (1974)
	Strawberry Yellow Edge	Miller and Belkengren (1963)
	Vein Banding	Miller and Belkengren (1963)
	Yellow virus complex	Miller and Belkengren (1963)
	Vein Chlorosis	Smith et al. (1970), Vine (1968)
	Yellow Edge	Mullin et al. (1974)
Freesia sp.	FrMV	Brants and Vermeulen (1965)
	Freesia Virus I	Paludan (1971)
	Phaseolus Virus 2	Brants (1968)
Gladiolus spp.	Unidentified viruses	Simonsen and Hildebrandt (1971)
Glycine max (soybean)	SMV	White et al. (1977)
Hippeastrum spp. (amaryllus)	Mosaic	Nowicki and O'Rourke (1974)
Humulus lupulus (hop)	Hop Latent and Necrotic Ringspot	Adams (1975), Gippert et al. (1974), Vine and Jones (1969)
	Mosaic	Schmidt (1974)
Hyacinthus spp.	HyMV	Asjes et al. (1974) Van Slogteren (1966)
	Lily Symptomless	Asjes et al. (1974)
Hydrangea macrophylla	Hydrangea Ringspot	Paludan and Christensen (1973)
Ipomoea batatas (sweet potato)	Feathery Mottle	Mori (1971a,b),
	Hanmon Mosaic	Ikegami et al. (1964)
	Internal Cork	Mori (1971a, b), Nielsen (1960)
	Rugosa Mosaic	Mori (1971a, b), Mori and Hamaya (1966)
	Synkuyo Mosaic	Mori et al. (1963a)
	Unidentified	Alconero et al. (1975), Over De Linden and Elliott (1971)
Iris spp.	IMV	Baruch and Quak (1966), Mori (1971a), Mori and Hamaya (1966), Paludan (1971)
	Unidentified	Mori (1971b)
Lavendula spp.	Dieback	Walkey (1978)
Lilium spp.	CMV	Mori (1971b), Mori and Hamaya (1966)
	MyMV, Lily Symptomless	Asjes et al. (1974)
	Latent	Paludan (1971)
	LMV	Asjes et al. (1974), Mori (1971b), Mori and Hamaya (1966)
	Unidentified	Allen (1974), Nishizawa and Nishi (1966)
Lolium multiflorum (ryegrass)	RgMV	Dale (1977)

TABLE 15.1 (*continued*)

Plant species	Virus eliminated[b]	Reference
Malus sp. (apple)	Latent viruses	Campbell (1962)
Malus pumila (apple)	ACSLV	Hansen and Lane (1985)
Manihot sp. (Cassava)	African Cassava Mosaic, Cassava Brown Streak Mosaic	Kaiser and Teemba (1979)
	Unidentified	Kartha and Gamborg (1975)
Musa sp. (banana)	CMV, unidentified	Kartha et al. (1974a)
Musa acuminata × *M. balbisiana*	MMV	Berg and Bustamante (1974)
Narcissus tazetta	AMV	Gupta (1986)
	Narcissus Degeneration	Paludan (1971), Stone (1973), Stone et al. (1975)
Nasturtium officinale (watercress)	CMV, CIMV, TuMV	Stone (1973), Stone et al. (1975)
Nerine sp.	Nerine Latent, Unidentified	Walkey and Thompson (1978)
Nicotiana rustica	AlfMV, CMV	Hakkaart et al. (1975)
	ChLRV, AMV, Tobacco Ringspot	Walkey and Cooper (1975)
N. tabacum	Dark-green islands of TMV	Walkey (1978)
	TMV	Murakishi and Carlson (1976)
Ornithogalum	OMV	White et al. (1977)
Pelargonium sp.	CMV, Tomato Black Ringspot	Veelar et al. (1992)
	Tomato Ringspot	Gippert and Schmelzer (1973)
	Unidentified	Gippert and Schmelzer (1973), Paludan (1971), Pillai and Hildebrandt (1968)
Petunia sp.	TMV	Hakkaart and Hartel (1979), Pillai and Hildebrandt (1968)
	Tobacco Necrosis	Mori (1971a,b), Mori and Hamaya (1966)
Polyanthes tuberosa	Mosaic	Walkey (1978)
Ranunculus asiaticus	Unidentified	Wang and Hu (1980)
Rheum rhaponticum (rhubarb)	Tobacco Rattle, CMV, ChLRV, Strawberry Latent Ringspot, TuMV	Maia et al. (1973)
Ribes grossularia (gooseberry)	Vein Banding	Walkey (1968)
		Jones and Vine (1968)

Rubus ideaus (raspberry)	Mosaic	Putz (1971)
Saccharum officinarum (sugarcane)	Mosaic	Leu (1972), Mori (1971a,b), Mori and Hamaya (1966)
Solanum melongena (eggplant)	EMCV	Raj et al. (1991)
Solanum tuberosum (potato)	Leaf Roll	Mori (1971a,b), Mori et al. (1963b), Mori and Hamaya (1966), Wang and Loo (1973)
	Paracrinkle	Kassanis (1957)
	PAMV	Dhingra et al. (1982)
	PSTV	Lizarraga et al. (1980)
	PVA	Kassanis and Varma (1967), Morel and Martin (1955), Morel et al. (1968), Sip (1972), Wang and Huang (1975), Mori and Hamaya (1966)
	PVG	Mori (1971a,b) Phillips and Danielson (1961), Mori and Hamaya (1966)
	PVM	Morel et al. (1968), Pett (1974), Wang and Loo (1973)
	PVS, PVX	Mellor and Stace-Smith (1970), Mori and Hamaya (1966), Murakishi and Carlson (1976), MacDonald (1973), Stace-Smith and Mellor (1968)
	PVY	Mori and Hamaya (1966), Wang and Loo (1973)
Vitis vinifera (grapevine)	GFLV	Monette (1986a)
	AMV	Monette (1986a)
Xanthosoma brasiliensis (cocoyam)	Unidentified	Hartman (1974), Startisky (1974) Walkey and Webb (1968)
Zingiber officinale (ginger)	Mosaic	Wang and Hu (1980)

[a]The main sources of information provided in this table are Quak (1977), Walkey (1978) and Wang and Hu (1980).

[b]ACLSV, Apple Chlorotic Leafspot Virus; AlfMV, Alfalfa Mosaic Virus; AMV Arabis Mosaic Virus; CbBRSV, Cabbage Black Ringspot Virus; ChLRV, Cherry Leaf Roll Virus; CIMV, Cauliflower Mosaic Virus; CMV, Cucumber Mosaic Virus; EMCV, Eggplant Mottled Crinkle Virus; GFLV, Grapevine Fanleaf Virus; GMV, Garlic Mosaic Virus; GYSV, Garlic Yellow Streak Virus; HyMV, Hyacinth Mosaic Virus; IMV, Iris Mosaic Virus; LMV, Lily Mosaic Virus; MMV, Musa Mosaic Virus; NDV, Narcissus Degeneration Virus; OMV, Ornithogalum Mosaic Virus; OYDV, Onion Yellow Dwarf Mosaic Virus; PAMV, Potato Aucuba Mosaic Virus; PSTV, Potato Spindle Tuber Viroid; PV, Potato Virus; RbRSV, Raspberry Ringspot Virus; RgMV, Ryegrass Mosaic Virus; SMV, Soybean Mosaic Virus; TMV, Tobacco Mosaic Virus; TuMV, Turnip Mosaic Virus.

gens whose absence has been established through reliable tests. In this text, for the sake of convenience, 'virus-free' has been used in the sense of 'specific virus-free'.

15.3. VIRUS ELIMINATION BY HEAT TREATMENT

Thermotherapy has been effectively used for a long time to obtain virus-free plants from infected individuals of diverse plant species (Hollings, 1965). The basic principle behind heat eradication of viruses is that at temperatures higher than normal many viruses in plant tissues are partially or completely inactivated with little or no injury to the host tissues (Baker, 1962) (see Fig. 15.1). The host plant also seems to contribute in some way to the in vivo inactivation of viruses during heat treatment (Harrison, 1956; Gay and Kuhn, 1967).

Heat treatment is given through hot water or hot air; whereas hot-water treatment has proved better for dormant buds, hot-air treatment has generally given better elimination of viruses and better survival of the host in actively growing shoots (Hollings, 1965). For hot-air treatment, which is comparatively easy to apply, actively growing plants are transferred to a thermotherapy chamber and exposed to a temperature of 35–40°C for a suitable period. The duration of the treatment varies from a few minutes to several weeks. Baker and Kinnaman (1973) eradicated all viruses from carnation shoot tips by continuous treatment of plants at 38°C for 2 months. On the other hand, Potato Virus X (PVX) required several months of treatment at 35°C to obtain some virus–free tips (Stace-Smith and Mellor, 1968). Small cuttings are taken from the shoot tips immediately after the heat treatment and grafted onto healthy rootstock.

In heat treatment the temperature of the air should be gradually raised during the first few days until the desired temperature is reached. If continuous exposure to the high temperature, required for inactivation of viruses, proves harmful to the host tissue it would pay to try diurnal cycles of low and high temperatures (see Section 15.4.3). An adequate supply of humidity and light should be maintained during heat treatment. Baker and Kinnaman (1973) have recommended that for carnation a relative humidity of 85–95% must be maintained during heat treatment. The plants, to be subjected to heat treatment, must have ample carbohydrate reserves which can possibly be achieved by pruning back of the plants (Wang and Hu, 1980). Hollings (1965) has also remarked that pruning back increases the ability of a plant to withstand heat treatment.

A major limitation of thermotherapy for virus eradication has been that not all viruses are sensitive to the treatment. For example, in potato

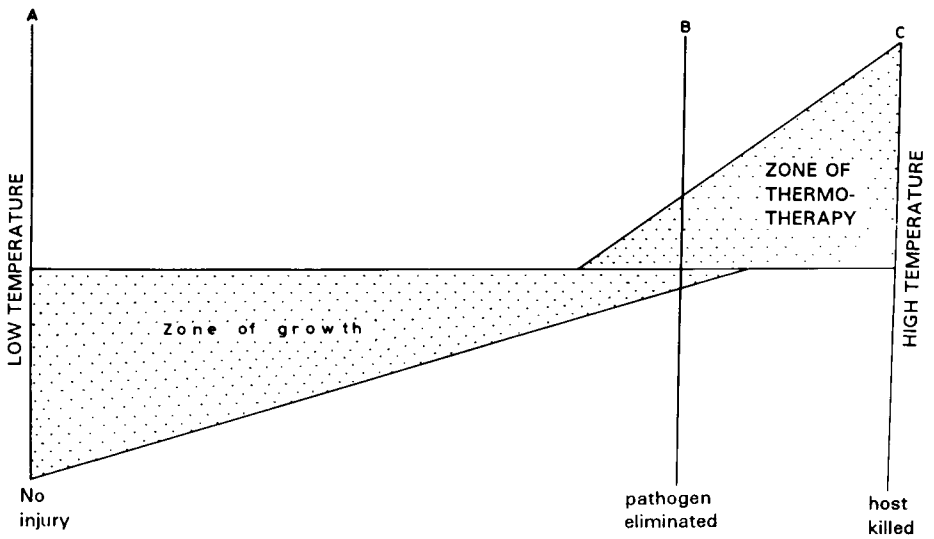

Fig. 15.1. Diagram showing the relationship of the zone of plant growth to the zone of thermotherapy. The area B–C is critical in heat treatment; the greater the spread between the thermal death point of the host (C) and the parasite (B), the greater are the chances for successful thermotherapy (after Baker, 1962).

only Leaf Roll Virus could be eradicated by this technique (Kassanis and Varma, 1967). Heat treatment has usually been effective against isometric and thread-like viruses and against diseases now known to be caused by mycoplasmas (Quak, 1977). Prolonged heat treatment of host plants may also inactivate the resistance factor(s) in the plants over the control (Mellor and Stace-Smith, 1970; Walkey, 1978). Moreover, only a small proportion of plants survive after heat treatment.

In contrast to thermotherapy alone, meristem-tip culture is widely applicable. Many viruses which could not be eradicated by heat treatment alone have been eliminated by shoot-tip culture alone or in combination with thermotherapy and/or chemotherapy (Raychaudhury, 1966; Mathews, 1970). Meristem-tip culture has, therefore, become a very popular approach to virus elimination.

15.4. VIRUS ELIMINATION BY MERISTEM-TIP CULTURE

15.4.1. Explant terminology

Horticulturists and plant pathologists who have attempted to recover pathogen-free plants through tissue culture techniques have indiscrimi-

nately designated the explants used to initiate cultures as 'shoot tip', 'tip', 'meristem' and 'meristem tip'. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium (Cutter, 1965); it measures up to about 100 μm in diameter and 250 μm in length (Quak, 1977). The apical meristem together with one to three young leaf primordia, measuring 100–500 μm, constitute the shoot apex (Cutter, 1965; Hollings, 1965). Although the chances of eradicating viruses is higher through 'meristem' culture, in most published works virus-free plants have been raised by culturing 100–1000 μm long explants which could be, according to the above definition, referred to as 'shoot tips'. To distinguish it from the in vivo technique of propagation through shoot-tip cuttings, the term 'meristem-tip' culture has been preferred for in vitro culture of small shoot tips.

15.4.2. Technique

Meristem-tip explants of the size recommended for virus elimination are too small to be prepared with unaided eyes. It requires a simple stereoscopic microscope (8–40× magnification) provided with a suitable light source. Besides other paraphernalia normally necessary for the aseptic culture of plant tissue (see Chapter 2), a set of needles and blades (may be fragments of razor blades with sharp and smooth edges) mounted on separate handles of suitable lengths are required to perform dissections. If a sterile hood is not available it may be possible, under certain conditions, to perform dissections in a dust-free room. The area to be used for excision of meristem-tips must be sprayed and the platform of the dissecting microscope and the working bench top swabbed with 75% ethanol.

Care must be taken to prevent desiccation of shoot tips due to a constant flow of air through the laminar flow cabinet. During dissections the exposure of the meristem tips should be as brief as possible. Use of a cool light illuminator (fluorescent lamp) or glass fibre lamp is more desirable. Performing dissections in a petri plate lined with sterile, moist filter paper also helps to retard desiccation of the small explants.

As with other types of tissue cultures, the first essential step in raising meristem-tip cultures is to obtain explants free of surface pathogens. Usually the meristem-tips are so well protected with overlapping leaf primordia that careful dissection should permit excision of aseptic explants without a surface sterilization treatment. Indeed, sometimes disinfection treatments increase the incidence of contaminated cultures (Hollings, 1965; D. Choen, pers. commun.). If possible, the experimental plants should be grown in a sterilized potting mix and maintained in a

glasshouse. It is important that the plants are watered directly on the soil rather than on the foliage. Periodic spraying of the plants with systemic disinfectants is recommended. Wang and Hu (1980) used a spray mixture comprising the fungicide Benlate (0.1%) and antibiotic streptomycin (0.1%). The application of such a mixture is especially important for field-grown materials. With some field-grown materials it may be possible to take small cuttings and grow them in Knop's solution in the laboratory. The shoots arising from axillary buds on these cuttings would be considerably superior to those taken directly from the field-grown plants in so far as the contamination problem is concerned.

Despite the high degree of sterility maintained around meristem tips, generally the shoot buds are surface-sterilized before excising explants. Wang and Hu (1980) have recommended that buds with densely covered leaves, such as chrysanthemum, pineapple, ginger and orchids, are surface-sterilized by a quick dip in 75% ethanol, and buds with loosely covered leaves, such as garlic, carnation and potato, are surface-sterilized with 0.1% sodium hypochlorite for 10 min. The ingenuity of the worker should enable him to suitably modify the recommendations (see Chapter 2) to suit the experimental system under study. For meristem-tip cultures of garlic, Bhojwani et al. (1982) followed a method in which cloves were dipped in 95% ethanol and flamed before dissecting out sterile shoot buds.

At the time of dissecting out meristem-tips the bud is held under the microscope in one hand with the help of a fine pair of forceps, and leaves and leaf primordia are removed using fine needles. The needles should be frequently sterilized. When approaching the apical meristem-tip separate sterile needles should be used to remove individual leaf primordium. To allow a needle to cool down sufficiently before reuse, a set of at least three needles must be available. Alternatively, the needle can be cooled by dipping in sterile distilled water. When the apical meristem, which appears as a shiny dome, is sufficiently exposed, excise the meristem with or without leaf primordia (see Fig. 15.2), giving a clean cut by sharp blade mounted on a large handle. The same instrument may be used to transfer the tip to culture medium. It is important to make sure, especially when the buds have not been surface-sterilized, that the excised meristem-tip explants do not come in contact with the older parts of the bud or the platform used for dissection or the forceps holding the bud. The orientation of meristem-tip on the medium does not seem to be critical (Wang and Hu, 1980).

The shoots derived from meristem-tips often root in the original medium but if this does not happen a different treatment has to be tried (for details of in vivo rooting of cultured shoots, see Chapter 16). Occasion-

Fig. 15.2. Photograph of a shoot tip of potato with two leaf primordia (after Morel, 1972).

ally, as experienced with *Dahlia* by Morel and Martin (1952), the shoots developed in cultures may fail to root under any treatment. In such cases whole virus-free plants have been obtained by grafting the virus-free shoots onto healthy rootstocks (see Section 15.5 and Appendix 15.II).

15.4.3. Factors affecting virus eradication by meristem-tip culture

Factors such as culture medium, explant size and culture storage, which effect plant regeneration from small (100–1000 μm) excised meristem-tips, will considerably influence virus eradication by this method. In addition, thermotherapy or chemotherapy during culture significantly influences the efficiency of this technique. The physiological stage of the explants also affects virus elimination by shoot-tip culture.

(i) *Culture medium.* The success in obtaining complete plants can be considerably improved by the right choice of the culture medium. The major features of the culture medium to be considered are its nutrients, growth regulators and physical nature.

During the early days of shoot-tip culture (Morel, 1948; Morel and Martin, 1952) the major elements of the media used for meristem-tip culture were derived from the recipes of White's (1943) and Gautheret's (1959) media. To these the minor elements of Berthelot's (1934) or Heller's (1953) medium were added. However, Morel et al. (1968) found that these media were poor in the levels of certain ions, especially K^+ and NH_4^+. In Gautheret's medium the shoot tips of potato did not grow more

than a few millimetres, and looked chlorotic. With a five-fold increase in the level of K^+ ions in the medium rapid development of sturdy shoots occurred. Most of the commonly used culture media are fairly rich in essential elements. For potato meristem-tip culture, Stace-Smith and Mellor (1968) tested the media after White (1954), Kassanis (1957), Murashige and Skoog (1962) and Morel and Muller (1964), and obtained survival frequencies of 16, 16, 67 and 36%, respectively. Shoots developed on Murashige and Skoog's medium were very healthy, while those on the other three media were slender and chlorotic. The suitability of MS medium for meristem-tip culture has also been emphasized by other workers (Kartha, 1975; Quak, 1977; Wang and Hu, 1980).

There has been no critical assessment of the role of various vitamins and amino acids normally added to culture media. Sucrose or glucose, in the range 2–4%, have been commonly used as the carbon source. The composition of some of the media used for virus elimination by meristem-tip culture is given in Table 15.2.

Large meristem-tip explants, measuring 500 μm or more in length, may give rise to some entire plants in a medium lacking growth regulators (Mellor and Stace-Smith, 1970) but generally the presence of a small amount (0.1–0.5 mg l^{-1}) of an auxin or a cytokinin or both is beneficial. In angiosperms the meristematic dome in the shoot tip is not autonomous for auxin and it is not the source of auxin in the plant (Smith and Murashige, 1970; Shabde and Murashige, 1977). Auxin is probably synthesized by the second pair of youngest leaf primordia. Accordingly, for the successful culture of meristem explants (without any leaf primordia) of *Coleus blumei*, *Daucus carota*, *Nicotiana tabacum*, *N. glauca*, *Tropaeolum majus* (Smith and Murashige, 1970) and *Lilium candidum* (Riviers, 1973) the presence of exogenous auxin was essential. Both cytokinin and auxin were required for the development of excised apical meristems of *Dianthus caryophyllus* (Shabde and Murashige, 1977). Probably the plants requiring only auxin have a high endogenous cytokinin level in their meristems. Among auxins the use of 2,4-D should be avoided because it generally promotes callusing of the explants. NAA and IAA have been widely used. Of these, NAA is considered more effective due to its better stability.

The role of GA_3 in meristem-tip cultures was emphasized by Morel et al. (1968). They reported that in *Dahlia* the presence of 0.1 mg l^{-1} GA_3 suppressed callusing but favoured better growth and differentiation. GA_3 in combination with BAP and NAA has been shown to be essential to raise full plants from excised meristem tips (200–500 μm) of cassava (*Manihot esculenta*; Kartha et al., 1974a). However, other workers have found GA_3 to be without an appreciable effect and, at higher concentra-

TABLE 15.2

Composition of some culture media used for shoot-tip culture of crop plants

Constituents	Media (amount in mg l^{-1})[a]							
	1	2	3	4	5	6	7	8
NH_4NO_3	–	–	–	–	–	60	60	–
KNO_3	125	125	125	200	125	–	–	125
$(NH_4)_2SO_4$	–	–	–	–	1000	–	–	–
KCl	–	–	–	–	1000	80	80	–
$CaCl_2 \cdot 2H_2O$	–	500	500	–	–	–	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	500	–	–	800	500	170	170	500
$MgSO_4 \cdot 7H_2O$	125	125	125	200	125	240	240	125
KH_2PO_4	125	125	125	200	125	40	40	125
$FeCl_3 \cdot 6H_2O$	–	–	–	–	1	–	–	–
Fe·citrate	–	–	–	–	5	5	–	–
$Fe(SO_4)_3$	–	25	–	–	–	–	27.8	25
Na_2·EDTA	–	–	–	–	–	–	37.3	–
$MnSO_4 \cdot 4H_2O$	↑	0.8	↑	–	0.1	–	22.3	1
$ZnSO_4 \cdot H_2O$	|	0.04	|	0.2	1	0.05	8.6	0.05
$NiCl_2 \cdot 6H_2O$	|	0.025	|	0.3	–	–	–	0.025
$MnCl_2 \cdot H_2O$	|	–	|	1.8	–	0.4	–	–
$CoCl_2 \cdot 6H_2O$	(10	0.025	(10	–	–	–	0.025	0.025
$CuSO_4 \cdot 5H_2O$	drops of	0.025	drops of	0.08	0.03	0.05	0.025	0.025
$AlCl_3$	Berthelot	–	Berthe–	–	0.03	–	–	–
$H_2MoO_4 \cdot H_2O$	soln.)[b]	–	lot	0.02	–	0.02	–	–
$Na_2MoO_4 \cdot 2H_2O$	|		soln.[b]	–	–	–	0.025	–
KI	|	0.25	|	–	0.01	–	0.83	0.25
H_3BO_3	↓	0.025	↓	2.8	1	0.6	6.2	0.025
Myo-inositol	0.1	0.001	0.1	–	100	0.1	0.1	–
Ca·pantothenate	10	0.001	10	–	1	10	10	–
Nicotinic acid	1	–	1	5	1	1	1	–
Pyridoxine·HCl	1	–	1	1	1	1	1	–
Thiamine·HCl		0.001	–	1	1	1	1	1
Biotin	0.1	0.001	0.01	–	0.01	0.01	0.01	–
Cystein	–	0.001	10	–	1	10	10	–
Adenine	–	–	–	–	0.1	5	5	–
$AdSO_4$	–	–	–	–	–	–	–	8
Casein hydrolysate	–	–	1	–	–	1	1	–
Sucrose	20000	–	20000	30000	20000	–	–	–
Glucose	–	40000	–	–	–	10000	30000	40000

[a]After: 1, Morel (1948); 2, Morel and Martin (1955); 3, Kassanis (1957); 4, Nielsen (1960); 5, Morel and Muller (1964); 6, Mori (1971b); 7, Wang and Huang (1975); 8, Baker and Kinnaman (1973).

[b]Berthelot solution contains (mg l^{-1}): $MnSO_4$ (2000), $NiSO_4$ (60), TiO_2 (40), $CoSO_4$ (60), $ZnSO_4$ (100), $CuSO_4$ (50), $BeSO_4$ (100), H_3BO_3 (50), $Fe_2(SO_4)_3$ (50 000), KI (50), H_2SO_4 (specific gravity 1.83) (1 ml).

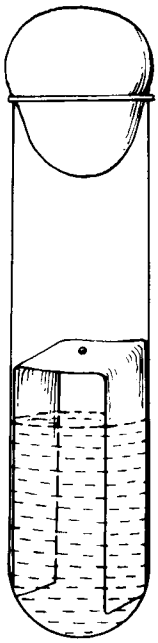

Fig. 15.3. Filter-paper bridge technique of culturing excised shoot tip in liquid medium.

tions, even inhibitory (Mellor and Stace-Smith, 1969; Shabde and Murashige, 1977).

Both liquid and semi-solid media have been tried for meristem-tip culture but, because of the convenience in handling, agar medium is generally preferred. However, in those cultures where agar medium induces callusing of the explants the use of a liquid medium is recommended (Stone, 1963; Baker and Kinnaman, 1973). For the liquid cultures a filter-paper bridge is prepared and inserted into the culture tube in such a way that the two arms are dipping into the medium and platform on which the explant is placed remains above the medium (see Fig. 15.3).

(ii) *Explant size.* Under optimal culture conditions the size of the explant determines the survival of the meristem tips. The larger the explant, the greater are the chances of plant regeneration. In cassava, only 200 μm explants formed complete plants (Kartha and Gamborg, 1975) smaller tips either callused or formed only roots. The small size of the explant may also have an adverse effect on the rootability of the shoots. In the present context, however, the survival of the explants cannot be treated independent of the efficiency with which virus elimination is achieved, which is inversely related to the size of the explant. Thus, explants should be small enough to eradicate viruses and large enough to be able to develop into a complete plant.

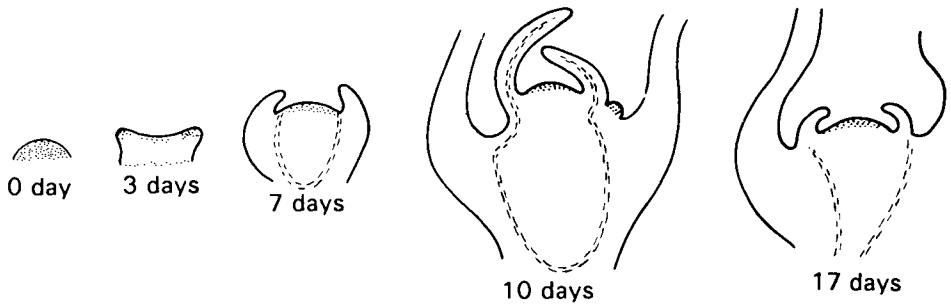

Fig. 15.4. Diagram showing plant development from excised apical meristem of carnation cultured on MS medium supplemented with kinetin and IAA; stages from 0 to 17 days after culture are shown. Root and shoot axis is established within 7 days after culture and multiple leaves are evident after 10 days (after the data of Shabde and Murashige, 1977).

Besides the size of the explant the presence of leaf primordia influences the ability of the meristems to form plants. In rhubarb it was essential to excise shoot meristems with two to three leaf primordia (Walkey, 1968). Smith and Murashige (1970) and Shabde and Murashige (1977), who demonstrated the potential of isolated shoot meristems (without a leaf primordium) of several plants for unlimited growth and development into full plants, have suggested that leaf primordia supply auxin and cytokinin to the meristem necessary for its growth and differentiation. In a culture medium containing essential growth regulators the excised meristem domes develop bipolar axes very quickly during reorganization. Root initiation, which occurs before the differentiation of the leaf primordia, is axial rather than lateral (see Fig. 15.4). Once the root-shoot axis is established further development follows the same pattern as that of seedlings. Although, it is possible to culture apical domes it does not seem to be a practical method for virus eradication. As remarked by Murashige (1980), 'the chances of eradicating viruses by culturing the meristems may not be substantially higher than that by a properly executed culture of larger meristem tips.'

(iii) *Storage conditions.* For meristem-tip cultures light incubation has generally proved better than dark incubation (Huth and Bode, 1970; Dale, 1980; Wang and Hu, 1980). Working with *Lolium multiflorum*, Dale (1980) observed that 59% of the meristem tips incubated in light (6000 lx) regenerated plants as against 34% in the dark. The optimum light intensity for initiating tip cultures of potato is 100 lx which should be increased to 200 lx after 4 weeks (Wang and Hu, 1980). When the shoots

have attained a height of 1 cm the light intensity is further increased to 4000 lx. Meristem-tip cultures of *Pelargonium* require a definite dark period, probably to minimize the inhibitory effect of polyphenols (Pillai and Hildebrandt, 1968). There is hardly any information on the effect of temperature on the regeneration of plants from excised meristem tips. The cultures are generally stored under standard culture-room temperature (25 ± 2°C).

(iv) *Physiological condition of the explant.* Meristem tips should, preferably, be taken from actively growing buds. In carnations (Stone, 1963) and chrysanthemums (Hollings and Stone, 1968) tips taken from terminal buds gave better results than those from axillary buds. However, Boxus et al. (1977) did not observe such a difference in strawberry. Even where the performance of axillary buds is poorer than the terminal bud, the available axillary buds, which are often several per shoot, must be used in order to increase the overall production of virus-free plants.

The time of excision of buds is also an important factor, especially with trees which display periodic growth. In temperate trees the growth of the plant is limited to only a very short period in the spring, after which the shoot apex stays dormant for a long time until the dormancy is broken by chilling or light. In such cases meristem tips should be cultured during the spring, or only after some suitable treatment during the dormancy period. Boxus and Quoirin (1974) have shown that in various *Prunus* species the stem must be maintained at 4°C for almost 6 months before excising the tips.

The efficiency of meristem-tip culture would depend on, in addition to the survival of the explants and shoot development, the rootability of the shoots and their freedom from virus(es). While the meristem-tip-derived shoots of carnation root more easily when the cultures are initiated in the winter, the frequency of virus-free plants obtained is maximum during the summer (Os, 1964). For most potato varieties studied the meristem tips taken in the spring and early summer root more readily than those taken later in the year (Mellor and Stace-Smith, 1969; Quak, 1977).

(v) *Thermotherapy.* Although the apical meristems are often free of viruses, this cannot be regarded as a phenomenon of universal occurrence. Some viruses actually invade the meristematic region of the growing tips (Sheffield, 1942; Langhans et al., 1977). In carnation 33% of the 100 μm terminal portion of shoot tips carried Carnation Mottle Virus (Hollings and Stone, 1964). All the plants raised from the callus derived from 300–600 μm meristem tips of chrysanthemum were infected (Earle and Langhans, 1974a,b). Other viruses known to invade the meristematic region of

shoot tips are TMV, Potato Virus X (Mori, 1977), Cucumber Mosaic Virus (CMV; Walkey and Cooper, 1972a) and Odontoglossum Ringspot Virus (Toussaint, 1985; cited in Debergh et al., 1990). In such cases also it has been possible to obtain virus-free plants by combining meristem-tip culture with thermotherapy. Heat treatment is given to the mother plant before excising the meristem tips or, alternatively, shoot-tip cultures are exposed to high temperatures. The first procedure has an added advantage. After heat treatment of mother plant relatively larger explants can be taken which greatly increases the proportion of the tips surviving and developing into virus-free plants (Stace-Smith and Mellor, 1968; Boxus et al., 1977; Wang and Hu, 1980). Mullin et al. (1974) have reported that a combination of heat treatment (36°C for 6 weeks) and meristem-tip culture was more effective than tip culture alone for eliminating Mild Yellow Edge strain from strawberry. In most strawberry varieties heat treatment also improves the growth rate of the plants.

Potato Virus S (PVS) and Potato Virus X (PVX) are two common viruses of potato. Neither of these viruses is readily eliminated by thermotherapy or meristem-tip culture alone. However, by taking meristem tips from heat-treated plants several potato cultivars have been freed from these most stable viruses (Stace-Smith and Mellor, 1968; Zaklukiewicz, 1971; MacDonald, 1973). Stace-Smith and Mellor (1968) found that PVS was more difficult to eradicate than PVX. The size of the meristem-tip explants was critical when they were excised from plants which had received heat treatment for only a short period or none at all, but this was not so with plants which were subjected to thermotherapy for longer periods. The frequency of PVX-free explants from White Rose cultivar of potato increased from 50% after heat treatment for 8 weeks to almost 100% after heat treatment for 18 weeks. Only 15% PVS-free plants were obtained under best conditions.

Eradication of Potato Spindle Tuber Virus (PSTV) required two consecutive cycles of heat treatment followed by meristem-tip culture (Mellor and Stace-Smith, 1970). In the first cycle 66 plants were regenerated of which 62 carried severe infection with PSTV. The other four plants, which carried mild infection, were again exposed to high temperature. Of the 248 plants regenerated from meristem tips of these plants 6 were completely free of PSTV.

The duration of heat treatment should be decided judiciously. Excessive exposure to high temperature may adversely affect the plant tissues. Hakkaart and Quak (1964) have reported that in chrysanthemum the increase in heat treatment from 10 to 30 days enhanced the percentage of virus-free plants from 9 to 90%. Treatment for 40 days or longer did not improve the yield of virus-free plants but markedly decreased the regen-

eration of plants from meristem tips. There is some indication that high temperature treatment may also inactivate the factor(s) for virus resistance in the host tissue (Walkey, 1978).

If continuous exposure to high temperature causes deterioration of the treated host tissue (Walkey, 1978), diurnal or daily cycles of high and low temperatures (Larsen, 1974; Walkey and Freeman, 1977; Walkey, 1978) can be tried. Hamid and Locke (1961) eradicated Potato Leaf Virus from potato eye pieces by subjecting them to a diurnal treatment of 40°C (4 h) +16–20°C (20 h). Continuous exposure to 40°C killed the eye pieces. To inactivate CMV in tissue cultures of *Nicotiana rustica* without appreciable damage to the host plant the optimal diurnal treatment programme is 40°C for 16 h and 22°C for 8 h per day or cycles of 2 days at 40°C and 2 days at 35°C (Walkey, 1978). Monette (1986a) applied thermotherapy to proliferating shoot cultures of *Vitis vinifera* and *V. piasezkii* infected with Grapevine Fanleaf Virus (GFLV) or Arabis Mosaic Virus (AMV) and cultured 2 mm shoot tips from the aseptic shoots to obtain virus-free plants. Daily treatment of the donor shoot cultures at 39°C/22°C for 6 h/18 h, for 28–40 days, was effective in eliminating GFLV but not AMV. Neither of these viruses could be eradicated simply by shoot-tip culture.

Rarely, has cold treatment been used to eradicate viruses through shoot-tip culture. Exposure of excised shoot tips to 5°C for 7.5 months proved valuable to eradicate Chrysanthemum Stunt Viroid and Chrysanthemum Mottle Viroid from tissue cultured *Chrysanthemum morifolium* (Paludan, 1985). Similarly, a severe strain of Potato Spindle Tuber Viroid was eliminated from a clone of *Solanum tuberosum* × *S. phureja* by a combination of low temperature treatment (5–8°C) of the infected plants followed by shoot-tip culture (Lizarraga et al., 1980).

(vi) *Chemotherapy*. It has been suggested that culture conditions play some role in the in vivo eradication of viruses. Hollings and Stone (1964) reported that in carnation the proportion of surviving meristem tips that developed into CMV-free plants was greater than what could be expected from the frequency of infected domes. Similarly, immunofluorescent studies of Mori (1973) revealed that in tobacco and petunia the region of the shoot tip 200 μm below the tip carried CMV. However, meristem-tip explants from these plants frequently regenerated virus-free plants (Wang and Hu, 1980). Some workers have ascribed the virus eradication during culture to the growth regulators in the medium (Johnstone and Wade, 1974; Quak, 1961), but there is no convincing evidence to support such a conclusion. According to Cohen and Walkey (see Walkey, 1978) growth substances may reduce the concentration of viruses in tissues but they are unlikely to eradicate them.

TABLE 15.3

Chemical compounds used to inactivate, suppress or eliminate plant viruses

Compound	Virus	Host	References
Ribavirin	CMV, PVY, TMV	Tobacco	Long and Cassells (1986)
	ACLSV	Apple	Hansen and Lane (1985)
	LSV, TBV	Lily	Blom-Barnhoorn and van Aartrijik (1984)
	ORSV	Cymbidium	Toussaint (1985; cited in Debergh et al., 1990)
	PVY, PVX, PVS, PVM	Potato	Cassells and Long (1982); Griffiths et al. (1990)
	EMCV	Eggplant	Raj et al. (1991)
Vidarabine	OMV	Ornithogalum	Veelar et al. (1992)
Malachite Green	PVX	Potato	Norris (1954)
2-Thiouracil	PVY	Tobacco	Kassanis and Tinsley (1958)
Actinomycin-D	TMV	Chinese cabbage	Renaudin and Bove (1977)

ACLSV, Apple Chlorotic Leafspot Virus; CMV, Cucumber Mosaic Virus; EMCV, Eggplant Mottled Crinkle Virus; LSV, Lily Symptomless Virus; OMV, Ornithogalum Mosaic Virus. ORSV, Ornithogalum Ring Spot Virus; PVM, Potato Virus M; PVS, Potato Virus S; PVX, Potato Virus X; PVY, Potato Virus Y; TBV, Tulip Breaking Virus; TMV, Tobacco Mosaic Virus.

Chemotherapeutic treatments of whole plants have generally failed to eradicate viruses from them (Tomlinson, 1982). However, a large number of chemicals like antibiotics, plant growth regulators, amino acids, purine and pyrimidine analogues, have been tested for inactivation of viruses or inhibition of virus multiplication in tissue cultures with some success (Table 15.3). As early as 1954, Norris had reported eradication of PVX from potato tissue cultures by Malachite green and thiouracil treatments. However, of all the compounds tested for virus eradication in cultured plant tissues, so far the nucleotide analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; trade name 'virazol') has been found to be the most efficient viracide for plant viruses. This broad spectrum antiviral agent, effective against a range of animal and plant viruses, was shown to eradicate PVX in tobacco plantlets regenerated from virus infected protoplasts (Shepard, 1977). Cassells and Long (1980) and Long and Cassells (1986) reported the elimination of PVY, CMV and TMV from tobacco explant cultures by ribavirin. Other examples of virus eradication by incorporation of ribavirin in the culture medium include Chlorotic Leaf Spot Virus (CLSV) in apple (Hansen and Lane, 1985),

Symptomless Virus and Tulip Breaking Virus in lily (Blom-Barnhoorn and Van Aartrijk, 1984), and Odontoglossum Ring Spot Virus (ORSV) in *Cymbidium* (Toussaint; cited in Debergh et al., 1990). Vidarabine (adenine arabinoside), another viracidal compound, reduced the titre of Ornithogalum Mosaic Virus (OMV) in the regenerated plants of *Ornithogalum* to an undetectable level up to 3 months after transfer to soil but at maturity all the plants tested OMV-positive (Veelar et al., 1992).

Antiserum in the culture medium has also been used to inactivate viruses in shoot tips or cultured tissues. Inouye (1983) found that a pretreatment of isolated *Cymbidium* meristems with the antiserum of ORSV for 2–24 h markedly increased the frequency of virus-free plants from shoot-tip cultures. Cyclohexamide-B (Alblas and Bol, 1977) and actinomycin-D (Renaudin and Bove, 1977) and a range of other compounds have been shown to reduce virus titre (Griffiths et al., 1990) but their utility in virus elimination has not been established. The effectivity of a compound may vary with the virus and the host genotype.

With a combination of thermotherapy and chemotherapy (ribavirin), Slack et al. (1987) could use nodal cuttings as an explant to eliminate PVS in potato. Griffiths et al. (1990) confirmed this observation and further demonstrated that by culturing nodal cuttings on MS medium containing 0.8 mM ribavirin and incubating the cultures in heat chambers programmed on continuously alternating 4 h light at 30°C and 4 h dark at 31°C, for 28 days, potato plants free from PVX, PVS and PVM could be obtained from infected individuals. The advantages of being able to use nodal cuttings instead of shoot tips for virus elimination are obvious. It is less tedious, has a higher survival rate and the regeneration time is shorter. Whereas time required to produce virus-free plants from nodal cuttings is 6 weeks it takes approximately 6 months from shoot-tip cultures.

15.5. VIRUS ELIMINATION THROUGH IN VITRO SHOOT-TIP GRAFTING

Sometimes the shoots developed in shoot-tip cultures do not form roots. In some such cases it may be possible to graft the shoots onto a virus-free rootstock derived from seedlings. The concept of shoot-tip grafting originated from the work of Morel and Martin (1952). In their pioneering studies to raise virus-free plants of *Dahlia* by shoot tip culture when their efforts to root the meristem derived shoots failed they established virus-free plants by grafting the shoots on healthy rootstock of the same plant. Micrografting is especially useful for woody species where shoot-tip culture is often impossible.

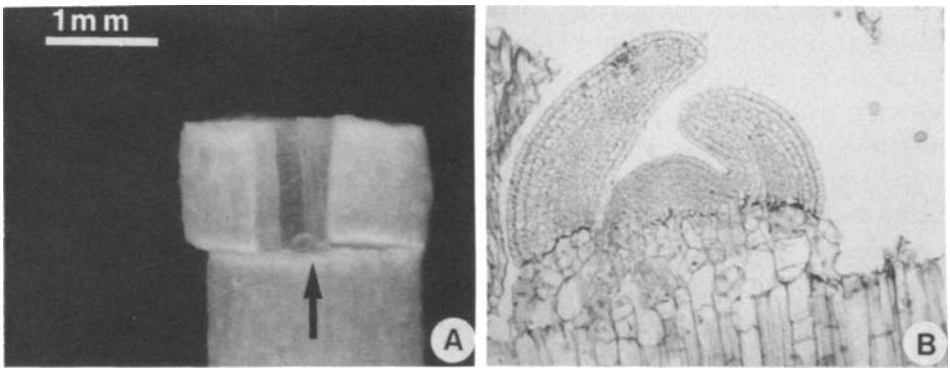

Fig. 15.5. In vitro shoot-tip grafting. (A) *Citrus sinensis* shoot-tip (arrow) freshly grafted on a 2-week-old Troyer citrange rootstock. (B) Histological section 5 days after grafting, showing callus formation at the graft union. Reprinted by permission from L. Navarro, 1992. In: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, pp. 327–339. © Springer-Verlag.

All attempts to develop *Citrus* plants from shoot tips have been unsuccessful (Navarro, 1992). Murashige et al. (1972) were able to obtain a few citrus plants by grafting shoot tips from diseased plants on young rootstock seedlings growing in vitro. Some of these plants were free of exocortis disease. This technique of shoot tip grafting in citrus was further improved by Navarro et al. (1975) and a routine protocol (see Appendix 15.II) that allowed 40–60% successful grafts was developed. Complete vascular connection between the shoot tip and the rootstock is established within 11 days after grafting (Fig. 15.5). Using this technique citrus plants have been freed of about 16 diseases caused by viruses, viroids, mycoplasma and fastidious bacteria (Navarro, 1992). Shoot-tip grafting technique has also been used to produce virus-free plants of peach (Martinez et al., 1979; Navarro et al., 1983; Juarez et al., 1988) and apple (Huth, 1978).

15.6. VIRUS ELIMINATION THROUGH CALLUS CULTURE

In calli derived from infected tissues not all cells uniformly carry the pathogen. Only 40% of the single cells mechanically separated from TMV-infected tobacco callus contained the virus (Hansen and Hildebrandt, 1966). That some cells were actually free of virus was confirmed by the regeneration of many TMV-free plants from infected calli. Virus-free plants have also been regenerated from shoot-tip calli of several other plant species (Pillai and Hildebrandt, 1968; Abo El-Nil and Hildebrandt, 1971; Simonsen and Hildebrandt, 1971). The two possible rea-

sons for the escape of some cells of a systemically infected callus from virus infection are: (a) virus replication is unable to keep pace with cell proliferation, and (b) some cells acquire resistance to virus infection through mutagenesis. Cells resistant to virus attack may even exist in the parent tissue together with susceptible ones (see Chapter 9). Murakishi and Carlson (1976) utilized the uneven distribution of virus in tobacco leaf to obtain TMV-free plants. In a TMV-infected leaf the dark green patches of tissues are either free or have a very low concentration of virus. Fifty percent of the plants regenerated from excised 1 mm explants from these tissues were virus-free.

Wang and Huang (1975) have reported that the frequency of PVX-free plants of potato was much higher in plants regenerated from the meristem-tip callus (46%) than those directly derived from meristem tips. However, while considering virus elimination through callus cultures, the genetic instability of cultured cells and lack of plant regeneration in callus cultures of several important crop species cannot be ignored.

15.7. VIRUS INDEXING

It is important to recognize that even after taking all precautions to excise small meristem tips and subjecting them to various treatments favouring virus eradication, only a proportion of the cultures yield virus-free plants. Every meristem tip or callus-derived plant should, therefore, be tested for specific viruses before using it as a mother plant to produce virus-free stock. Many viruses have a delayed resurgence period in cultured plants. This necessitates the indexing of plants several times during the first 18 months (Walkey, 1978), and only those individuals which give consistently negative results should be labelled as 'virus-tested' for specific virus(es) and released for commercial purposes. Since the virus-tested plants are susceptible to reinfection it is a useful practice to repeat virus indexing at various stages in propagation.

The simplest test for the presence or absence of viruses in plant tissues is to examine the leaves and stem for the visible symptoms characteristic of the virus. However, because symptoms may take a considerable time to develop on the host plant more sensitive tests are usually required. The sap transmission test (also called 'bioassay test' or 'infectivity test') for viruses is the most sensitive of all the methods generally used for virus indexing (Baker and Kinnaman, 1973; Wang and Hu, 1980). It is also easy to perform on a commercial scale by a person proficient in symptom identification, a skill which is not very difficult to acquire. This traditional method for virus indexing is performed as follows. Leaves are taken from the test plant and ground in an equal volume (w/v) of 0.5 M phosphate buffer using

a mortar and pestle. Leaves of the indicator plant (a plant very susceptible to specific virus/es), dusted with 600-grade carborundum, is swabbed with the leaf sap from the test plant. Rubbing of the indicator plant should be hard enough to infect the surface cells of the leaf but should not damage the leaf. After about 5 min the inoculated leaves are gently washed with water to remove the residual inoculum. The inoculated indicator plants are maintained in a glasshouse or aphid-proof cages, separate from each other and from other plants. Depending on the nature of the virus and the virus titre in the sap it may take from 6–8 days to several weeks for the symptoms to appear on the indicator plants.

Although the sap transmission method is still being used to detect some viruses and viroids, it is a slow process requiring days, weeks or months to provide proof of the presence of a pathogen. Moreover, it is not suitable to detect latent viruses which do not show any visible symptoms on the host, and has the disadvantage of different strains of a virus produce markedly different symptoms on the same host (Lawson, 1986). Therefore, more rapid serological methods have been introduced which permit quick detection of important virus pathogens with reliability and feasibility to handle several thousand samples in a short time. However, these tests are performed in addition to the transmission test.

The most commonly used serological test, whose sensitivity equals or exceeds infectivity test is the enzyme-linked immunosorbant assay (ELISA). This test, which relies on the use of antibodies prepared against the viral coat protein (for details see Appendix 15.III), requires only a small amount of antiserum and can be performed with simple equipment. The technique has become more popular by the development of machines, such as ELISA readers. Many agricultural crops can now be routinely tested with ELISA for the presence of one or more virus diseases. Serological method can also be combined with electron microscopy (immunosorbent electron microscopy; ISEM; see Appendix 15.IV).

The limitations of ELISA are: (a) it is not applicable to viroids and viruses which have lost their coat proteins, and (2) in diseases which involve several related luteoviruses, such as Potato Leaf Role Virus, all viruses may not react with the antiserum prepared against the main virus (Mellor and Stace-Smith, 1987). Such agents can be detected through cDNA/cRNA probes. The basic principle underlying the detection based on the nucleic acid hybridization technique is that the strand of viral nucleic acid will specifically bind with the complimentary cDNA/cRNA, and the detection of the hybridized form is facilitated by labelling the cDNA/cRNA strand with radioactive material. However, the nucleic acid hybridization and ISEM methods of virus detection require special facilities and expertise which are not readily available with nurserymen.

15.8. MAINTENANCE OF VIRUS-FREE STOCKS

As mentioned earlier, virus-freed plants have no additional resistance to diseases and may become quickly reinfected. To overcome this problem virus-free stocks are maintained in sterilized soils in a glasshouse or insect-proof cages. Large-scale multiplication of these plants can be done by growing them in fields in isolated areas where chances of reinfection are minimal or none at all. Alternatively, meristem-tip derived, virus-tested plants can be multiplied (see Chapter 16) and maintained (see Chapter 18) more easily and cheaply in cultures.

Although the plants derived from shoot-tip cultures are expected to exhibit very little, if any, genetic variability, it is good practice to check virus-freed plants for their trueness-to-type. Minor physiological variations as a result of virus eradication have been reported in apple (Campbell, 1974; cited in Walkey, 1978) and rhubarb (Case, 1973).

15.9. EFFECTS OF VIRUS ELIMINATION

Virus-free plants are useful experimental materials for studying the effects of specific viruses on host plants. More importantly, development of virus-free plants, mainly by shoot-tip culture, has shown that many viruses cause considerable damage to the vigour, quality and yield of host plants. More accurate estimates of yield losses have been made by introducing known viruses into virus-free plants and comparing the infected plants with virus-free plants of the same clone (Mellor and Stace-Smith, 1977).

Stone (1973) developed a clone of *Narcissus tazetta* cv. Grand soleil d'Or free of Arabis Mosaic Virus and Narcissus Degeneration Virus by meristem-tip culture. The virus-free bulbs grew rapidly and showed greater vigour than the ordinary stock. The flowers were larger, richer in colour and more per stem than those obtained from infected plants. In rhubarb the petiole yield increased by 60–90% as a consequence of virus eradication (Walkey and Cooper, 1972b).

15.10. IN VITRO ERADICATION OF PATHOGENS OTHER THAN VIRUSES

So far, meristem-tip culture and callus cultures have mainly helped in the eradication of viruses but there is some evidence to suggest that they may also contribute to eradication of other pathogens such as mycoplasmas, bacteria and fungi. Jacoli (1978) had shown that in repeated transfers of carrot explants, infected with Aster Yellow (mycoplasma-like bod-

ies), the pathogen undergoes gradual degeneration and disappears within 80 days. Bacteria and fungi borne by cultured plant tissues normally show up readily due to their luxuriant growth on the nutrient medium. Meristem-tip culture has been used to eradicate such systemic bacteria from *Dieffenbachia* (Knauss, 1976) and *Pelargonium* (Beauchesne et al., 1977). Carnations have been cured of stem rot caused by *Fusarium roseus* (Baker and Phillips, 1962).

15.11. PRACTICAL METHOD FOR VIRUS ELIMINATION THROUGH MERISTEM-TIP CULTURE

To obtain and propagate virus-free plants of a cultivar the first thing to do is to acquire some background knowledge of the plant, especially the systemic pathogens that have been reported for the plants and how the plants were being propagated. The experimental plants should then be tested for suspected pathogens. The optimal size of the meristem tip and the culture conditions that would give most rapid growth and greatest potential for pathogen freedom are determined. Finally, the plants derived from meristem-tip cultures are to be tested for the suspected pathogens several times and those found free of the desired viruses are propagated under suitable conditions which prevent any chance reinfection.

15.12. CONCLUDING REMARKS

Although genetic engineering methods to breed virus resistant plants have been developed (see Chapter 14), shoot-tip culture continues to play an important role in the production of disease-free plants. With frequent introduction of new varieties and the emergence of new strains of pathogens it is virtually impossible to achieve stable resistance of all cultivars of horticultural species through genetic engineering. The benefits of virus elimination by shoot-tip culture have already been realized by the horticulture industry. On the one hand, it has led to qualitative and quantitative improvement of crop plants and, on the other, it has facilitated the importation and exportation of live plant materials (Button, 1977). The latter application of the technique will gain increasing importance as more and more countries restrict the importation to only certified disease-free plants.

Although pathogen eradication by meristem-tip culture appears to be a simple technique, the overall operation involving the production, multiplication and maintenance of pathogen-free plants (see Fig. 15.6) requires a good knowledge of plant pathology and greenhouse maintenance, in addition to tissue culture techniques.

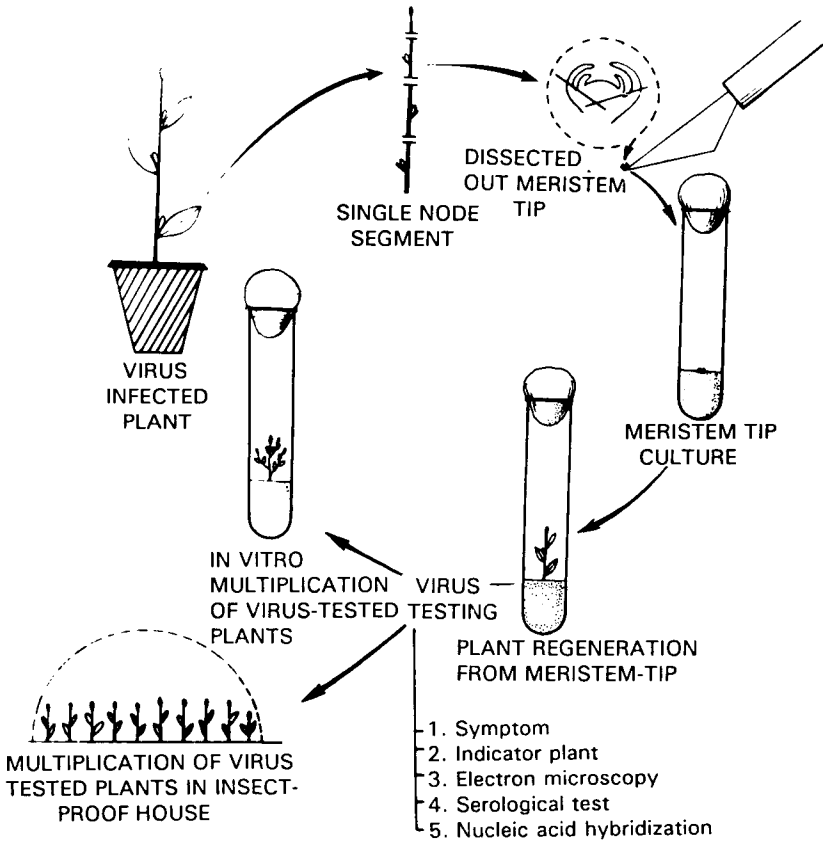

Fig. 15.6. Production of virus-free plants through shoot-tip culture: diagrammatic summary.

The beneficial effects of virus eradication may be partly offset by the increased susceptibility of the host plant to attack by more severe viruses and fungi. The phenomenon of virus cross-protection, in which the presence of one virus makes the host plant resistant to attack by another virus, is now well known (Fulton, 1986). Jones and Mullin (1974) found that PVX-free potato tubers when left in the ground for 2–3 weeks after top-kill were more susceptible to *Fusarium* dry rot than comparable PVX-infected tubers. In Japan, replacement of PVX-infected potato cultivars by virus-free stocks raised by meristem-tip culture resulted in the outbreak of severe mosaic diseases (Wang and Hu, 1980). The increased susceptibility to pathogens may be due to the altered nutritional and physiological state of the host plant as a result of virus eradication. Hossain et al. (1991) reported that the virus-freed potato plants did not produce tubers.

APPENDIX 15.I: PROTOCOL FOR VIRUS ERADICATION IN POTATO BY SHOOT TIP CULTURE (AFTER MELLOR AND STACE-SMITH, 1977)

(a) Plant a single-eyed tuber piece in soil under normal temperature and light conditions. After the first sprout is about 15 cm tall take a tip cutting 6–8 cm long. Remove the two lower leaves. Apply rooting hormone to the cut surface and plant the cutting in a 10 cm peat pot of sterilized soil. Cover it with a glass beaker for 10 days.

(b) Three to four weeks after planting move the cutting to a growth chamber where the light is 3000–4000 lx for 16 h a day and the air temperature is about 36°C during the day and about 33°C at night.

TABLE 15.4

Composition of Mellor and Stace-Smith's (1977) medium for shoot-tip culture of potato

Constituents	Amount (mg l^{-1})
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
$FeSO_4 \cdot 7H_2O$	27.85
Na_2.EDTA	37.25
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25
KI	0.83
H_3BO_3	6.2
Inositol	100
Nicotinic acid	0.5
Pyridoxine·HCl	0.5
Thiamine·HCl	0.1
Glycine	2
Kinetin	0.04
IAA	0.5
GA_3	0.1
Activated charcoal (optional)	2857

(c) Two weeks later pinch out the tip of the young plant to promote the growth of axillary shoots.
(d) After 6 weeks of heat treatment remove one axillary shoot for bud excision, leaving at least two leaves on the plant to encourage further growth. Cut the leaves from the excised shoot in such a way that small basal portions of the petioles are left on the stem. Enfold the shoot in wet paper to prevent wilting.
(e) Dissect out the meristem tip with the aid of a binocular (sterilization of shoots is unnecessary). Pull back the petiole stump to expose the axillary bud and, with a dissecting needle break off the rudimentary leaves.
(f) When the two youngest leaf primordia are left, cut off the meristem tip (300–600 μm long) with a fine fragment of blade fixed in a suitable holder, and transfer it to the culture medium (see Table 15.4).
(g) Store the cultures at about 23°C under a 16 h photoperiod.
(h) When rooted shoots reach 3 cm or more in length transfer it to the soil. Initially maintain the plants under high humidity. In varieties where the shoots fail to root, grafting onto the healthy seedling rootstock may be tried.
(i) Index the plants immediately after transferring out of cultures and again after several weeks. Indicator plants used for potato viruses are *Gomphrena globosa* and *Chenopodium amaranticolor*.

APPENDIX 15.II: PROTOCOL FOR SHOOT-TIP GRAFTING TO ELIMINATE VIRUSES IN *CITRUS* (AFTER NAVARRO, 1992)

Step I: Preparation of rootstock. Raise aseptic seedlings by culturing surface sterilized decoated seeds of Troyer citrange (*Poncirus trifoliata* × *Citrus sinensis*) on semi-solid medium containing the salts of MS medium, in the dark, at 27°C. Use 12–16 days old seedlings, 3–5 cm long and 1.6–1.8 mm across stem, to prepare the stock. Remove the seedling from the culture tube and, under aseptic conditions decapitate it leaving 1.5 cm of the epicotyl. Cut the root to a length of 4–6 cm. Remove the cotyledons and their axillary buds.

Step II: Preparation of scion. Completely defoliate the infected citrus plants growing in pots in a warm greenhouse. After 8–15 days take about 3 cm long flushes and strip them of the large leaves, cut to about 1 cm long, surface sterilize and use them for excision of shoot-tip scion (flushes produced by budwood cultured in vitro on a medium containing MS salts and solidified with 1.2% agar, at 32°C, and exposed to 16 h photo-

period with 80 μE m^{-2} s^{-1} illumination can also be used to excise shoot-tip scion).

Step III: Grafting. Make an inverted T incision on the decapitated stock seedling by giving a 1 mm long vertical incision from the point of decapitation and 1–2 mm wide horizontal incision at the base of the vertical incision. The incisions should be through the cortex. Under stereoscope remove the remaining large leaves and dissect 0.1–0.2 mm shoot-tip, comprising the meristem and 3 leaf primordia, with a razor blade sliver attached to a surgical handle. Place the shoot tip inside the slit on the root stock by slightly lifting the flaps of the incisions. The cut end of the shoot-tip should be in contact with the cortex exposed by the horizontal incision (Fig. 15.5).

Step IV: Culture of grafted plant. Culture the grafted plant in a liquid medium containing MS salts, White's vitamins and 7.5% sucrose in test tubes. Use filter paper bridges perforated in the centre and insert the root portion of the stock in the hole. Incubate the cultures at 27°C, under a 16 h photoperiod with 40–50 μE m^{-2} s^{-1}.

Step V: Transfer to soil. After 4–6 weeks the graft should be well established and 2–4 expanded leaves would have developed. At this stage transfer the micrografted plant to a pot containing steam sterilized artificial soil mix. Enclose the pot in a polythene bag with the help of a rubber band. Place the pot in a shaded area in the greenhouse at 18–25°C. Open the bag after 8–10 days and after another 8–10 days remove the bag and grow the plant under regular greenhouse conditions.

APPENDIX 15.III: PROTOCOL FOR VIRUS INDEXING BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TECHNIQUE (AFTER LAWSON, 1986; REID ET AL., 1992)

1. Apply to each well of a plastic microtitre plate 200 μl of appropriate antivirus γ globulin (IgG) in coating buffer[1], seal with cling-film and incubate at 30°C for 2–4 h to immobilize the IgG.
2. Rinse the plate 3 times with the PBS-T buffer[2].

[1] 1 l of the coating buffer contains: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 dissolved in distilled water (pH 9.6).

[2] 1 l of the PBS-T buffer contains: 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 8 g NaCl, 0.2 g KH_2PO_4, 0.2 g NaN_3 and 0.5 ml Tween-20 dissolved in distilled water (NaN_3 is excluded from the rinsing buffer).

3. Charge wells with 200 μl of plant extract prepared in PBS-T buffer containing 2% polyvinylpyrrolidone (PVP, MW 44 000). Incubate the plate overnight at 4°C to allow trapping of any viral antigen by the antibody adsorbed to the surface of the wells.
4. Rinse the plate 3 times with PBS-T buffer.
5. Charge wells with 200 μl of alkaline phosphatase-IgG conjugate, in PBS-T buffer and incubate at 30°C for 3 h to allow binding to any antigen previously complexed with the antibody.
6. Rinse the plate 3 times with the PBS-T buffer.
7. Add 200 μl of substrate buffer[3] containing 4 substrate tablets to the wells. Leave the plate for 30–180 min at room temperature (monophosphate substrate, such as *p*-nitrophenyl phosphate, may be used).
8. Add 50 μl of 3 M NaOH to each well to halt the colour reaction.
9. Record or measure the coloured enzyme reaction product with a spectrophotometer (colourless *p*-nitrophenyl phosphate is converted to yellow *p*-nitrophenol).

APPENDIX 15.IV: PROTOCOL FOR DETECTION OF VIRUSES BY IMMUNOSORBENT ELECTRON MICROSCOPY (ISEM). (AFTER BARBARA AND CLARK, 1986)

1. Dilute the virus-specific antiserum using 0.05 M sodium phosphate buffer (pH 6.5) in the ratio of 1:1000 (if the antiserum has low titre) or 1:2000 (if the titre is high).
2. Place the antiserum droplets (ca. 50 μl) on parafilm, stretched over a glass slide, in a moist chamber and coat the grids (grids coated with carbon or carbon-coated cellulose nitrate and with 400–600 mesh size are appropriate) with antibody by placing the carbon-coated surface of the grid onto the surface of the droplet. Leave for 3–5 h at 5°C.
3. Wash away surplus antibody with 5–6 droplets of phosphate buffer.
4. Place the grid, with the carbon coated side downwards on a droplet of the homogenate of plant tissue in distilled water. Incubate for 1–2 h, depending on the titre of the virus particles, at 5°C. The antiserum clumps the virus particles and coats the particles if the two are related.

[3] 1 l of the substrate buffer contains: 97 ml diethanolamine and 0.2 g NaN_3 dissolved in distilled water.

5. Wash as in step 3.
6. Stain the preparation using a negative stain, such as 2% aqueous uranyl acetate and dry. Observe in the microscope at a magnification of 18 000–35 000, depending on the morphology of the virus.

Chapter 16

Clonal Propagation

16.1. INTRODUCTION

Since most fruit trees and ornamental plants are highly heterozygous their seed progeny is not true-to-type. Asexual reproduction, on the other hand, gives rise to plants which are genetically identical to the parent plant and, thus, permits the perpetuation of the unique characters of the cultivars. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation and a plant population derived from a single individual by asexual reproduction constitutes a clone. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative reproduction (regeneration of new plants from vegetative parts).

Apomixis being restricted to only a few species, horticulturists have adopted the methods of vegetative reproduction for clonally multiplying selected cultivars. For crop plants, such as some cultivars of banana, grape, fig, double petunias and chrysanthemums, which produce little or no viable seeds, vegetative multiplication is the only method for propagation. Other advantages of vegetetative propagation over sexual reproduction are: (a) in plants with long seed dormancy, vegetative propagation may be faster than seed propagation, and (b) the undesirable juvenile phase associated with seed-raised plants in some cultivars does not appear in the vegetatively propagated plants from adult material.

Tissue culture has become a popular method for vegetative propagation of plants. The most significant advantage offered by this aseptic method of clonal propagation, popularly called 'micropropagation', over the conventional methods is that in a relatively short time and space a large number of plants can be produced starting from a single individual (see also Section 16.5). For orchids, micropropagation is the only commercially viable method of clonal propagation.

General interest in the use of tissue culture for clonal propagation of plants originates from the initial success with orchids, the credit for which goes to G. Morel. Because of the historic interest, and the fact that the pattern of in vitro growth and multiplication of orchids is fairly distinct from other plants, the micropropagation of orchids is discussed before dealing with the general techniques of in vitro clonal propagation.

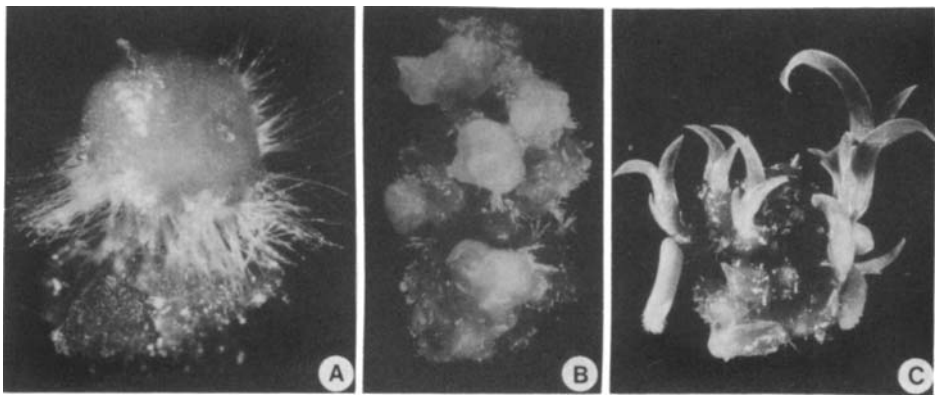

Fig. 16.1. Micropropagation of orchids. (A,C) *Cymbidium*; (B) *Cattleya*. (A) Magnified view of a protocorm with rhizoids derived from an excised shoot tip. (B) Group of protocorms developed in culture from a single shoot tip. (C) Differentiation of leafy shoots from in vitro-formed protocorms (courtesy of J. Margara, France).

16.2. ORCHID PROPAGATION

Cultivated orchids are very complex hybrids. Some of them combine the genomes of three or four different genera. *Sophrolaeliocattleya*, for example, is a triple hybrid in which *Sophronitis*, *Laelia* and *Cattleya* are involved (Morel, 1972). The only in vivo method to clonally multiply these plants is what is called 'back-bulb propagation', which involves separating the oldest pseudobulbil to force the development of dormant buds. This is an extremely slow process allowing, at best, doubling the plant number every year. Moreover, the back-bulk splitting method for clonal multiplication is applicable to only sympodial orchids; monopodial orchids do not form lateral bulbs. Traditionally, therefore, the orchid growers preferred seed propagation despite a high degree of heterogeneity in the progeny, leading to considerable economic losses. The best plants were crossed and the seedlings painstakingly raised at great expense in heated glasshouses (Morel, 1972). It takes almost 3–5 years for a plant raised from seed to reach the flowering stage.

In 1960, Morel described an in vitro method for clonally multiplying orchids at a fantastic rate. Cultured excised shoot tips of *Cymbidium*, instead of developing into a leafy shoot, formed a spherule-like body with rhizoids at the base (Fig. 16.1A). These structures, hereafter called protocorms, were morphologically similar to the protocorms formed by the embryo during seed germination. In shoot-tip cultures the protocorms originated from epidermal or sub-epidermal cells of the leaf (Champagnat et al., 1966). Some of these protocorms proliferated and formed a cluster of

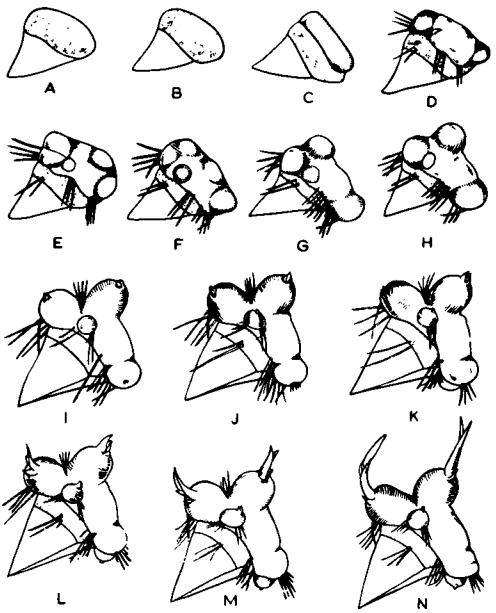

Fig. 16.2. Diagram showing the regeneration of several protocorms (B–H) followed by multiple shoot formation (I–N) from a fragment of protocorm (A) of *Cymbidium* (after Morel, 1972).

up to a dozen protocorms. Morel cut the protocorm mass into three to six pieces and planted them on a fresh Knudson C medium. Each piece formed additional protocorms (Figs. 16.1B, 16.2) by the activity of the superficial cells; the internal tissue of a protocorm lacks the regeneration potentiality. A single piece produced up to 12 protocorms in less than a month. This process of protocorm multiplication could be repeated indefinitely by regularly chopping the protocorm mass and planting the pieces on fresh medium. If chopping was stopped each protocorm developed into a full plantlet on the original medium (Figs. 16.1C, 16.2). From each shoot tip 3–5 protocorms were formed in 2 months. After this each protocorm could be cut into four to six pieces, each one of which gave rise to three to five protocorms in a month. At this rate of multiplication several million plants can be produced in a year starting from a single shoot tip measuring less than 1 mm (Morel, 1960, 1972).

The technique developed by Morel was rapidly adopted by orchid growers because of its tremendous practical applications. Today, tissue culture is the most popular method for clonal propagation of orchids. Micropropagation techniques are being applied on a commercial scale to sympodial as well as monopodial orchids (Table 16.1). Most of the economically important orchids are presently clonable in vitro (Murashige, 1978).

TABLE 16.1

List of orchids which have been clonally propagated in vitro

Plant	Explant	Reference[a]
Anacamptis pyramidalis	Shoot tip	Morel (1970)
Aranda	Shoot tip, axillary bud	Goh (1973)
	Inflorescence segment	Goh and Wong (1990)
(*Arachnis hookeriana* × *Vanda lamellata*)	Shoot tip	Cheah and Sagawa (1978)[a]
Aranthera	Shoot tip	Cheah and Sagawa (1978)[a]
Arundina bombusifolia	Shoot tip (from young seedling)	Mitra (1971)
Ascofinetia	Inflorescence segment (with flower primordia)	Intuwong and Sagawa (1973)[a]
Brassocattleya	Axillary bud	Kako (1973)
Calanthe	Shoot tip	Bertsch (1967)
Cattleya	Shoot tip	Lindemann et al. (1970)
	Axillary bud	Morel (1970)
	Lateral bud	Scully (1967)
	Leaf base	Champagnat et al. (1970)
	Leaf tip	Arditti et al. (1971, 1972), Ball et al. (1971, 1972, 1973)
Cymbidium	Shoot tip	Morel (1960, 1963, 1964a,b, 1970), Wimber (1963), Champagnat et al. (1966, 1968), Sagawa et al. (1966), Fonnesbech (1972)
Dendrobium	Shoot tip	Sagawa et al. (1966), Sagawa and Shoji (1967), Kim et al. (1970)
	Nodal segment	Arditti et al. (1973), Mosich et al. (1973, 1974)
	Flower stalk segment (with vegetative buds)	Singh and Sagawa (1972)
Epidendrum	Leaf tip	Churchill et al. (1970, 1972, 1973), Arditti et al. (1971, 1972)
Laelia	Axillary bud	Arditti (1977)
Laeliocattleya	Axillary bud	Arditti (1977)
Lycaste	Shoot tip	Arditti (1977)
Miltonia	Shoot tip	Arditti (1977)
Neostylis	Inflorescence segment (with flower primordia)	Arditti (1977)
Neottia nidus-avis	Root	Champagnat (1971)

Odontioda	Shoot tip	Arditti (1977)
Odontoglossum	Shoot tip	Arditti (1977)
Odontonia	Shoot tip	Arditti (1977)
Oncidium	Shoot tip	Bertsch (1967)
Oncidium papilio	Flower-stalk segment (with dormant apical buds)	Fast (1973)
Phajus	Shoot tip	Arditti (1977)
Phalaenopsis	Flower-stalk segment	Rotor (1949), Intuwong et al. (1972), Pieper and Zimmer (1976), Tanaka et al. (1988)[a]
	Shoot tip	Intuwong and Sagawa (1974)
	Leaf segment, stem segment, root segment	Pieper and Zimmer (1976)[a]
Pleione	Shoot tip	Weatherhead and Harberd (1980)[a]
Rhynchostylis gigantea	Shoot tip, lateral buds	Vajrabhaya and Vajrabhaya (1970)
R. retusa	Leaf segment	Vij et al. (1984)
Schomburgkia superbiens	Lateral bud	Arditti (1977)
Vanda (Terete-leaf)	Shoot tip	Kunisaki et al. (1972)
	Stem section	Sagawa and Sehgal (1967)
Vanda (Strap-leaf)	Shoot tip	Teo et al. (1973)
Vanda hybrid (*V. teres* × *V. hookeriana*)	Shoot tip, axillary bud, root segment	Goh (1970)
Vanilla planifolia	Root tips	Philips and Nainar (1986)[a]
Vascostylis	Inflorescence segment (with flower primordia)	Arditti (1977)
Vuylstekeara	Shoot tip	Arditti (1977)

[a]For references not marked thus, refer to Arditti (1977), which is the main source of information included in the table.

Cultures of *Cymbidium* and other sympodial orchids are commonly initiated from shoot tips excised from terminal or axillary buds. The size of the shoot-tip explant determines whether it would form protocorms or continue organized growth. Generally, shoot tips smaller than 1.5 cm produce a protocorm whereas larger explants develop into a plant (Kunisaki et al., 1972; Intuwong and Sagawa, 1973). However, a smaller shoot-tip piece may take 2–4 months to form the first protocorm. This should not frustrate the worker because further proliferation of protocorms is comparatively rapid (Morel, 1965b; Kunisaki et al., 1972). The commercial growers use 5–10 mm long shoot apices to initiate cultures (Murashige, 1974).

For monopodial orchids (e.g. *Vanda*, *Phalaenopsis*), where excising the terminal bud means sacrificing the selected plant, pieces of floral stalk with vegetative buds may be used to start the cultures (Intuwong and Sagawa, 1973). Such aerial explants should be good even for sympodial orchids because: (a) the procedure is relatively easy, (b) contamination of cultures is low, and (c) the percentage of cultures forming protocorms is high.

Compositions of media used for clonal propagation of some orchids are given in Appendices 16.II.1–16.II.4. For *Vanda*, liquid medium is reported to be better than semi-solid medium (Kunisaki et al., 1972). In liquid cultures shaking the medium facilitates the separation of newly developed protocorms, thus saving the manual chopping of the protocorm clumps at each subculture.

16.3. GENERAL TECHNIQUES OF MICROPROPAGATION

Micropropagation generally involves four stages: Stage 1, initiation of aseptic cultures; Stage 2, multiplication; Stage 3, rooting of in vitro formed shoots, and Stage 4, transfer of plants to greenhouse or field conditions (transplantation). Debergh and Maene (1981) introduced the Stage 0, making micropropagation a five stage process. Each stage has its special requirements.

16.3.1. Stage 0: preparative stage

This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in Stage 1. To reduce the contamination problem in Stage 1 the mother plants should be grown in a glasshouse and watered so as to avoid overhead irrigation. It would not only help minimize the incidence of infection in Stage 1 but may also reduce the need for a harsh sterilization treatment. Before the introduction of Stage 0 *Cordyline* cultures could be initiated only from apical bud; all the nodal segments got infected or did not respond favourably. With the introduction of Stage 0 both apical and all nodal segments could be used as primary explants (Debergh and Read, 1991). Senawi (1985) succeeded in obtaining responding cultures of *Theobroma cacao* only from the stock plants grown under glasshouse conditions.

Stage 0 also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. Red light-treated plants of *Petunia* provided leaf explants

which produced up to three times as many shoots as did the explants from untreated plants (Read et al., 1978). In woody and bulbous plants suitable temperature treatments should help in breaking bud dormancy and provide more responsive explants.

16.3.2. Stage 1: initiation of cultures

(i) *Explant.* The nature of the explant to be used for in vitro propagation is, to a certain extent, governed by the method of shoot multiplication to be adopted (see Section 16.3.3). For enhanced axillary branching only such explants are suitable which carry a pre-formed vegetative bud. When the objective is to produce virus-free plants from an infected individual it becomes obligatory to start with sub-millimeter shoot tips (Chapter 15). However, if the stock plant is virus-tested or, alternatively, virus eradication is not desired, the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem-tip culture may also result in the loss of certain horticultural characters which are controlled by the presence of virus, such as the clear-vein character of the geranium cv. Crocodile (Cassells et al., 1980; see Fig. 16.3).

Generally, sub-terminal and older segments withstand the toxic effects of sterilizing agents much better than the terminal cuttings. For clonal propagation of cauliflower, Crisp and Walkey (1974) used pieces of curd which provide numerous meristems capable of reverting back to vegetative buds in cultures and forming leafy shoots. For rhizomatic plants, such as strawberry and Boston fern, runner tips have been commonly used.

The physiological state of the parent plant at the time of explant excision has a definite influence on the response of the buds. Explants from actively growing shoots at the beginning of the growing season generally give best results (Seabrook et al., 1976; Yang, 1977; Anderson, 1980). The seasonal fluctuations in the response of shoot buds may be minimized by maintaining the parent plants under light and temperature conditions required for continual vegetative growth, in glasshouse or growth cabinets. Bulbs, corms, tubers and other organs should be subjected to the temperature and/or photoperiodic treatments required to break dormancy before excising the bud.

For multiplication through adventitious bud formation, with or without callusing, explants are derived from root, stem, leaf or nucellus based on their natural capacity to form adventitious buds. In monocots the meristems of leaves and scales are located at the proximal end where they are joined with the basal plate. Leaf-base and scale-base explants

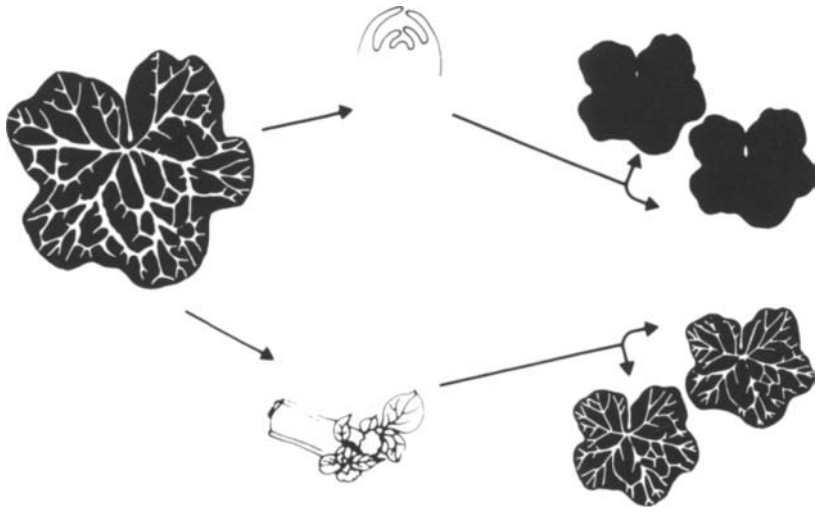

Fig. 16.3. Shoot-tip culture (top) and petiole culture (bottom) of the geranium cv. Crocodile, in which clear vein character has been attributed to infection by a virus or virus-like agent. Note that the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture (after Cassells et al., 1980).

should include a small piece of basal plate (Hussey, 1980). Embryogenic cultures of woody and other species are readily established from immature zygotic embryos. Nucellar explants of citrus and mango are highly embryogenic.

(ii) *Sterilization*. Standard methods of aseptic culture of plant tissues and organs are described in Sections 2.3.2 and 15.4.2. Special precautions need to be taken when explants are derived from field-grown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in a glasshouse. For species hard-to-root from cuttings, the growing branches on the tree may be loosely covered in a large polythene bag and the newly developed shoots, protected from wind-borne contaminants, taken to start cultures. Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination. Chances of contamination are much higher in the cultures of terminal cuttings and whole buds than that in the cultures of 0.5–1 mm shoot tips excised after removing several layers of older leaves. Shoot tips with sufficient covering of mature leaves, or

scales from the centre of the bulbs, may be dissected out sterile by wiping the buds or bulbs, respectively, with 70% ethanol and gently peeling off the outer covering (Hussey, 1980).

(iii) *Browning of medium.* A serious problem with the culture of some plant species is the oxidation of phenolic substances leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is especially common with the adult tissues from woody species (see Section 16.6.4).

16.3.3. Stage 2: multiplication

This is the most crucial stage, and is the point at which most of the failures in micropropagation occur. Broadly, three approaches have been followed to achieve in vitro multiplication (Fig. 16.4).

(i) *Through callusing.* The potentiality of plant cells to multiply indefinitely in cultures, and their totipotent nature permit a very rapid multiplication of several plant types. Differentiation of plants from cultured cells may occur via shoot-root formation (Chapter 5) or somatic embryogenesis (Chapter 6). Where applicable, this is often the fastest method of multiplication. However, there are several drawbacks in this method and, as far as possible, it should be avoided in clonal propagation of a cultivar. The most serious objection against the use of callus cultures for shoot multiplication is the genetic instability of their cells (see Chapter 9).

Between organogenesis and somatic embryogenesis the latter approach is more appealing from a commercial angle. A somatic embryogenic system once established lends itself to better control than organogenesis. Since somatic embryos are bipolar structures, with defined root and shoot meristems (see Chapter 6), the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds (see Section 6.9; Fig. 16.5), offering cost advantages from labour savings, which accounts for 60–70% of direct costs in micropropagation in developed countries (Onishi et al., 1994; Redenbaugh, 1993; Sakamoto et al., 1995). Unlike microcuttings, somatic embryos can also be stored through cold storage, cryopreservation or desiccation for prolonged periods (see Chapter 18), thus providing flexibility in scheduling production and transplantation. These characteristics make somatic embryogenesis potentially a less

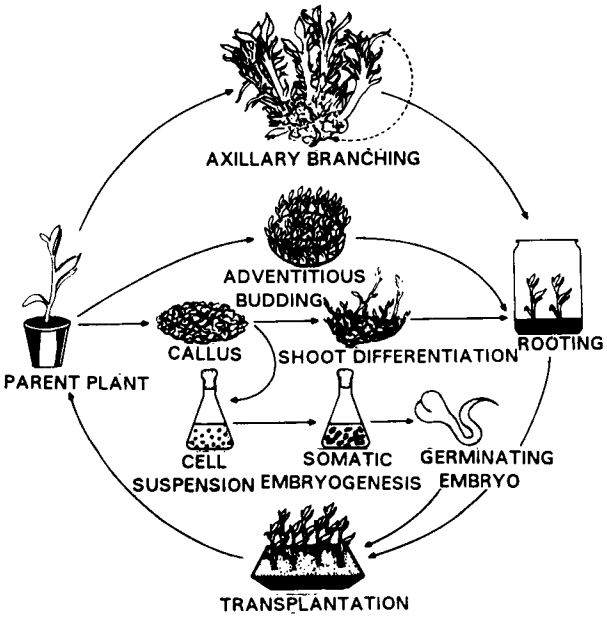

Fig. 16.4. Diagrammatic summary of steps involved in aseptic multiplication of plants. For details see text (after Dhawan and Bhojwani, 1986).

expensive and flexible system for micropropagation (Cervelli and Senaratna, 1995). For some plants, such as oil palm, somatic embryogenesis appears to be the only possible pathway to clonal propagation (Duval et al., 1995).

However, despite these advantages, somatic embryogenesis is not being used for commercial production of plants (Cervelli and Senaratna, 1995). Some of the problems currently limiting commercialization of this remarkable process are: (1) synchronization of embryogenesis is inadequate; (2) field conversion frequencies of embryos or artificial seeds are still very low (15–25%); and (3) it generally involves a callus phase. When these problems are overcome somatic embryogenesis is the method of choice for micropropagation.

(ii) *Adventitious bud formation.* Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. Strictly speaking, the shoots differentiated from calli should also be treated as adventitious buds. In this chapter, however, the use of the term adventitious buds is restricted to only those buds that arise directly from a plant organ or a piece thereof without an intervening callus phase.

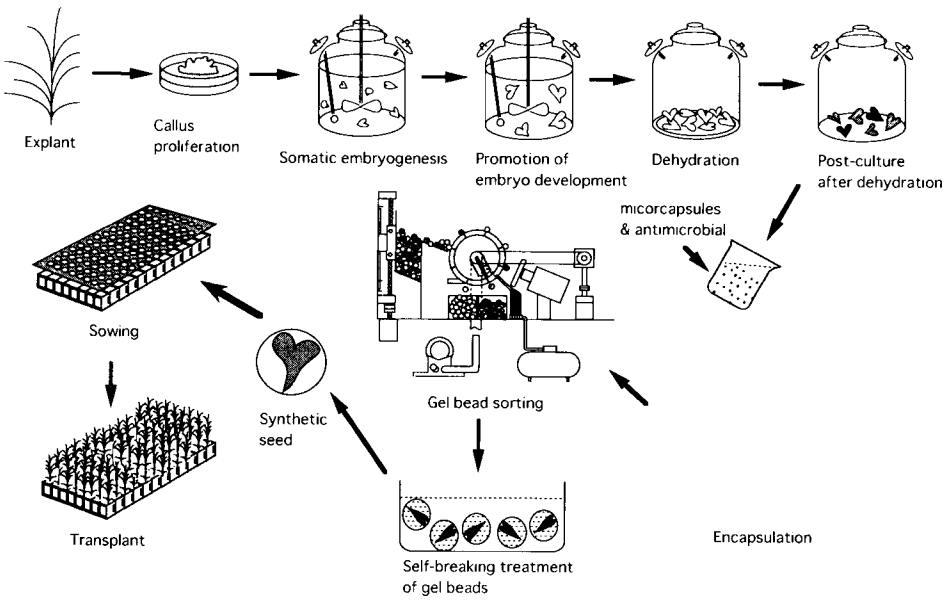

Fig. 16.5. Schematic representation of carrot synthetic seed production system. Leaf callus was initiated on gelled SH medium containing 1.0 μM 2,4-D, 0.5 μM kinetin, 3% sucrose and 0.2% gelrite and multiplied for 3 months by monthly subculture. Suspension cultures derived from this callus were transferred to liquid SH medium containing 1% sucrose and 8% mannitol, in a bioreactor, to induce embryogenesis. The somatic embryos which passed through 0.84 mm mesh were transferred to a medium containing SH basic salts, 1% sucrose and 10% mannitol for promotion of embryo development. After 7 days the embryos were put on filter papers for dehydration at 20°C, under 16 h photoperiod and 14 μE m^{-2} s^{-1} irradiation, for 7 days. The embryos were again cultured for 14 days in a medium containing SH salts, 2% sorbitol, and 0.01 mg l^{-1} each of BAP and GA_3, in air enriched with 2% CO_2, under a 16 h photoperiod, at 20°C, so that the embryos become autotrophic. The encapsulatable embryos so obtained were suspended in 1.5% (w/v) alginate solution containing 3% sucrose microcapsules and the fungicide Topsin M (0.1%) and fed to an encapsulation machine. After the capsules had been hardened in 100 mM $CaCl_2$ solution for 10 min they were washed and the beads with green embryos were sorted out with the help of a gel-bead sorting machine. A self-breaking feature was added to the capsules (see Section 6.9.2). The synthetic seeds thus produced were sown on synthetic substrate, in trays, and placed under humid conditions to allow self-breaking of the gel beads (courtesy of T. Hirosawa, Kirin Brewery, Japan).

In many crop plants vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (*Begonia*, *Crassula*, *Peperomia*, *Saintpaulia*) cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. In *Begonia*, for example, buds normally originate only along the cut ends but in a medium containing BAP

the bud formation is so profuse that the entire surface of a cutting becomes covered with shoot buds (Reuther and Bhandari, 1981). Takayama and Misawa (1982) described an in vitro method for mass propagation of *Begonia* × *hiemalis* which allowed the production of over 10^{14} plants in a year from a single 7 × 7 mm segment of a young leaf. A liquid shake culture method for rapid multiplication of this species by adventitious bud formation has been described by Simmonds and Werry (1987). *Pinguicula moranensis* is also normally propagated from leaf pieces. By the conventional technique only one plant is produced by each leaf but in cultures it increases 15-fold (Adams et al., 1979a). In cultures, explants as small as 20–50 mg in weight, which fail to survive in nature, are able to produce adventitious buds (Hussey, 1978). Under the influence of an appropriate combination of growth regulators, in cultures, adventitious buds can be induced on the leaf and stem cuttings of even those species which are normally not propagated vegetatively (Kartha et al., 1974b; Roest and Bokelmann, 1975; Behki and Lesley, 1976; Murray et al., 1977).

Barlass and Skene (1978, 1980a,b) described a propagation method for grapes in which excised shoot tips measuring 1 mm in length (with two to three leaf primordia), were cut into nearly 20 pieces. In cultures each shoot-tip fragment formed multiple shoots adventitiously from the swollen leaf bases (Barlass and Skene, 1980a). About 8000 plants could be produced from a shoot apex in 3–4 months. All the plants multiplied by this method were normal diploid. The technique of shoot-fragment culture has also been used for the micropropagation of *Asclepias rotundifolia* (Tideman and Hawker, 1982).

A remarkable capacity to form adventitious buds in vitro is displayed by certain ferns. In a simplified method of in vitro multiplication of *Davallia* and *Platycerium*, the tissue pieces obtained by aseptically homogenizing the plants in a blender produced numerous new plants (Cooke, 1979).

For most bulbous plants adventitious bud formation is the most important mode of multiplication, and the best explants are obtained from bulb scales. Micropropagation techniques are available for many bulbous species but large scale micropropagation programmes are functional only for lilies (Capellades et al., 1991). Propagation by adventitious bulblet formation directly from aseptically cultured bulb-scale segments (see Fig. 16.6) was described by Hackett (1969) and Gupta et al. (1978). In the cultures of whole scales about 10 bulblets were formed within 15 days. The number of bulblets per bulb-scale could be increased considerably by culturing small pieces of the scales. Gupta et al. (1978) reported that within 30–45 days each of the six segments taken from the basal half of a bulb-

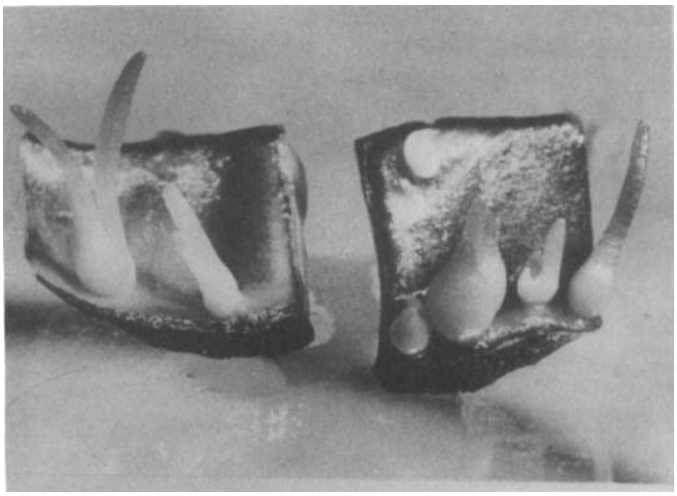

Fig. 16.6. Adventitious bulblet formation on lily bulb scales in culture (courtesy of D. Cohen, New Zealand).

scale produced, on average, 18 bulblets. Thus, almost 100 bulblets could be produced from a single scale. Maximum regeneration capacity is shown by the outer scales, and it decreases progressively among the inner scales of the bulb (Hackett, 1969). During the last decade several groups have described a fermentor technique for mass multiplication of lilies (Levin et al., 1988; Takayama et al., 1991; Takahashi et al., 1992a,b). Mitsui Petrochemical, Japan is producing virus-free bulblets of *Lilium longiflorum* in 2-l stirred drum tanks (Takahashi et al., 1992a).

Adventitious shoot formation directly from the explants is definitely a better approach than the callus method for clonal propagation of plant species. Often, where calli have produced cytologically abnormal plants, adventitious shoots have formed uniformly diploid individuals (Hussey, 1976; Krul and Myerson, 1980). This should not imply that this method always produces true-to-type plants. Westerhof et al. (1984) observed 18 types of variations after three cycles of in vitro propagation of *Begonia* × *hiemalis*. Somaclonal variants have been detected in plants regenerated directly from the explants of potato (Cassells et al., 1983) and *Brassica juncea* (Bhojwani and Arora, 1992).

A serious problem may arise when this method of propagation is applied to varieties which are genetic chimeras. Adventitious bud formation involves the risk of splitting the chimeras leading to pure type plants (Bush et al., 1976; Skirvin, 1978; Cassells et al., 1980; Johnson, 1980).

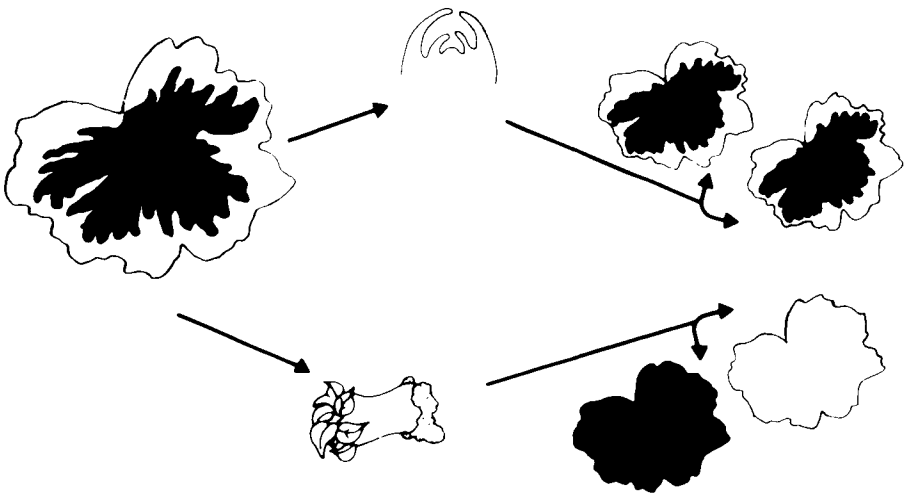

Fig. 16.7. Shoot-tip culture (top) and petiole-segment culture (bottom) of the variegated geranium cv. Mme Salleron. The chimera is perpetuated in meristem culture but broken down in petiole culture (after Cassells et al., 1980).

Some plants derived from adventitious buds induced on internodal regions of disbudded trees of apple differed from the parental clones in growth habits, fruiting characteristics and fruit pigmentation. This was ascribed to the complex chimeral nature of some of the apple clones (Dayton, 1969, 1970). Mme Salleron cultivar of geranium, with variegated leaves, is a genetic chimera. Plants derived from adventitious buds formed directly on petiole segments, without a callusing phase, never showed variegation; the plants were either green or albino. In contrast, all the plants raised from shoot-tip cultures showed the typical variegation (see Fig. 16.7) (Cassells et al., 1980). Plantlets produced in vivo or in vitro from leaf cuttings of 'Rober's Leman Rose' cultivar of geranium were of four or five different types (Skirvin and Janick, 1976b; Janick et al., 1977).

(iii) *Enhanced axillary branching.* Axillary buds are usually present in the axil of each leaf, and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods depending on the growth pattern of the plant. In species with a strong apical dominance the removal or injury of the terminal bud is necessary to stimulate the next axillary bud to grow out into a shoot. This phenomenon of apical dominance is controlled by the interplay of growth regulators. Application of a cytokinin to the axillary buds can overcome the api-

Fig. 16.8. Cluster of shoots developed from a nodal segment of *Pyrus serotina* (after Bhojwani et al., 1984b).

cal dominance effect and stimulate the lateral buds to grow in the presence of the terminal bud, but this effect is only temporary. The lateral shoot stops growing as the effect of the exogenous growth regulator diminishes (Sachs and Thimann, 1964).

The conventional method of vegetative propagation by stem cuttings utilizes the ability of axillary buds to take over the function of the main shoot in the absence of a terminal bud. The number of cuttings that can be taken from a selected plant in a year is extremely limited because, in nature, the vegetative growth is periodic, and a minimal size of the cutting (24–30 cm) is necessary in order to establish a plant from it.

In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration, with or without an auxin. Due to the continuous availability of cytokinin, the shoots formed by the bud, a priori present on the explant (nodal segment or shoot-tip cutting), develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches (Fig. 16.8). There is a limit to

which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, however, if shoots are excised and planted on a fresh medium of the same composition the shoot multiplication cycle can be repeated. This process can go on indefinitely, and can be maintained throughout the year.

In some plants, such as leguminous tree species (Dhawan and Bhojwani, 1985), *Feijoa* (Bhojwani et al., 1987), Japanese persimon (Fukui et al., 1989) and potato (McCown and Joyce, 1991), it may not be possible to break apical dominance by manipulating the hormonal composition of the medium, and the bud present a priori on the initial explant grows into an unbranched shoot. The rate of shoot multiplication in such cases would depend on the number of nodal cuttings that can be excised from the shoot at the end of each passage. Even with this alternative method of enhanced axillary branching, 3–4-fold shoot multiplication every 4–6 weeks could be achieved in the plants with strong apical dominance.

The enhanced axillary branching method of shoot multiplication may be initially slower than the other methods but with each passage the number of shoots increases logarithmically and within a year astronomical figures can be achieved. This method is currently the most popular approach to clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes under culture conditions. Chimeras, whose breakdown is common during adventitious bud development, are perpetuated in shoot-bud culture (Cassells et al., 1980; see Fig. 16.7). Moreover, the adventitious bud formation and callusing methods require de novo differentiation of shoot-buds which is not always possible. However, the plants multiplied by forced axillary branching may show some epigenetic changes. Excessive dose or wrong choice of cytokinin can be responsible for such changes. Bushiness, observed in micropropagated plants of *Gerbera jamesonii*, characterized by excessive leaves, limited number of flowers and short peduncle, is probably a consequence of the use of BAP (Debergh and Read, 1991). Unlimited subcultures in *Fragaria* result in disorders such as absence or weak rhizogenesis, excessive flowering, smaller and deformed fruits, and heterogeneous plants (Rancillac et al., 1987). Some of these abnormalities could also be due to adventitious bud formation which often occurs in older cultures on axillary shoot proliferation medium.

16.3.4. Stage 3: rooting of shoots

Somatic embryos carry a pre-formed radicle and may develop directly into plantlet. However, these embryos often show very poor conversion

into plantlets, especially under ex vitro conditions. They require an additional step of maturation to acquire the capability for normal germination (see Section 6.5).

Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt composition. The number of shoot multiplication cycles after which the rooting exercise is to be started is governed by the number of plants to be produced through micropropagation and the available nursery facilities.

For rooting, individual shoots measuring about 2 cm in length are excised and transferred to the rooting medium. However, it would not only be convenient but also economical if shoot clusters formed after a shoot multiplication cycle, can be handled as units during the rooting and transplantation stages, and individual plants separated at a later stage, as in garlic (Bhojwani, 1980b; Bhojwani et al., 1982).

It is common practice to root shoots in vitro but many commercial laboratories prefer to treat the in vitro multiplied shoots as microcuttings and root them in vivo (ex vitro), if possible. In vivo rooting offers many advantages (Debergh and Read, 1991; Preece and Sutter, 1991): (1) in vitro rooting is labour intensive and expensive, accounting for 35–75% of the total cost of micropropagation (Debergh and Maene, 1981). In vivo rooting combines the rooting and acclimatization stages and, thus, reduces aseptic handling. (2) In vivo formed roots are structurally and functionally of better quality than those developed in vitro. The in vitro formed roots are often thick and lack root hairs and good vasculature. These roots frequently die or collapse after the plantlets are removed from cultures and new, functional lateral and adventitious roots are formed during acclimatization. Under in vivo conditions, callusing at the base of the shoot occurs only rarely ensuring a continuous vascular connection between root and shoot. (3) The in vitro developed roots may get damaged during transplantation. (4) For difficult-to-root species it is easier and cheaper to create good rooting conditions in vivo than in vitro.

16.3.5. Stage 4: transplantation

The ultimate success of commercial micropropagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied in vitro are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients and growth regulators, sucrose as carbon source, high humidity,

low light and poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under in vivo conditions. The two main deficiencies of in vitro grown plants are: (i) poor control of water loss, and (ii) heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. During acclimatization the in vitro formed leaves do not recover but the plant develops normal leaves and functional roots. The major deficiencies of in vitro grown plants and the methods used or recommended for their transfer out of culture are briefly described in this section.

(i) *Deficiencies*. Scant deposition of protective epicuticular wax on the leaves developed under highly humid in vitro environment is regarded as one of the most important factors responsible for excessive loss of water by cuticular transpiration leading to poor transplantation survival (Grout and Aston, 1977; Sutter and Langhans, 1979, 1982; Fuchigami et al., 1981; Brainerd and Fuchigami, 1981, 1982; Wetzstein and Sommer, 1982; Dhawan and Bhojwani, 1987). Even the chemical nature of the wax deposited under in vitro conditions differs from that formed under natural conditions. The former has a higher proportion of polar compounds which are less hydrophobic and, therefore, allow excessive diffusion of water from in vitro formed leaves (Sutter, 1984).

Poor anatomical differentiation of leaves, greater stomatal frequency (Wetzstein and Sommer, 1982), and impaired stomatal structure and movement (Brainerd and Fuchigami, 1982; Wetzstein and Sommer, 1983; Ziv et al., 1987a,b) have also been implicated in the water imbalance exhibited by micropropagated plants after removal from culture. The stomata on in vitro developed leaves remain wide open and do not respond to the stimuli, such as dark, ABA and CO_2, that normally induce their closure (Brainerd and Fuchigami, 1982; Wardle and Short, 1983; Conner and Conner, 1984; Ziv et al., 1987a,b). Abnormally large stomata were observed in cultured plants of geranium, rose (Reuther, 1988), apple (Blanke and Belcher, 1989) and carnation (Ziv et al., 1983, 1987a,b). Structural changes in the guard cells are accompanied by lower levels of cellulose, pectins and cutin and elevated levels of callose (Ariel, 1987; Werker and Leshem, 1987; Marin et al., 1988; Ziv and Ariel, 1992, 1994).

In most of the species investigated, the in vitro developed leaves show poor mesophyll differentiation and weak vasculature. The palisade tissue is lacking or is poorly developed (Grout and Aston, 1978; Wetzstein and Sommer, 1982; Dhawan and Bhojwani, 1987), and the mesophyll tis-

sue is mainly composed of spongy parenchyma with large intercellular spaces. The chloroplasts are poorly developed, with low chlorophyll and protein contents and disorganized grana (Wetzstein and Sommer, 1983; Ziv et al., 1983; Lee et al., 1985; Capellades et al., 1990a; Ziv and Ariel, 1992).

The stem of in vitro developed plants is hypolignified. Cells are thin, and there are large intercellular spaces with a limited development of vascular tissue and considerably less collenchyma and sclerenchyma (Donnelly et al., 1985; Ziv, 1995). In carnation, the stem vascular bundles lacked normal organization (Werker and Leshem, 1987) and in cauliflower the root-stem vascular connection was incomplete (Grout and Aston, 1977a).

The low light, high humidity, lack of adequate CO_2 and fair amount of sucrose in the medium force the in vitro growing plants into heterotrophic mode of nutrition. Consequently, these plants are poor in chlorophyll content, and the enzymes responsible for photosynthesis are inactive or absent altogether (Grout and Aston, 1977a; Wetzstein and Sommer, 1982; Donnelly and Vidaver, 1984).

Thus, peculiar leaf anatomy, incomplete development of chloroplasts (Wetzstein and Sommer, 1982) and low net CO_2 uptake and fixation are some of the characteristics of in vitro raised plants.

(ii) *Acclimatization. Ex vitro:* Traditionally, transplantation follows the in vitro rooting stage but in vivo rooting is gaining popularity for obvious reasons of economy and the quality of roots (Section 16.3.4). In the latter case the rooting and acclimatization stages are combined.

While transferring out shoots/plants from culture, their lower part is gently washed to remove the medium sticking to them. The individual shoots or plantlets are then transferred to potting mix and irrigated with low concentration of inorganic nutrients (Knop's or 1/4 strength MS salt solution). A variety of potting mixes, such as peat, perlite, polystyrene beads, vermiculite, fine bark, coarse sand, etc. or their mixtures in different proportions are used for transplantation. For the initial 10–15 days, it is essential to maintain high humidity (90–100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to the ambient level over a period of 2–4 weeks. Thus, in the first phase of acclimatization the main environmental stress to the plants is the change from a substrate rich in organic nutrients to one providing only inorganic nutrients. This probably recommissions the photosynthetic machinery of the plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions.

Several methods have been used to build up high humidity around the plants during the early phase of transplantation. The most primitive approach has been to cover the plants with clean, transparent plastic bags and make small holes in them for air circulation. To reduce humidity inside the plastic bag the holes are gradually enlarged over a period of time. Another simple method to acclimatize in vitro raised plants is to gradually remove the cap of the culture vial over a period of 7–10 days (Ripley and Preece, 1986; Ziv, 1986); slightly loosen the cap for the initial 1 or 2 days, completely unscrew the cap but leave it on the vessel for the next 3–4 days, and leave the plants in the uncapped jar for about 7 days for the plants to get gradually acclimatized to low humidity. The plants are then transplanted in a suitable potting mix after removing the agar and irrigated with a suitable nutrient solution.

Acclimatization of micropropagated plants on a large scale is generally carried out in a polyhouse or glasshouse where a gradation of high-to-low humidity and low-to-high levels of irradiance are maintained. High humidity is maintained by a misting or fogging system. The latter, although expensive, is preferred because mist leaches nutrients, causes the medium to become too wet, allows the plantlets themselves to dry and creates an environment favourable for the growth of algae and some fungi and bacteria. Fogging, avoids many of these problems (see Preece and Sutter, 1991). It produces droplets less than 20 μm in diameter by forcing water through fine nozzles. This ensures that the droplets float and saturate the air rather than sink and over-wet plants and potting mix.

In tropical countries, a gradient of humidity in the hardening facility can be achieved by fixing exhaust fans at one end and cellulose or polyester pads at the opposite end. As the pads are constantly kept wet, the air sucked through them, from the outside, is fully saturated to begin with but gradually its moisture content decreases thus creating a humidity gradient from the pad end to the fan end. In summer, this system also reduces the temperature inside the glasshouse by 5–6°C, compared to the outside. The micropropagated plants are put in protrays/flats during acclimatization. Over a period of 4–6 weeks the trays are moved from a high humidity-low light zone to low humidity-high light zone. By this time the plants should be fully acclimatized and are removed to a nethouse or field for better growth. During the acclimatization phase the plants need to be fed regularly, by drenching of the potting medium, with a liquid fertilizer. A robust growth of roots during this phase would help in better survival and growth of the in vitro plants.

Some other methods recently recommended to improve the transplantation success, presumably, at lower costs are: (i) in vitro acclimatization, and (ii) in vitro formation of storage organs.

In vitro: Ex vitro acclimatization of tissue culture raised plants is labour intensive and involves high capital investment. It also restricts the market for supplying in vitro grown plants to specialized growers with the expertise and facilities to harden the micropropagated plants. A number of treatments applied during in vitro culture have been shown to improve the quality of transplants and, thus, make their transfer to greenhouse or field simple and reduce losses (Roberts et al., 1990b; Ziv., 1995). During in vitro hardening, the roots of micropropagated plants, particularly of tree species, can be infected with *Rhizobium* or other microorganisms which make symbiotic association with them, to ensure better survival and growth of the plants after transfer to field (Perinet and Lalonde, 1983; Bertrand and Lalonde, 1985; Dhawan and Bhojwani, 1987).

The high relative humidity (RH) in the headspace of the culture vial and water potential of the medium influence plant growth and development. The culture of plants at relatively low humidity reduces their wilting after transfer to soil by improving stomatal movement (Wardle et al., 1983; Short et al., 1987), and increasing the deposition of epicuticular wax on the leaves (Wardle et al., 1983; Maene and Debergh, 1986; Short et al., 1987). The methods that have been used to reduce humidity inside the culture vial include the use of desiccants (Wardle et al., 1983; Ziv et al., 1983; Short et al., 1987), use of culture vials with microporous closure to allow gaseous exchange (Short et al., 1987), and cooling the bottom of the culture vessels (Maene and Debergh, 1987). By increasing the irradiance from 20 μmol m^{-2} s^{-1} to 80 μmol m^{-2} s^{-1} and reducing the RH inside the culture vessel from 100 to 75% it was possible to induce the in vitro growing rose plants to acquire morphological and anatomical characteristics similar to those observed during ex vitro acclimatization (Capellades et al., 1990b).

Certain growth retardants have been reported to promote in vitro root and shoot growth (Chin, 1982; Khunachak et al., 1987), reduce shoot elongation and leaf area, increase chlorophyll content and improve stress resistance of in vitro grown plants by promoting epicuticular wax deposition and stomatal response and development of thick roots (Ziv, 1991a, 1992; Novello et al., 1992; Roberts et al., 1992). Addition of paclobutrazol (0.5–4 mg l^{-1}) to the rooting medium enhanced desiccation tolerance of micropropagated chrysanthemum, rose and grapevine plants (Smith et al., 1990; Roberts et al., 1992). The effect of this antigibberellin compound was further enhanced if the plants were cultured in cellulose plugs irrigated with sucrose-free nutrient medium, probably because of reduced damage to the root system during transplantation (Roberts et al., 1990b). In *Philodendron*, application of paclobutrazol or ancymidol during shoot

proliferation enhanced ex vitro survival of micropropagated plants (Ziv and Ariel, 1991).

Autotrophic micropropagation, a plant propagation method based on tissue cultures deriving all their carbon requirement from CO_2 (Kozai, 1991d), is also being explored to produce plants suitable for transfer to glasshouse or field without prolonged ex vitro hardening (Kozai, 1991a,c). Some of the advantages (realized or potential) of photoautotrophic micropropagation are: development of structurally and physiologically normal plants, simplified rooting, reduced use of growth regulators, reduced problem of microbial contamination due to lack of sucrose in the medium and, facilitation of automation, robotization and computerization of micropropagation (Kozai, 1991a,c). Fujiwara et al. (1988) and Hayashi et al. (1988b) developed a microcomputer controlled acclimatization chamber in which levels of CO_2, RH, irradiance, temperature and air flow to plants in the culture vessel could be monitored.

In several plant species an increase in plant growth and photoautotrophic behaviour could be achieved by reducing or deleting sucrose from the medium, increasing CO_2 level (to 350 ppm) around the plant and increasing irradiance (to 200 μmol m^{-1} s^{-1}). CO_2 level in vitro could be enhanced either by using gas permeable films, such as microporous polypropylene, or culture vessels of fluorocarbon polymer (Kozai, 1991c,d), which also reduces relative humidity over the plants. However, photoautotrophic micropropagation is still very much at the laboratory stage and many aspects, including its economic feasibility and practicality, have to be worked out before it can be taken up at commercial level.

In vitro formation of storage organs. The plant species which normally form storage organs, such as tubers, corms and bulbs, can be induced to form such organs under in vitro conditions for direct planting in the greenhouse or field, cutting down the tedious step of acclimatization and reducing transplantation losses. It may even eliminate the rooting step as the storage organs can be formed by unrooted shoots. Moreover, the storage organs can be stored or readily shipped and planted either manually or by machines.

In vitro storage organ formation by manipulating the sugar and/or growth regulator contents of the culture medium and light and temperature conditions has been reported in several species, including *Crocus* sp. (Plessner et al., 1990), *Dioscorea* spp. (Forsyth and van Staden, 1984), *Freesia* (Hirata et al., 1995), *Gladiolus* (Dantu and Bhojwani, 1995), lilies (Takayama et al., 1991) and potato (Dodds et al., 1992; Alchanatis et al., 1994). Bulblet formation in *Muscari armeniacum* was promoted by 1% activated charcoal (Peck and Cumming, 1986).

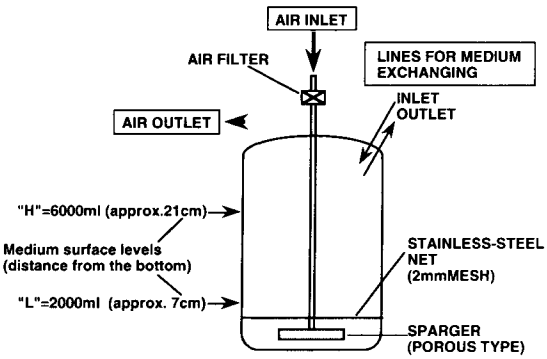

Fig. 16.9. Diagram of a jar fermentor used for mass propagation of potato tubers (after Akita and Takayama, 1993).

Most extensive work on in vitro formation of storage organs pertains to microtuber formation by nodal cuttings of potato. It is a two step process (see Appendix 16.I.1). Shoots are generally multiplied on a medium containing 3% sucrose, a cytokinin and an auxin, in light, at 25°C. Potato shoot cultures do not respond well to the classical cytokinin stimulation of axillary shoot proliferation and produce only a single multinodal shoot even in the presence of a cytokinin. Therefore, single node cuttings are used for recurrent shoot multiplication. In the second step the nodal segments are induced to form microtubers. A high concentration of sucrose (6–10%) and the presence of a cytokinin in the medium, low temperature (15–18°C) and dark are critical factors for in vitro tuberization. Addition of 500 mg l^{-1} of CCC to MS medium containing 8% sucrose and 5 mg l^{-1} BAP was shown to induce tuber formation in a broad range of genotypes (Schilde-Rentschler et al., 1984). Recently, Alchanatis et al. (1994) reported that the addition of 0.25–0.5 mg l^{-1} of ancymidol to the shoot multiplication medium (1/4 MS salts + 8% sucrose + 5 mg l^{-1} kinetin) considerably enhanced the number of nodes formed and improved the quality of shoots so that the tubers formed in the second step were in greater number and larger than those formed otherwise.

Despite the various advantages offered by microtuber formation, it has not been commercialized because the plants produced by this method are more expensive than those produced by rooted microcuttings (McCown and Joyce, 1991; Akita and Takayama, 1993). To obviate this problem attempts have been made to achieve mass production of microtubers in bioreactors. Akita and Takayama (1993) used an 8-l airlift fermentor (Fig. 16.9) and a two-step method (Fig. 16.10) to micropropagate potato via microtuber formation (Fig. 16.11). In step I, 60 single node segments from stock shoot cultures, established and multiplied on semi-solid MS (3% sucrose) medium, were transferred to the reactor containing 2 l of

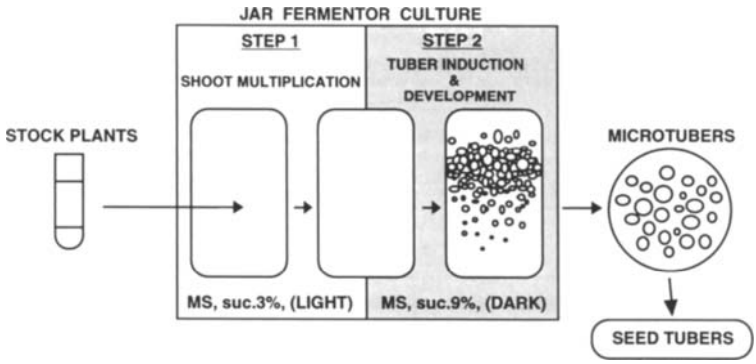

Fig. 16.10. Diagrammatic representation of the two-step culture scheme for mass propagation of potato tubers in a jar fermentor (after Akita and Takayama, 1993).

liquid MS medium with 3% sucrose. After 4 weeks of culture under continuous light (2–5 W m^{-2}), when numerous elongated shoots were formed, the culture medium was replaced by liquid MS medium containing 9% sucrose. After 5 weeks, in the dark, about 270 microtubers were formed. Small Potato Inc. (USA) has developed a cylindrical reactor that is rotated on its horizontal axis, intermittently bathing the shoots held at the outside of the reactor chamber (see McCown and Joyce, 1991). In this system the shoot multiplication is carried out under conditions which cause shoot tip necrosis resulting in the breaking down of apical dominance. As the next axillary shoot develops, it also suffers shoot tip necrosis. Thus, after some time multiple shoots are formed.

The microtubers formed in vitro are similar in colour, shape and electrophoretic pattern of storage proteins to field-produced tubers (Espinoza et al., 1986; Dodds et al., 1992), and the field grown plants derived from microtubers, in vitro produced microcuttings or field produced tubers formed similar numbers of tubers (Wang and Hu, 1982; Wattimena et al., 1983).

Micropropagated shoots of gladiolus could be induced to form corms in vitro by increasing sucrose concentration in liquid medium (Dantu and Bhojwani, 1987, 1995) or in the presence of certain growth retardants (Ziv, 1991a). The cold-treated in vitro formed corms of cvs Friendship, Gold Finch's and Her Majesty showed 100% germination in the field, and the resulting plants were morphologically and cytologically comparable to those produced by in vivo formed corms (Dantu and Bhojwani, 1995). Following in vitro formation of storage roots, in high-sucrose rooting medium, *Asparagus officinalis* plants required only 3–4 days of hardening under high humidity tents before their transfer to an open greenhouse bench (Conner et al., 1992).

Fig. 16.11. A potato plant bearing microtubers formed in the jar fermentor shown in Fig. 16.9 (after Akita and Takayama, 1993).

16.4. FACTORS AFFECTING IN VITRO STAGES OF MICROPROPAGATION

16.4.1. Culture initiation and shoot multiplication

The salt mixture of Murashige and Skoog's (1962) medium has proved satisfactory for many crop plants. It is often possible to use the same

medium for culture initiation and shoot multiplication. A basal medium containing inorganic salts of MS medium, 170 mg l^{-1} $NaH_2PO_4 \cdot H_2O$, 80 mg l^{-1} adenine sulphate dihydrate, 0.4 mg l^{-1} thiamine·HCl, 100 mg l^{-1} inositol and 3% sucrose has been successfully used for the micropropagation of a large number of plant species (Murashige, 1977). For some plants, however, the level of salts in the MS medium is either toxic or unnecessarily high (Anderson, 1975; Adams et al., 1979a). Blueberry shoots, for example, grow extremely well in a medium with MS salts reduced to one-quarter strength; higher levels are either toxic or without any beneficial effect (Cohen and Elliott, 1979). Similarly, *Dendrocalamus longispathus* showed better shoot proliferation on 1/2 or 1/4 strength MS medium than on full MS (Saxena and Bhojwani, 1993). Leaf explants of *Pinguicula moranenesis* died even on half-strength salts mixture of the LS medium (Linsmaier and Skoog, 1965) which is identical to the MS medium (Adams et al., 1979a). For their micropropagation the salt concentration had to be reduced to one-fifth.

The promoting effect of diluted mineral salt solution on shoot proliferation is probably due to reduced nitrogen level. In several Gymnosperms shoot bud differentiation was promoted by lowering nitrogen level in the MS medium (Ellis and Bilderback, 1984; Flinn et al., 1986; Perez-Bermudez and Sommer, 1987; Tuskan et al., 1990). Flinn et al. (1986) demonstrated that NH_4^+ ions accounted for much of the difference in morphogenesis in embryo cultures of *Pinus strobus* on MS and SH media. SH basal medium was better than MS medium for shoot bud differentiation. When NO_3^- level in SH medium was raised to 150% it did not affect the caulogenic response, but increase in NH_4^+ concentration to a level comparable to MS adversely affected the response.

Organic nutrients of MS or LS media are generally adequate for the micropropagation of most species.

The requirement for growth regulators varies with the system and the mode of shoot multiplication. The general concept given by Skoog and Miller (1957) that organ differentiation in plants is regulated by an interplay of auxins and cytokinins, is universally applicable and should work as a guide when developing a medium for a new plant type. According to this hypothesis the nature of organogenic differentiation is determined by the relative concentrations of the two hormones. A higher cytokinin-to-auxin ratio promotes shoot formation and a higher auxin-to-cytokinin ratio favours root differentiation. This should not imply that for adventitious shoot formation or enhanced axillary branching both the hormones must be included in the medium. The exogenous requirements of the hormones depend on their endogenous levels in the plant system which is variable with the tissues, plant type, and the phase of plant

growth. Consequently, for shoot multiplication the presence of an auxin in the medium is not obligatory. In a number of cases a cytokinin alone is enough for optimal shoot multiplication (Lane, 1979; Stoltz, 1979; Bhojwani, 1980b; Garland and Stoltz, 1981). Hormonal control of organogenesis and somatic embryogenesis in callus cultures is discussed in Chapters 5 and 6, respectively.

The qualitative and quantitative requirements for cytokinin and auxin for the maximum, but safe, rate of shoot multiplication for a new plant type should be determined by a series of experiments. A range of cytokinins (kinetin, BAP, 2-ip, and zeatin) has been used in micropropagation work. BAP, a synthetic cytokinin, is the most useful and reliable (also the cheapest) cytokinin and should be tested first for a new system. For hybrid willow (Bhojwani, 1980a) and white clover (Bhojwani, 1981) 2-ip, either autoclaved or filter-sterilized, was completely ineffective in inducing shoot multiplication whereas BAP supported the best multiplication. Anderson (1975) observed that for rhododendrons 2-ip was the best cytokinin. BAP supported a poor rate of shoot multiplication and it was toxic to the shoots; 40–70% of the rhododendron shoots died in the presence of 2.5–20 mg l^{-1} BAP. 2-ip also proved to be the best cytokinin for shoot multiplication in blueberry (Cohen, 1980) and garlic (Bhojwani, 1980b). Zeatin is not preferred because of its high cost. However, in Japanese persimmon both BAP and 2-ip were ineffective, and best shoot growth and multiplication occurred in 1/2 strength MS medium containing 1×10^{-5} M zeatin (Fukui et al., 1989). Since 1982, thidiazuron (a urea derivative) has been used as a cytokinin in several studies on shoot multiplication (see Lu, 1993). It is especially more effective than the other cytokinins (with purine ring) with recalcitrant woody species.

Cytokinins have been used in the range of 0.5–30 mg l^{-1} but, generally, 1–2 mg l^{-1} is adequate for most systems. Thidiazuron is effective at much lower concentration (0.0022–0.088 mg l^{-1} is recommended for micropropagation). At higher levels cytokinins tend to induce callusing and/or adventitious bud formation (McComb, 1978; Zimmerman and Broome, 1980) which can endanger the clonal nature of the micropropagated plants in some systems. High cytokinin concentration may also bring about morphological abnormalities and cause hyperhydration (Section 16.6.1).

With higher levels of cytokinins the number of shoots formed may be more but the growth of individual shoots remains arrested. This may require an additional in vitro step of shoot elongation on a medium with a lower concentration of cytokinin. A good growth of the shoot is especially critical when shoot multiplication through axillary branching is

achieved by taking nodal segments at each subculture. In such cases the rate of shoot multiplication is directly related to the elongation of the shoots and the number of nodal cuttings available at the end of each passage.

Of the various auxins, IAA is the least stable in the medium. Therefore, synthetic auxins such as NAA and IBA have been preferred for use in tissue culture media. For shoot multiplication their concentration ranges from 0.1 to 1 mg l^{-1}. Because of its strong tendency to induce callusing 2,4-D is avoided when shoot multiplication is attempted through axillary branching or adventitious bud development. For somatic embryogenesis, however, 2,4-D is the most effective auxin (see Chapter 6).

In some woody species GA_3 has been used in the shoot proliferation medium to improve shoot elongation (Wochok and Sluis, 1980; Brand and Lineberger, 1992). GA_3 (2.5 μM) improved the rate of multiplication, growth and quality of shoots in *Gardenia* (Economou and Spanoudaki, 1986). Debergh et al. (1993) reported stimulation of shoot multiplication in *Cordyline terminalis* by carbendazim (40–160 mg l^{-1}), a fungitoxic ingredient of some fungicides.

Since semi-solid cultures are easier to handle and maintain, the media for micropropagation are traditionally gelled with 0.6–0.8% agar. However, for several systems liquid medium has proved either critical for their survival in culture or beneficial for multiplication (Simmonds and Cummings, 1976; Snir and Erez, 1980; Molnar, 1987; Viseur, 1987; Saxena and Bhojwani, 1993). In *Cattleya* (Appendix 16.II.1) and most of the bromeliads (Hosoki and Asahira, 1980), for example, cultures could be started only in liquid medium (see Murashige, 1974). In pear the number of axillary shoots produced in liquid medium was much higher (>15 per explant) than that on semi-solid medium (1–3 per explant) but in liquid medium the shoots got vitrified (Viseur, 1987). In such cases the double-phase culture system of Maene and Debergh (1985), in which liquid medium is poured over the semi-solid medium, has been found useful (Viseur, 1987). The vitrification problem in liquid medium could also be obviated by suppressing leaf growth with the addition of paclobutrazol to the medium (Ziv, 1989; Ziv and Ariel, 1991).

Generally the pH of the medium is set at 5.8 but for some calcifuge (*Magnolia* × *soulangiana*) and acid loving (*Disanthus cercidifolius*) species, low pH is required. *Magnolia* shoot production increased from 3.7 to 7.2 by reducing pH from 4.5 to 3.5 and that of *Disanthus* from 2.3 to 7.2 with change in pH from 6.5 to 4.5 (Howard and Marks, 1989). Lowering the pH to 4, with doubling of Ca^{+2} and Mg^{+2}, promoted shoot multiplication and elongation in chestnut (Chevre et al., 1983).

Despite being green the in vitro growing shoots do not rely on photosynthesis for their food. They grow as heterotrophs, deriving all their nourishment (organic as well inorganic) from the medium. Light is required by these cultures only for certain morphogenic processes. A light intensity of 1000–5000 lx is adequate for this purpose (Hussey, 1980). Optimum light intensity for shoot multiplication in *Gerbera* and many other herbaceous species was reported by Murashige (1974) to be 1000 lx. In low light intensities, the shoots are greener and taller (Murashige, 1977). In future it may be possible to develop autotrophic cultures under high light intensity. Photoperiodism is, strictly speaking, not critical. A diurnal illumination regime of 16 h day and 8 h night is found satisfactory (Murashige, 1977).

Cultures are usually maintained at a constant temperature around 25°C. However, for tropical species a higher temperature (30°C) may be beneficial (Dhawan and Bhojwani, 1985; Rahman and Blake, 1988).

16.4.2. Rooting

For in vitro rooting, nutrient salts in the medium have a dramatic effect both on rooting percentage and root number per microcutting. Often, where shoot multiplication was induced on full-strength MS medium, the salt concentration was reduced to half (Garland and Stoltz, 1981; Zimmerman and Broome, 1981) or a quarter (Skirvin and Chu, 1979) for rooting. Bulblets and shoots of *Narcissus* rooted only when the culture medium contained MS salts at half strength (Seabrook et al., 1976). Similarly, Anderson (1984) reported that rooting of *Rhododendron* microcuttings increased from 19% on full strength medium to 77% on half strength medium. Best rooting of *Leucopogon obtectus* microcuttings occurred on agar-water medium without an auxin, and the response worsened with increasing MS salt level (Bunn et al., 1989).

As for the shoot multiplication, the promoting effect of diluted mineral salt solution on rooting is probably due to reduced nitrogen level. Driver and Suttle (1987) observed that preconditioning of walnut microcuttings on a medium with lower nitrogen and high sucrose content was beneficial for in vitro rooting. All the three apple scion cultivars (Gala, Royal Gala, Jonagold), studied by Sriskandarajah et al. (1990) showed best rooting, in response to 9.8 μM IBA, when NH_4NO_3 was deleted from MS medium. Reduction of NH_4NO_3 level to 1/2 or 1/4 strength in MS medium also significantly increased the rooting response in cvs Gala and Royal Gala but not in Jonagold. Generally, however, nitrogen must be supplied both as NO_3 and NH_4; the optimal ratio between these ions is 3 for rose cut-

tings (Hyndman et al., 1982) and 4 for *Cynara scolymus* (Moncousin, 1982).

The rooting of microcuttings requires continuous availability of free sugars (Haissig, 1974; Thorpe, 1978, 1982b, 1984; Gaspar and Coumans, 1987) which favours the differentiation of a good vascular system (Sommer and Caldas, 1981) and accelerates lignification (Thorpe, 1978; Thorpe and Biondi, 1981; Driver and Suttle, 1987). Pre-treating microcuttings of *Juglans regia* × *J. hindsii* with 3% solution of sucrose for 7 days induced 80.5% rooting compared to 55.5% with 2% sucrose (Driver and Suttle, 1987).

Kaneko et al. (1988) investigated the effect of CO_2 on adventitious root formation by lettuce cuttings. On 1/10th MS medium, without sucrose, and under 120 μmol m^{-2} s^{-1} irradiance, in an environment enriched with 0.9% CO_2 the rooting percentage was 75–90% as against 10% under non-enriched conditions.

Occasionally, as in *Gladiolus* (Hussey, 1977), *Narcissus* (Seabrook et al., 1976), and *Fragaria* (Boxus, 1974) shoots are readily rooted on a hormone-free medium. For most other species a suitable auxin is required to induce rooting. Most often IAA, IBA and NAA (0.1–1 mg l^{-1}) have been used for this purpose, but the former two seem to be more effective (George and Sherrington, 1984).

In some woody species, rooting of microcuttings may require a high concentration of auxin. Prolonged exposure to high auxin levels, however, has some undesirable effects, such as callusing, leaf chlorosis, inhibition of root elongation and quiescence or dormancy in the shoot tip which is difficult to overcome in the acclimatization stage (Maynard et al., 1991). A possible solution to this problem is to treat the cuttings with a high concentration (100–500 mg l^{-1}) of a suitable auxin for a short period (4 h–5 days) and then plant them in an auxin-free medium in vitro (Riffaud and Cornu, 1981) or ex vitro (Maynard et al., 1991; Brand and Lineberger, 1991). Microcuttings of *Prunus serotina* were rooted on 1/5th strength MS salt medium after a quick dip in 3 mM IBA (Riffaud and Cornu, 1981). Similarly, with pre-dip in 200 mg l^{-1} IBA solution mature material of *Liquidamber styraciflua* could be rooted at a rate of 70–90% (Brand and Lineberger, 1992).

Riboflavin is reported to improve the quality of the root system in *Eucalyptus ficifolia*. Several chemicals, often termed 'auxin synergists' or rooting 'co-factors', have been found to enhance the rooting response of applied auxins (Jarvis, 1986). For example, Smith and Thorpe (1977) showed that exogenously supplied aromatic amino acids and simple phenolics could enhance the formation of root primordia in *Pinus radiata*. Pythoud et al. (1986) also observed a synergistic effect between vitamin D

and IBA on adventitious root formation in *Populus tremula* (Gorst and De Fossard, 1980).

Dark treatment, particularly during the beginning (3–7 days) of the rooting stage, and raising the temperature during this period from 25 to 30°C have been reported to improve the rooting response of several apple scion cultivars (Zimmerman, 1984; Zimmerman and Fordham, 1985). In some of the hard-to-root cultivars these treatments helped achieve up to 100% rooting.

In tree species, the rootability of shoots may improve with the number of shoot multiplication cycles. For example, *Eucalyptus citriodora* shoots formed during the first two multiplication cycles failed to root but after the third passage rooting could be induced (Gupta et al., 1981). The time needed to approach maximum rooting may vary with the material. Whereas Minnesota azalea selections required four subcultures to reach over 95% rooting (Economou and Read, 1986) apple cv Delicious took 31 subcultures to reach 79% rooting (Sriskandarajah et al., 1982). The in vitro conditions seem to induce rejuvenation of the material, the effect of which may last for some time even after their transfer out of culture (Marks, 1991a,b).

The time required for in vitro rooting of shoots may vary from 10 to 15 days. It has been experienced that handling of plants during transplantation is made convenient if the roots are below 5 mm in length. Longer roots may break during transplantation and, thus, diminish the chances of survival of the plants.

For ex vitro rooting, the size of microcuttings may be critical. In the case of *Cordyline terminalis* 2.5–5 cm long shoots were best. Treatment of the microcuttings with root inducing growth regulators (auxins) or a commercial rooting powder may be necessary for their rooting in vivo. An intermediate approach between in vitro and in vivo rooting is to apply the rooting treatment in vitro and transfer cuttings out of culture before roots appear. This approach has been successful with apple (Welander, 1983; Zimmerman and Fordham, 1985). Driver and Suttle (1987) harvested 3–10 cm long microshoots of walnut and peach and placed them on a basal nutrient medium with elevated sucrose and auxin levels and reduced nitrogen concentration for 1–2 weeks and then transferred to non-sterile medium. During auxin treatment the shoots were placed under higher light intensity (66 μmol m^{-2} s^{-1}), shorter photoperiod (17 h) and lower temperature (19°C) than during shoot multiplication to promote lignification. The in vitro multiplied shoots of grape placed under 1200 ppm CO_2 during ex vitro rooting grew more rapidly than those growing continuously with 350 ppm CO_2, probably because of increased root growth (Lakso et al., 1986). When combining rooting and acclimati-

zation the same environmental considerations apply as when acclimatizing rooted plantlets (see Section 16.3.5).

16.5. APPLICATIONS OF MICROPROPAGATION

In vitro methods can be used to produce, maintain, multiply and transport pathogen-free plants safely and economically. Currently, the most popular application of micropropagation is the mass clonal multiplication of desirable genotypes of plants. Through tissue culture, over a million plants can be grown from a small, even microscopic, piece of plant tissue within 12 months. Such a prolific rate of multiplication cannot be expected by any of the in vivo methods of clonal propagation. An advantage of tissue culture propagation is that the shoot multiplication cycle is very short (2–6 weeks), each cycle resulting in a logarithmic increase in the number of shoots. Additionally, in tissue cultures plant multiplication can continue throughout the year irrespective of the season. A large number of horticultural, plantation and forest species are being propagated in vitro on commercial scale.

An enhanced rate of plant multiplication in vitro should reduce considerably the period between the selection and the release of a new cultivar. The amount of plant material released from quarantine being usually limited, micropropagation should also hasten the introduction of new crops following their passage through quarantine. In vitro conservation and propagation of endangered plants is another important application of this aseptic technique (Fay, 1992; Bhojwani and Arumugam, 1993).

Tissue culture provides reliable and economical methods for maintaining pathogen-free plants in a state that can allow rapid multiplication when needed, irrespective of the time of the year. In commercial nurseries tissue culture can be used to minimize the growing space usually provided for the maintenance of stock plants. Several thousand million plants can be maintained inside culture vials on a shelf space built into a room of about $3 \times 3 \times 5$ m.

Vegetative propagation is extremely important in the case of dioecious species where seed progeny yields 50% males and 50% females but plants of one of the sexes are more desirable commercially. In *Asparagus officinalis*, for example, male plants are more valuable than the female plants but their clonal propagation by stem cuttings is not possible. However, it can be cloned in vitro (Desjardins, 1992). In papaya, another dioecious crop, clonal propagation of established female plants (Jordan, 1992) would save the losses suffered due to discarding a large number of male plants in a seed-raised orchard which is possible only after the plants have reached the flowering stage.

16.6. LIMITATIONS OF MICROPROPAGATION

In vitro clonal propagation of plants is a commercial technique, being practised for a wide range of herbaceous and woody species by nurserymen all over the world. However, satisfactory protocols for micropropagation of adult materials of many important hardwood and softwood tree crops, whose propagation by conventional methods is difficult, have not been developed so far.

Once a method has been evolved and aseptic cultures established it may be tempting to continue to propagate from it for many generations. This may lead to bulking up of any off-types (sport or accidental change) that might have arisen in cultures at an early stage. To offset this real problem it is suggested that in vitro multiplication should be restricted to 5–10 cycles and only a few thousand plants raised from a single explant. These and some other major problems associated with micropropagation are discussed in this section.

16.6.1. Hyperhydration

The term hyperhydration, earlier called vitrification (Debergh et al., 1992b), refers to the morphological, physiological and metabolic derangements frequently affecting herbaceous and woody plants during their in vitro culture. Hyperhydration occurs only during intensive shoot multiplication and never during the rooting stage (Gaspar, 1991). Various terms, viz. glassiness, glauciness, translucency and vitrescence have been used to describe these malformations (Gaspar, 1991; Ziv, 1991b).

Since the first description of hyperhydration by Phillips and Mathews (1964), considerable literature has accumulated on the various manifestations, causal factors and preventive measures of this phenomenon (Gaspar, 1991; Ziv, 1991b; Debergh et al., 1992a).

(i) *Characteristics.* Besides the common structural and functional abnormalities shown by in vitro growing plants (see Section 16.3.5), hyperhydrated shoots have short internodes and appear thick, brittle, glassy, and water-soaked. Leaves are the immediate organ to be affected by these abnormalities.

Hyperhydrated shoots generally show poor growth, become necrotic and, finally, die (Gaspar, 1991). On solid medium, most of the hyperhydrated shoots turn into callus after two or three subcultures (Leshem et al., 1988). Hyperhydrated shoots do not root easily either in vitro or in vivo and adversely affect the growth of new shoots (Gaspar, 1991). The plants showing hyperhydration have low survival rate during acclimati-

zation (Ziv, 1991a,b). Therefore, such shoots are discarded during multiplication. However, in some conifers the hyperhydrated shoots have shown a better rate of multiplication. For example, Bornman and Vogelmann (1984) observed that vitrified cultures of *Picea abies* produced 10 times as many buds and shoots as normal shoots. It would be worthwhile to examine if it is applicable to other tree species.

(ii) *Causes*. The physical and chemical nature of the culture medium, and the atmosphere around the cultured plant material are responsible for hyperhydration. Liquid medium causes more hyperhydration than solid medium, and in the latter case the degree of hyperhydration is influenced by the concentration and type of gelling agent. It decreases with increase in the concentration of the gelling agent. Compared to agar, gelrite or phytagel causes more hyperhydration (Zimmerman et al., 1991).

Plants are more prone to hyperhydration on media rich in mineral salts, such as MS medium (Installe et al., 1985; Paques and Boxus, 1987a,b). Reducing NH_4^+ ions in the medium increased lignification and reduced hyperhydration in willow, plum, petunia and cacti species (Beauchesne, 1981; Daguin and Letouze, 1986; Leonhardt and Kandeler, 1987; Zimmerman et al., 1991). Elevated Ca^{2+} ions reduced hyperhydration in both herbaceous (Ziv et al., 1987b) and woody (Kreutmeier et al., 1984) species.

In several cases high cytokinin concentration is reported to promote hyperhydration. In apple, normal plants transferred from solid medium to liquid medium containing BAP became hyperhydrated (Paques and Boxus, 1987b). Removal of BAP from liquid medium induced reversal to normal shoots as long as hyperhydration was not very advanced (Gaspar et al., 1987). In carnation and conifers lowering the cytokinin level led to decreased hyperhydration, which was stimulated more by high BAP than kinetin (Dencso, 1987).

The size of the culture vessel, the type of closure, and the climate parameters of the culture room all influence hyperhydration (Hakkaart and Versluijs, 1983; Debergh and Maene, 1984). High relative humidity above the cultures accelerates the hyperhydration process (Wardle et al., 1983; Ziv et al., 1983; Debergh and Maene, 1984; Bottcher et al., 1988).

In the early days of plant tissue culture, cotton plugs were used as closure for the culture tubes which allowed good aeration of the cultured material. Commercialization of this technique introduced the use of a variety of plastic caps which do not allow good gaseous exchange and, consequently, create hyperhydration-inducing conditions inside the culture vial, viz. build-up of high humidity and accumulation of various gases. To overcome this problem new plastic lids with a vent covered by a

bacteria-proof membrane have been produced. A cheaper but equally effective alternative, being practised routinely in some commercial laboratories in India, is drilling a hole in the plastic lids and closing it with a cotton plug.

(iii) *Preventive measures.* Hyperhydration can be reduced and the quality of in vitro plants improved by lowering the humidity in the headspace of the culture vial by: (1) increasing the concentration of agar (Debergh et al., 1981); (2) using an agar with higher gelling strength (Debergh, 1983); (3) improving the aeration of the container (Dillen and Buysens, 1989; Rossetto et al., 1992);(4) overlaying of medium with paraffin (Wardle et al., 1983); (5) using a desiccant such as $CaSO_4$ (Ziv et al., 1983) and silica gel (Wardel et al., 1983); and (6) bottom cooling of the culture vial (Maene and Debergh, 1987; Vanderschaeghe and Debergh, 1988). Other treatments which may help reduce hyperhydration are: lowering cytokinin levels or replacing one type of cytokinin by another (Werner and Boe, 1980; Beauchesne, 1981; Debergh et al., 1981; Wilkins and Dodds, 1983), lowering NH_4^+ concentration (Zimmerman et al., 1991), diluting MS medium or replacing it with Heller's medium (Riffaud and Cornu, 1981; Letouze and Daguin, 1983; Vieitez et al., 1985), and addition of phlorizin, phloroglucinol (Hegedus and Phan, 1983, 1987) or $CoCl_2$ (Gaspar and Kevers, 1985) to the medium.

16.6.2. Off-types

Appearance of off-types in tissue culture-raised plants (see Chapter 9) is a major limitation in wide-spread acceptance of this technology for clonal propagation of crop plants. The abnormalities induced in vitro are influenced by the choice of explant, composition of culture medium (particularly the nature and concentration of phytohormones), length of time in vitro, the level of organization during culture, and genotype of the material. Generally, the systems involving a callus phase show maximum aberrations. Therefore, the callus phase, unless unavoidable, must be kept to a bare minimum. Direct differentiation of adventitious buds or embryos from the explants may also induce abnormalities, particularly in chimeric plants by disturbing the chimeric arrangement. The abnormalities observed in systems multiplied by axillary bud proliferation mainly result due to the development of some adventitious shoots, lasting effect of in vitro environment (particularly growth regulators) and/or, in perennial species, by the appearance of juvenile traits.

Oil palm is a well known example of developmental abnormalities observed in micropropagated plants (Duval et al., 1995). The most common

abnormality shown by this crop plant is feminization (Paranjothy et al., 1990; Duval et al., 1995). Staminodes in the female flowers develop into a mantle of fleshy carpels around the fruit, leading to the formation of abnormal fruits or to partial or complete sterility, depending on the extent of abnormality. In severe cases, even the male flowers show the development of stamens into pseudocarpels. In this system, micropropagated via somatic embryogenesis, the age and nature of callus affected the degree of abnormalities; it increased with age of the callus (Corley et al., 1986). Whereas all the plants regenerated from fast growing callus exhibited severe abnormalities, the slow growing nodular callus produced a limited number (3.1%) of abnormal plants (Duval et al., 1988, 1995).

Banana is another example where several research groups have described the appearance of off-types in micropropagated plants. The most common type of abnormality is the development of dwarf phenotypes, which are not only shorter in height but also show small fruits, closer packing of the hands on the bunches and chocking of the branches (failure to emerge fully from the pseudostem when temperature remains below 15°C). This aberration ranged from 3% in Taiwan (Hwang and Ko, 1987) and Australia (Smith and Drew, 1990a,b) and 9% in Israel (Reuveni et al., 1985) to 25% in Jamaica (Stover, 1987).

Increased cytokinin level in the medium may help enhance the rate of shoot multiplication but it also induces some undesirable features, probably by promoting the differentiation of adventitious buds in systems propagated by axillary branching (see Section 16.3.3). In strawberry, frequent subculture of shoots promotes formation of adventitious buds (Zimmerman, 1991). Micropropagated plants of chrysanthemum show juvenile characteristics, such as forming basal shoots and delayed flowering. Therefore, micropropagated plants of this species are more suitable as mother plants for propagation by cuttings because this basitonic behaviour disappears after one or a few generations of in vivo propagation.

Any in vitro method adopted for commercial purpose must ensure high genetic fidelity of the propagated plants. In tree species, where several years may be required to check trueness-to-type based on morphological features, at least cytological and molecular tests (RFLP, RAPD, isozyme profiles) can be made in the initial stages.

16.6.3. Contamination

Contamination is one of the most serious problems in commercial micropropagation as it can cause a disastrous situation if it occurs at an advanced stage of the production schedule. There is a growing belief that

tissue cultures are only rarely totally free of contaminants. There are many causes of contamination of established cultures. Slow growing bacteria carried with the explant may remain latent initially and show up at a later stage. Fungal spores which occur in the air with high concentration may be carried into the laboratory on body hairs of the workers or vectors like mites and thrips, which have been occasionally observed inside culture containers (Debergh et al., 1990). It is, therefore, extremely important to maintain high standards of hygiene in and around the sterile area. Spore concentration in the laboratory can be controlled by regular vaporization of formaldehyde or a fungicide such as thiobendazole (Debergh et al., 1990). Californian thrips can be controlled by a daily treatment with Baygon™ or two treatments, at an interval of 5 days, with 10% dichloorvos (Debergh et al., 1990).

16.6.4. Oxidative browning

A problem generally faced in culturing adult tissues from woody species and some other plants is the browning/blackening of the culture medium and/or the explant. The injury caused during the excision of explants induces the cells to leach out phenolic compounds which are readily oxidized to produce quinones and cause discoloration. The oxidation products of phenols can be phytotoxic and cause necrosis and, eventually, death of the explant. Several different methods have been used to overcome this problem (Preece and Compton, 1991).

Quick transfer of explants within the same vessel or to fresh medium 2 or 3 times, at short intervals, is the simplest and a fairly successful method to protect the explants from the detrimental effects of oxidative browning (Kotomari and Murashige, 1965; Morel, 1972; Preece and Compton, 1991). During this period the cut end of the explant may become sealed up and the leaching of phenols stop. Keeping the cultures initially in the dark may also help to reduce the browning problem (Monaco et al., 1977; Adams et al., 1979b) by preventing or reducing the activity of the enzymes concerned with both biosynthesis and oxidation of phenols (George and Sherrington, 1984).

For the micropropagation of a 20-year-old tree of *Eucalyptus citriodora* it was essential to grow the shoot buds, taken directly from the parent plant, in a liquid medium (at 15°C, in continuous light of 500 lx) for 3 days before planting on a semi-solid medium (Gupta et al., 1981). Cultures raised directly on a semi-solid medium did not survive. Zimmerman (1978) followed a similar approach with apples and *Rubus* sp. The initial culture in liquid medium probably helps in getting rid of the phenolic compounds and other growth inhibitors.

When the problem of medium browning persists at each subculture the addition of antioxidants, such as cysteine·HCl (100 mg l^{-1}), ascorbic acid (50–100 mg l^{-1}) or citric acid (150 mg l^{-1}) to the culture medium is recommended (Sondahl and Sharp, 1977; Skirvin and Chu, 1979). Immersion of shoot pieces, for 24 h, in a solution of antioxidants before planting them on a medium containing activated charcoal helped establish healthy cultures of *Strelitzia reginae* which otherwise died due to oxidative browning of tissue exudates (Ziv and Halvey, 1983). Polyvinylpyrrolidon (Polycar AT or PVP), which can adsorb phenolic compounds, has also been used to save tissues from the toxic effects of the oxidized phenols (Walkey, 1972; Gupta et al., 1980). For *Tectona grandis* insoluble PVP was better than soluble PVP (Gupta et al., 1980).

16.6.5. Recalcitrance of adult-trees

During the last 10 years efficient methods have been developed for in vitro clonal propagation of several tree species, of which some have been propagated on a large scale. These include ornamentals (rhododendron, cherry), fruit trees (apple, pear), plantation crops (oil palm, date palm), and hardwood (poplars, willows, eucalyptus) and softwood (radiata pine, redwood) forest tree species (Thorpe et al., 1991). For many other tree species (sandalwood, birch, teak, loblolly pine, walnut) promising protocols have been published. In 1989, Enso-Gutzeit (Finland) produced several thousand elite birch plants through micropropagation (Anonymous, 1991).

A spectacular development has been the achievement of somatic embryogenesis in a large number of woody species. However, this success is restricted to zygotic embryos and young seedling explants (Gupta et al., 1993). In many woody species even propagation by shoot bud proliferation has been successful only with juvenile tissues (Thorpe et al., 1991). By the time trees are old enough for assessment of desirable traits they become recalcitrant for tissue culture. There are two options to overcome this problem: (1) select the most juvenile tissues (e.g. current year's basal sprouts in redwood) from adult trees, and (2) rejuvenate parts of the desired plant by special treatments prior to excision of explants, such as repeated spraying of selected branches with cytokinin (particularly BAP; Abo El-Nil, 1982), and etiolation of shoots before excising shoot tip (Ballester et al., 1989). Repeated grafting of scion from mature trees onto seedling rootstocks accentuated and prolonged juvenile behaviour in scions of *Hevea*, *Eucalyptus* and *Psudotsuga* (Franclet et al., 1987). Repeated subculture of shoot apices in cytokinin medium induced reactivation of meristem in many species, including *Prunus*, *Eucalyptus*, *Pinus*

pinaster and *Sequoia* (Thorpe et al., 1991). The degree of rejuvenation increases with the number of subcultures.

Another problem encountered with temperate woody hardwood species is the episodic growth pattern observed in culture (McCown and McCown, 1987). In the culture of mature explants the shoot developed in vitro may become quiescent for varying periods (sometimes several years) depending on the degree of maturity of the selected material.

16.6.6. High costs

Although tissue culture is being used on commercial scale for several ornamental and other horticultural crops, it is generally more expensive than other forms of clonal propagation, using cuttings or seeds (Pierik, 1991). It is labour intensive, involving manual handling at 3 or 4 stages. Therefore, micropropagation can be justified only when the conventional methods of clonal propagation do not work or the product is of high value. For some crops, micropropagation can only be economical to produce pathogen-free foundation stock which can be used to produce a number of generations in glasshouse or field. Carnations and chrysanthemums can be easily propagated in vitro and with high propagation rates but so far straight production of these crops from tissue culture is regarded as uneconomical (Debergh and Read, 1991). Similarly, large scale micropropagation protocols for strawberry are well established and have been used all over the world. However, the micropropagated plants of this fruit crop being expensive, and the fact that they produce small fruits compared to the runner propagated crop, are rarely used for fruit production. They generally serve as mother plants for runner production (Zimmerman, 1991).

Since labour charges account for 60–70% of the cost of production of tissue culture plants in developed countries there is considerable interest in automating the process partly or completely (Vasil, 1991; Aitken-Christie et al., 1995). To some extent, automation has been introduced at the media preparation stage by using dispensing machines. Bioreactors are being used to automate the multiplication stage in some cases (see Sections 16.3.3 and 16.3.5). Somatic embryogenesis is potentially the most amenable system to automation, not only at the production stage but also for mechanized planting in the field as synthetic seeds. Although considerable progress has been made in this area yet there are several problems that must be resolved before somatic embryogenesis becomes a viable method for mass clonal propagation of plants.

An alternative approach being currently followed by some of the developed countries in order to reduce the cost of tissue culture propagated

plants is to sub-contract in vitro multiplication of their materials in the developing countries where labour is comparatively very cheap. This has resulted in the establishment of several commercial tissue culture laboratories in developing countries. Whether such laboratories, with huge investments, exclusively for export purpose would be economically viable is a big question. Air freighting of highly perishable ex vitro plants for delivery at right times while meeting the strictly prescribed quality standards may result in high rejection rates. India has at least half-a-dozen such laboratories.

16.7. CONCLUDING REMARKS

From the commercial viewpoint, micropropagation is the most important aspect of plant tissue culture. Industrial application of this technique started in the late 1960s and early 1970s, first with orchids and later with other crop plants. According to one estimate, 350 million plants are produced annually through micropropagation (Tormala, 1989), mainly including ornamentals (begonia, ferns, ficus sp., gerbera, philodendron, rhododendron, saintpaulia, syngonium), and some fruit (strawberry, banana, apple, pear) and forest (poplars, eucalyptus, redwood, radiata pine) species. Protocols for several other crops have been published. However, all research reports do not provide commercially viable protocols. Often the information regarding the rates of multiplication provided in such papers is inadequate for their commercial use. Moreover, each species and, in many cases, each variety may require a different combination of hormones for successful multiplication.

The micropropagation industry is capital and labour intensive. During the 1980s many companies and entrepreneurs became fascinated by this novel technique for clonal propagation of plants and took it up as a business but only a few survived. Micropropagation should be regarded as yet another method of plant propagation and not as a business in itself. Rapid multiplication of plants under disease-free in vitro conditions is often easy but it is not enough to survive in the business. It requires the production of quality plants at competitive price and according to the demands of the market. It is extremely important in this business to plan production according to market demand rather than to have a product first and then search for a market (Cervelli and Senaratna, 1995).

Any attempt to reduce the cost of production by accelerating the rate of multiplication or automation should not be at the expense of the quality of the product. Pushing a system beyond a limit with regard to the rate of multiplication or the life-span of a culture may introduce genetic aberrations which, if not identified and discarded at the right time, would get

rapidly multiplied with normal type. For this a trained human eye is probably indispensable. Mezitt (1989), who observed several abnormalities (foliar and floral variation, abnormal flowering, increased susceptibility to disease) in his rhododendron cultivars micropropagated in other laboratories, remarked that tissue culture laboratories run by individuals unfamiliar with horticulture and with very little or no association with the plant industry run the risk of allowing off-types to go through the production line under the economic pressure to accomplish too much too quickly.

In developed countries the labour charges account for 50–70% of the cost of production of tissue culture plants. Therefore, considerable research efforts are being made to reduce costs by introducing automation at different stages of production (Vasil, 1991; Aitken-Christie, 1991; Aitken-Christie et al., 1995). Somatic embryogenesis is considered as the ultimate mode of micropropagation because it may allow automation not only at the multiplication stage but also for their field planting as synthetic seeds (Cervelli and Senaratna, 1995; Sakomoto et al., 1995). Several problems must be solved before this objective can be realized.

APPENDIX 16.I: MICROPROPAGATION PROTOCOLS FOR SOME CROP PLANTS

16.I.1. Potato (based on Dodds et al., 1992)

(a) Collect shoot pieces from 5 to 10-week-old greenhouse grown stock plants, remove the leaves, and surface sterilize in 10% household chlorine bleach with 0.05% Tween 20 as a wetting agent.

(b) If the stock plant is a certified virus-free material, cut the shoot into 2 or 3 node segments and plant on MS basal medium containing 3% sucrose and 0.8% agar. If the parent plant is virus-infected, dissect 0.1 mm shoot tips under a stereoscopic microscope and culture on the medium as above. Incubate the cultures in continuous light, at 25–30°C.

(c) After the solitary shoot derived from an axillary bud or apical bud has attained a height of 7–10 cm, subculture it on MSA medium (see Appendix 16.II.5). Discard the terminal node and place the shoot horizontally on the medium.

(d) Repeat step (c) 3 or 4 times at weekly intervals to build-up the number of shoots. By the fourth passage uniform shoot growth and multiplication should be established. In the case of uncertified material, suitable indexing of the shoots must be carried out

and only those individuals found free of the specified viruses further multiplied.

(e) Cut the shoots into single node segments and transfer about 30 segments per 250 ml Erlenmeyer flask containing M-9 medium (see Appendix 16.II.5). The flasks may be shaken at 80 rev. min^{-1} on an orbital horizontal shaker. Incubate the cultures under a 16 h photoperiod at 22°C.

(f) After 3–4 weeks, 26–30 vigorous plants develop per flask. Individual plantlets can be transferred to the greenhouse following the standard method of ex vitro acclimatization. Microcuttings 3–5 cm long can also be readily rooted in peat-perlite medium in the glasshouse.

(g) Alternative to step (f), the plantlets can be induced to form microtubers by replacing the M-9 medium with MT medium (see Appendix 16.II.5) and storing the cultures in the dark at 15–20°C. B_5 medium may be better than MS medium.

(h) Harvest the microtubers, break their dormancy by storing in sterile petri plates sealed with parafilm, in diffuse light, at 4°C, for 4 weeks.

(i) The microtubers may be planted in pots in greenhouse to produce pathogen-tested mother plants for conventional rapid multiplication programme or can be planted at high density (up to 100 tubers m^2) in nursery beds, inside an insect-proof nethouse to produce high quality seed tubers.

16.I.2. *Gladiolus* (after Dantu and Bhojwani, 1992, 1995)

(a) Dehusk the corms and scoop out individual axillary buds along with some corm tissue. Trim the corm tissue to 5 mm^3.

(b) Rinse the excised buds in 90% ethanol, air dry for 15 min, dip in a 0.5% solution of Cetavlon (or any other surfactant) for 5 min and wash thoroughly under running water for 60 min. Surface sterilize the buds in a 2.5% (w/v) solution of sodium hypochlorite for 15 min and give three quick washes in sterile distilled water under aseptic conditions.

(c) Trim the peripheries of the corm tissue and plant the bud on MS + 0.5 mg l^{-1} BAP (BAP concentration might have to be changed depending upon cultivar being used) and incubate the cultures at 25°C under continuous light (10 W m^{-2} s^{-1}).

(d) After 2 passages of 4 weeks each on the medium as in (c), when multiple shoots have developed, cut the cluster of buds into pieces, each bearing more than one bud and culture them indi-

vidually on shoot multiplication medium (see Appendix 16.II.6). At the end of 4 weeks cut the cluster of shoot buds into small propagules, each with 10–20 shoot buds, and transfer them individually to fresh medium for shoot multiplication. Incubate the cultures in continuous dark, at 25°C.

(e) Cut the shoot clusters into small pieces, each bearing *ca* 10 buds, and transfer them to liquid MS medium containing 0.5 mg l^{-1} BAP, for shoot elongation. Incubate the cultures under conditions as in (c).

(f) Separate 10 cm long shoots from 4-week-old cultures in shoot elongation medium, and individually transfer them to MS liquid medium with sucrose level raised to 6–10% (depending on cultivar). Incubate the cultures in continuous light. Corms will be ready for harvest in 10–12 weeks.

(g) Remove the corms, wipe off the medium, and store them at 5°C for 6–8 weeks.

(h) Sow the corm directly in the field following the standard planting procedures for this crop. The corms will sprout within a fortnight.

16.I.3. Banana (after Drew et al., 1991)

(a) Excise 20 × 30 mm sections containing the shoot tip from 0.4–1.0 m high sword suckers and surface sterilize them in 3.5% solution of sodium hypochlorite containing a few drops of Tween 80, for 15 min. Wash it in sterile distilled water and resterilize in 1% solution of sodium hypochlorite containing Tween 80 for 5 min.

(b) Wash the material in sterile distilled water and trim it to prepare a 5 mm^3 explant carrying the shoot tip, ensheathing leaf bases and 2–3 mm of basal stem tissue.

(c) Culture the explants on MS medium supplemented with 2.25 mg l^{-1} BAP, 2% sucrose, and 0.8% Difco Bacto agar.

(d) Multiply the shoots on the above medium by 6–8 weekly subcultures.

(e) For rooting, transfer the shoots to hormone-free MS basal medium.

(f) Harden the plantlets using standard procedures.

16.I.4. *Pyrus communis* (after Chevreau et al., 1992)

(a) Excise single node segments (2 cm) from actively growing grafted plants in the greenhouse and surface sterilize by immersion in

sodium hypochlorite (0.5–2.5%) containing 0.01% Tween-20, for 5–30 min. Rinse three times in sterile distilled water.

(b) Culture the nodal cuttings on Lepoivre mineral medium (see Appendix 16.II.8) supplemented with 1–2 mg l^{-1} BAP, 0.1 mg l^{-1} IBA or 0.01 mg l^{-1} NAA. For shoot proliferation maintain the cultures at 24°C and subculture at 30-day intervals.

(c) Multiplication and shoot elongation rates may show a progressive decline with the number of subcultures. Give 45 days of cold treatment, at 8°C, to reinitiate growth activity.

(d) For rooting, transfer individual shoots to MS medium (full or 1/2 strength) supplemented with IBA or IAA (0.025–2 mg l^{-1}). A dark treatment during the initial 7–10 days improves the rooting percentage. Liquid medium supports better rooting but such plants show poor survival after transplantation than those rooted on semi-solid medium.

(e) Transfer the rooted plants to vermiculite and maintain them under high humidity initially. Apply GA_3 spray (100–200 mg l^{-1}) every 4–5 days to improve plant growth.

16.I.5. Walnut (after Leslie and McGranahan, 1992)

(a) Collect vigorously growing softwood shoots or stump sprouts.

(b) Remove the foliage, wash the stem in soapy water and cut them into 3–4 cm nodal segments. Wash the segments in running tap water.

(c) Give a quick (5–10 s) rinse in 95% ethanol, and then surface sterilize in 10% solution of commercial bleach (0.5% sodium hypochlorite) containing 1–2 drops of detergent, with constant stirring.

(d) Rinse each explant in separate container of sterile water and plant individually in separate culture vials containing multiplication medium (see Appendix 16.II.9). Culture under continuous cool white fluorescent light, at 25°C.

(e) Transfer each explant to fresh medium daily for 1 week and thereafter at least once per week until new axillary shoot emerges.

(f) Excise the shoots when 2 cm long and culture them separately, transferring every week.

(g) Multiply shoots by cutting long shoots into segments or excising individual axillary shoots. Transfer weekly and remove basal callus every 3–4 weeks.

(h) For rooting, cut shoots 5–10 cm in length and transfer to pre-rooting medium (see Appendix 16.II.9) for 1 week.

(i) Remove the shoot from the pre-rooting medium, wash thoroughly to remove medium, dip the shoot bases in 2% IBA (w/w) in talc powder, plant in a well drained potting mix, and treat with a fungicide. Maintain the plants under high humidity for 2–8 weeks until new shoots emerge.

16.I.6. *Simmondsia chinensis* (after Chaturvedi and Sharma, 1989)[1]

(a) Collect single-node segments from field grown plants and wash in running tap water for 30 min, followed by 20 min of washing in 5% Teepol solution. Dip the segments in 95% ethanol for 5 s and then surface sterilize in 0.1% $HgCl_2$ for 10 min followed by a 5 min rinse in chlorine water. Despite this only about 10% aseptic cultures are obtained.

(b) Culture the nodal segments in a modified SH medium (MSH) supplemented with 1 mg l^{-1} each of BAP and IAA (MSH-I) to induce bud break and shoot proliferation. Maintain the cultures under a 15 h light regime at 27°C.

(c) Multiply the shoots on the modified SH medium containing 0.5 mg l^{-1} BAP, 1 mg l^{-1} IAA and 15 mg l^{-1} adenine sulphate (MSH-II). New shoots continue to differentiate and proliferate from the parent nodal axis.

(d) For rooting, transfer individual shoots to a modified SH medium with reduced salts and supplemented with 7 mg l^{-1} NAA and 1 mg l^{-1} caffeic acid, and pH adjusted to 5.0 (MSH-III). As soon as the shoots form roots transfer them to MSH with reduced auxin level (2 mg l^{-1} IBA and 1 mg l^{-1} caffeic acid). After 15 days about 90% shoots are expected to show rooting. At this stage transfer them to an auxin-free medium.

(e) Transfer the plantlets to pots containing coarse sand and sandy soil (1:3) and cover the plant with a glass cylinder to maintain high humidity. Gradually, over a period of 15 days, remove the glass cylinder completely.

[1] For the composition of culture media see Appendix 16.II.10.

APPENDIX 16.II: COMPOSITION OF MEDIA FOR MICRO-PROPAGATION OF SOME CROP PLANTS

16.II.1. *Cattleya*[a]

Constituents	Media (amounts in mg l^{-1})		
	Initiation[b]	Maintenance[b,c]	Rooting[d]
Inorganic nutrients			
$MgSO_4 \cdot 7H_2O$	120	120	250
KH_2PO_4	135	135	250
$Ca(NO_3)_2 \cdot 4H_2O$	500	500	1000
$(NH_4)_2SO_4$	1000	1000	500
KCl	1050	1050	–
KI	0.099	0.099	–
H_3BO_3	1.014	1.014	0.056
$MnSO_4 \cdot 4H_2O$	0.068	0.068	7.5
$ZnSO_4 \cdot 7H_2O$	0.565	0.565	0.331
MoO_3	–	–	0.016
$CuSO_4 \cdot 5H_2O$	0.019	0.019	–
$CuSO_4$	–	–	0.040
$AlCl_3$	0.031	0.031	–
$NiCl_2$	0.017	0.017	–
$FeSO_4$	–	–	25
$FeC_6H_5O_7 \cdot 3H_2O$	5.4	5.4	–
Organic nutrients			
Inositol	–	18.00	–
Nicotinic acid	–	1.22	–
Pyridoxine·HCl	–	0.21	–
Thiamine·HCl	–	0.34	–
Folic acid	–	4.4	–
Biotin	–	0.024	–
Ca-pantothenate	–	0.48	–
Glutamic acid	–	15.0	–
Asparagine	–	13.0	–
Guanylic acid	–	182.0	–
Cytidylic acid	–	162.0	–
Growth regulators			
Kinetin	0.2	0.22	–
NAA	0.1	0.18	–
GA_3	–	0.35	–
Complex nutrients			
Coconut water	150 ml l^{-1}	50–150 ml l^{-1} } either	–
Casein hydrolysate	–	100 mg l^{-1} } one	

Sucrose	0.5%	2%	2%
Agar	–	–	1.2–1.5%

[a]Explant: shoot tip.
[b]After Lindemann et al. (1970); taken from Arditti (1977).
[c]Maintenance medium is for protocorm multiplication and shoot development.
[d]Modified Knudson C (1946) medium; taken from Arditti (1977).

16.II.2. *Cymbidium*[a,b]

Constituents	Media (amounts in mg l^{-1})		
	Wimber[c]	Fonnesbech[d]	Modified Knudson C[e]
Inorganic nutrients			
KNO_3	525	–	–
$MgSO_4 \cdot 7H_2O$	250	250	250
KH_2PO_4	250	250	250
K_2HPO_4	–	212	–
$(NH_4)_2SO_4$	500	300	500
$Ca(NO_3)_2 \cdot 4H_2O$	–	400	1000
$CaHPO_4$	200	–	–
H_3BO_3	–	10	0.056
$MnSO_4 \cdot 4H_2O$	–	25	7.5
$ZnSO_4 \cdot 7H_2O$	–	10	0.331
$Na_2MoO_4 \cdot 2H_2O$	–	0.25	–
$CuSO_4 \cdot 5H_2O$	–	0.025	0.040
MoO_3	–	–	0.016
$Fe_2(C_4H_4O_6)_3$	300	–	–
$FeSO_4 \cdot 7H_2O$	–	27.9	25
Na_2.EDTA	–	37.8	–
Organic nutrients			
Inositol	–	100	–
Nicotinic acid	–	1	–
Pyridoxine·HCl	–	0.5	–
Thiamine·HCl	–	0.5	–
Glycine	–	2	–
Growth regulators			
Kinetin	–	0.215	–
NAA	–	1.86	–
Complex nutrients			
Casamino acid	–	2000–3000 (either)	–
Tryptophan	2000	3000–4000 (one)	–
Coconut water	–	100–150 ml l^{-1}	–
Banana, ripe	–	–	15%

Sucrose	2%	3–4%	2%
Agar	–	0.8%	1.2–1.5%

[a]Explant: shoot tip.
[b]Any one of the media is enough for all the three stages of micropropagation.
[c]After Wimber (1963); taken from Arditti (1977).
[d]After Fonnesbech (1972).
[e]Taken from Arditti (1977).

16.II.3. *Dendrobium*[a,b]

Constituents	Amount[c] (mg l^{-1})
Inorganic nutrients	
KNO_3	525
$MgSO_4 \cdot 7H_2O$	250
KH_2PO_4	250
$(NH_4)_2SO_4$	500
$Ca_3(PO_4)_2$	200
$MnSO_4 \cdot 4H_2O$	7.5
$Fe_2(C_4H_4O_6)_3$	28
Coconut water	150 ml l^{-1}
Sucrose	2%
Agar[d]	0.8%

[a]Explant: axillary bud.
[b]After Kim et al. (1970).
[c]This medium is suitable for all the in vitro stages of micropropagation.
[d]Omitted when a liquid medium is used.

16.II.4. *Epidendrum*[a,b]

Constituents	Media (amounts in mg l^{-1})		
	Initiation	Callus multiplication	Differentiation
Inorganic nutrients			
NH_4NO_3	1650	1650	–
KNO_3	1900	1900	–
$CaCl_2 \cdot 2H_2O$	440	440	–
$MgSO_4 \cdot 7H_2O$	370	370	250
KH_2PO_4	170	170	250
$(NH_4)_2SO_4$	–	–	500
$Ca(NO_3)_2 \cdot 4H_2O$	–	–	1000
KI	0.83	0.83	–

H_3BO_3	6.2	6.2	0.056
$MnSO_4 \cdot 4H_2O$	22.3	22.3	–
$ZnSO_4 \cdot 7H_2O$	–	9	0.331
$ZnCl_2$	3.93	–	–
$Na_2MoO_4 \cdot 2H_2O$	–	0.25	–
MoO_3	–	–	0.016
$CuSO_4 \cdot 5H_2O$	0.025	0.025	–
$CuSO_4$	–	–	0.040
$CoCl_2 \cdot 6H_2O$	0.025	0.025	–
$FeSO_4 \cdot 7H_2O$	27.8	27.8	25
Na_2EDTA	74.6	37.3	–
Organic nutrients			
Inositol	–	100	–
Thiamine·HCl	0.4	0.4	–
Glycine	2	–	–
Growth regulators			
BAP	0.5	–	–
Kinetin	–	0.1	–
2,4-D	1	–	–
IAA	–	2	–
Banana	–	–	15%
Sucrose	3%	3%	2%
Agar	–	1%	1.2–1.5%

[a]Explant: leaf tip.
[b]After Churchill et al. (1970); taken from Arditti (1977).

16.II.5. Potato (after Dodds et al., 1992)

Constituents	Amounts (mg l^{-1})		
	MSA[a]	M–9[b]	MT[c]
Inorganic nutrients			
NH_4NO_3	1650	1650	1650
KNO_3	1900	1900	1900
$CaCl_2 \cdot 2H_2O$	440	440	440
$MgSO_4$	370	370	370
KH_2PO_4	170	170	170
H_3BO_3	6.2	6.2	6.2
$MnSO_4 \cdot 4H_2O$	16.9	16.9	16.9
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6
KI	0.83	0.83	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
Na_2EDTA	37.3	37.3	37.3

$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8
Organic nutrients			
Inositol	100	100	100
Thiamine·HCl	0.4	0.4	0.4
Pyridoxine·HCl	0.5	–	–
Nicotinic acid	0.5	–	–
Glycine	2.0	–	–
Ca-pantothenate	–	2.0	–
Growth regulators			
Gibberellic acid	0.1	0.4	–
Benzylamino purine	–	0.5	5.0
Naphthalene acetic acid	–	0.01	–
Chlorocholine chloride	–	–	500
Sucrose	0.3%	2%	8%
Gelrite	1.9	–	–

[a]Semi-solid medium, for initiation of shoot cultures and initial 3–4 cycles of shoot multiplication.
[b]Liquid medium for shoot multiplication.
[c]Tuber formation medium.

16.II.6. *Gladiolus*, shoot multiplication medium (after Dantu and Bhojwani, 1992)

Constituents	Amounts (mg l^{-1})
Macronutrients	
NH_4NO_3	825
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	400
$MgSO_4 \cdot 7H_2O$	370
$NaH_2PO_4 \cdot 2H_2O$	300
Micronutrients	
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.125
$CuSO_4 \cdot 5H_2O$	0.0125
$CoCl_2 \cdot 6H_2O$	0.0125
Na_2EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
Organic nutrients	
myo-Inositol	100.0
Nicotinic acid	0.5
Pyridoxine·HCl	0.5

Thiamine·HCl	0.1
Glycine	2.0
Growth regulator	
BAP	0.5
Sugar cubes	30000

16.II.7. Papaya (after Drew et al., 1991)

Constituents	Amounts (mg l^{-1})			
	Initiation[a] of shoot culture	Shoot[b] multiplication	Rooting[c]	Single[d] shoot
Inorganic nutrients				
NH_4NO_3	1600	1600	800	1600
KNO_3	2022.20	2022.20	1011.10	2022.2
$NaH_2PO_4 \cdot 2H_2O$	312.02	312.02	156.01	312.02
$CaCl_2 \cdot 2H_2O$	441.06	441.06	294.04	441.06
$MgSO_4 \cdot 7H_2O$	739.41	739.41	369.70	739.41
H_3BO_3	9.27	9.27	3.09	9.27
$MnSO_4 \cdot H_2O$	16.90	16.90	8.45	16.90
$ZnSO_4 \cdot 7H_2O$	11.50	11.50	5.75	11.50
$CuSO_4 \cdot 5H_2O$	0.37	0.37	0.02	0.37
$Na_2MoO_4 \cdot 2H_2O$	0.24	0.24	0.02	0.24
$CoCl_2 \cdot 6H_2O$	0.23	0.23	0.11	0.23
KI	0.83	0.83	0.41	0.83
$FeSO_4 \cdot 7H_2O$	27.80	27.80	13.90	27.80
Na_2EDTA	37.22	37.22	18.61	37.22
Organic nutrients				
Inositol	108.12	108.12	54.06	108.12
Nicotinic acid	4.92	4.92	2.46	4.92
Pyridoxine·HCl	1.23	1.23	0.61	1.23
Thiamine·HCl	13.49	13.49	0.67	13.49
Biotin	0.24	0.24	0.04	0.24
Folic acid	0.88	0.88	0.44	0.88
Ca-pantothenate	2.38	2.38	0.47	2.38
Riboflavin	3.76	3.76	0.37	3.76
Ascorbic acid	1.98	1.98	0.19	1.98
l-Cysteine·HCl	37.58	37.58	18.79	37.58
Glycine	3.75	3.75	0.37	3.75
Choline chloride	1.39	1.39	0.13	1.39
Growth regulators				
BAP	0.22	0.45	–	–
NAA	0.18	0.09	–	–

IBA	–	–	2.03	–
Sucrose	20000	20000	20000	20000
Agar	8000	8000	8000	8000

[a]A good method of forcing lateral buds is to put the whole node, with lateral buds attached, onto a simple medium of high minerals, 2% sucrose and 0.8% agar. Smith and Drew (1990c) have suggested that healthy lateral shoots for culture initiation can be obtained by decapitating 30 cm plantlets growing in glasshouse and application of lanolin paste containing 225 mg l^{-1} BAP to the cut surface.
[b]Lateral shoot growth can also be promoted by pruning roots and removing the apical bud before placing the plantlets on multiplication medium. Subculture every 2–3 weeks. Alternate culture on BAP-containing and BAP-free media may help in maintaining the quality of the shoots (Smith and Drew, 1991).
[c]As soon as roots are initiated transfer the plantlets to single-shoot medium.
[d]On this medium shoots with strong apical dominance and healthy root system will be produced.

16.II.8. *Pyrus communis* (composition of Lepoivre medium)[a]

Constituents	Amount (mg l^{-1})
Inorganic nutrients	
NH_4NO_3	400
$Ca(NO_3)_2 \cdot 4H_2O$	1200
KNO_3	1800
KH_2PO_4	270
$MgSO_4 \cdot 7H_2O$	360
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.2
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
Organic nutrients	
myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine·HCl	0.5
Thiamine·HCl	0.4
Sucrose	30000
Agar	7000
pH	5.0

[a]After Quoirin et al. (1977).

16.II.9. Walnut (after Leslie and McGranahan, 1992)

Constituents	Amounts (mg l^{-1})	
	Shoot multiplication	Prerooting treatment
Inorganic nutrients		
NH_4NO_3	1416.0	456.2
$Ca(NO_3)_2 \cdot 4H_2O$	1968.0	634.0
K_2SO_4	1559.0	1559.0
$MgSO_4 \cdot 7H_2O$	740.0	740.0
$CaCl_2 \cdot 2H_2O$	149.0	149.0
KH_2PO_4	265.0	265.0
$Zn(NO_3)_2 \cdot 6H_2O$	17.0	17.0
$MnSO_4 \cdot H_2O$	33.5	33.5
$CuSO_4 \cdot 5H_2O$	0.25	0.25
H_3BO_3	4.8	4.8
$Na_2MoO_4 \cdot 2H_2O$	0.39	0.39
$FeSO_4 \cdot 7H_2O$	33.8	33.8
Na_2EDTA	45.4	45.4
$NiSO_4 \cdot 6H_2O$	0.005	0.005
Organic nutrients		
myo-Inositol	100	100
Thiamine·HCl	2.0	2.0
Nicotinic acid	1.0	1.0
Glycine	2.0	2.0
Growth regulators		
BAP		
IBA	0.01	0.15
Sucrose	3%	3%
Gelrite	0.2%	0.24%

16.II.10. *Simmondsia chinensis* (after Chaturvedi and Sharma, 1989)

Constituents	Amounts (mg l^{-1})		
	MSH-I (Initiation)	MSH-II (Multiplication)	MSH-III (Rooting)
Inorganic nutrients			
NH_4NO_3	500	500	–
KNO_3	2500	2500	500

$MgSO_4 \cdot 7H_2O$	400	400	200
$NH_4H_2PO_4$	300	300	300
$CaCl_2 \cdot 2H_2O$	200	200	100
$MnSO_4 \cdot H_2O$	10	10	10
H_3BO_3	5	5	5
$ZnSO_4 \cdot 7H_2O$	1	1	1
KI	1	1	1
$CuSO_4 \cdot 5H_2O$	0.2	0.2	0.2
$CoCl_2 \cdot 6H_2O$	0.1	0.1	0.1
$NaMoO_4 \cdot 2H_2O$	0.1	0.1	0.1
$FeSO_4 \cdot 7H_2O$	15	15	15
Na_2EDTA	20	20	20
Organic nutrients			
Inositol	100	100	100
Thiamine·HCl	0.1	0.1	0.1
Nicotinic acid	0.5	0.5	0.5
Pyridoxine·HCl	0.1	0.1	0.1
Growth regulators			
BAP	1	0.5	–
IAA	1	1.0	–
IBA	–	–	7
NAA	–	–	1
Adenine sulphate	–	15	–
Caffeic acid	–	–	1
Sucrose	30000	30000	70000
Agar	6000	6000	6000
pH	5.8	5.8	5.0

Chapter 17

Production of Secondary Metabolites

17.1. INTRODUCTION

Higher plants produce a great variety of secondary products which play a minor role in the basic life processes of the plant but often have an ecological role, such as attractant of pollinators and chemical defence against microorganisms, insects and higher predators (Wink, 1988). Many of these natural products have been used as sources of a large number of industrial products, including agricultural chemicals, pharmaceuticals and food additives (Table 17.1). Although, some of the natural products have been replaced by synthetic substitutes because of cost considerations, a number of commercially important high value chemicals are still being extracted from plants (Sauerwein et al., 1992; Misawa, 1994). According to Lambie (1990), of the 30 medicinal alkaloids in use, 24 are obtained by extraction from plants.

Our dependence on plants for natural products is expected to continue because some compounds are difficult to synthesize due to their struc-

TABLE 17.1

Natural plant products of industrial importance

1. Pharmaceuticals	
a. Alkaloids	Ajmalicine, atropine, berberine, codeine, reserpine, vincristine, vinblastine
b. Steroids	Diosgenin
c. Cardenolides	Digitoxin, digoxin
2. Food and flavours	
a. Sweeteners	Stevioside, thaumatin
b. Bittering agent	Quinine
c. Pigment	Crocin
3. Pigments and perfumes	
a. Pigments	Shikonin, anthocyanins, betalins
b. Fragrances	Rose oil, jasmine oil, lavender oil
4. Agrochemicals and fine chemicals	
a. Agrochemicals	Pyrethrin, salannin, azadirachtin
b. Fine chemicals	Proteases, vitamins, lipids, latex, oil

tural complexity, and novel active compounds are still being detected in plant extracts as more and more hitherto unsurveyed plants are analyzed. Besides their direct application, the natural plant products serve as model compounds for the chemical synthesis of new, more potent analogues. In addition, secondary metabolites can be applied as starting compounds for further chemical modification. For example, podophyllotoxin obtained from *Podophyllum* sp. is used for the synthesis of the clinically applied antitumour agents etoposide and teniposide (Holthuis, 1988). The increasing consumer preference for natural food colours and flavours over their synthetic counterparts further increases our dependence on plants.

During the last 30 years there has been an increasing interest among scientists to produce high value natural plant products by cell culture which can overcome many of the problems associated with industrial production of these phytochemicals by extraction from field grown plants (mass cultivated or natural populations). In cultures, factory-type production of natural compounds can be carried out throughout the year, unaffected by the season. The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from natural populations are eliminated. Cell cultures not only provide means for de novo synthesis of natural products but also serve as 'factories' for bioconversion of low value compounds into high value products (see Section 17.2.6). Moreover, some novel compounds produced in cell cultures are not produced in intact plants (Table 17.2). At least 85 novel compounds including 23 alkaloids, 19 terpenoids, 30 quinones and

TABLE 17.2

Examples of compounds produced exclusively in tissue culture and not in the corresponding intact plants[a]

Compounds	Plant species	Reference
Epchrosine	*Ochrosia elliptica*	Pawelka et al. (1986)
Dehydrodiconiferyl-alcohol-γ-β-D-glucoside	*Plagiorhegma dubium*	Arens et al. (1985)
Paniculid A	*Andrographis paniculata*	Butcher and Connolly (1971)
Pericine	*Picralima nitida*	Arens et al. (1982)
Rutacultin	*Ruta graveolens*	Steck et al. (1971) Nahrstedt et al. (1985)
Tarennosid	*Gardenia jasminoides*	Ueda et al. (1981)
Voafrine A and Voafrine B	*Voacanga africana*	Stockigt et al. (1983)

[a]After Berlin (1988).

TABLE 17.3

Some examples where cell cultures have produced natural compounds in amounts equal to or higher than whole plants

Product	Plant species	Yield (% dry weight) Whole plant	Cell culture	Reference
Ajmalicine	*Catharanthus roseus*	0.3	1	Zenk et al. (1977)
Anthraquinones	*Morinda citrifolia*	2.2	18	Zenk et al. (1975)
Berberine	*Coptis japonica*	2.4	13.4	Murray (1984)
Caffeine	*Coffea arabica*	1.6	1.6	Anderson et al. (1986)
Catharanthine	*Catharanthus roseus*	0.0017	0.005	Kurz et al. (1981)
Diosgenin	*Dioscorea deltoidea*	2.4	7.8	Tal et al. (1982)
Ginsenoside	*Panax ginseng*	4.5	27	Misawa (1994)
Rosmarinic acid	*Coleus blumei*	3	23	Ulbrich et al. (1985)
Serotonin	*Peganum harmala*	2	2	Sasse et al. (1982)
Serpentine	*Catharanthus roseus*	0.26	2	Deus-Neumann and Zenk (1984)
Shikimic acid	*Galium mollugo*	2–3	10	Amrhein et al. (1980)
Shikonin	*Lithospermum erythrorhizon*	1–2	15–20	Fujita (1988)
Trigonelline	*Trigonella foenum-graecum*	0.4	5	Radwan and Kokate (1980)
Tripodiolide	*Tripterygium wilfordii*	0.01	0.2	Hayashi et al. (1982)
Vomilenine	*Rauwolfia serpentina*	0.004	0.214	Stockigt et al. (1981)

11 phenyl compounds have been isolated from some 30 different plant culture systems (Phillipson, 1990).

Since the early 1950s, when the concept of tissue culture production of natural compounds was conceived, many technological advances have been made and in several cases cell cultures have been shown to produce higher amounts of the products than the intact plants from which they are derived (Table 17.3). Of the various plant products produced by plant tissue culture, pharmaceuticals have received maximum attention (Fig. 17.1). The two countries which have made substantial contribut-

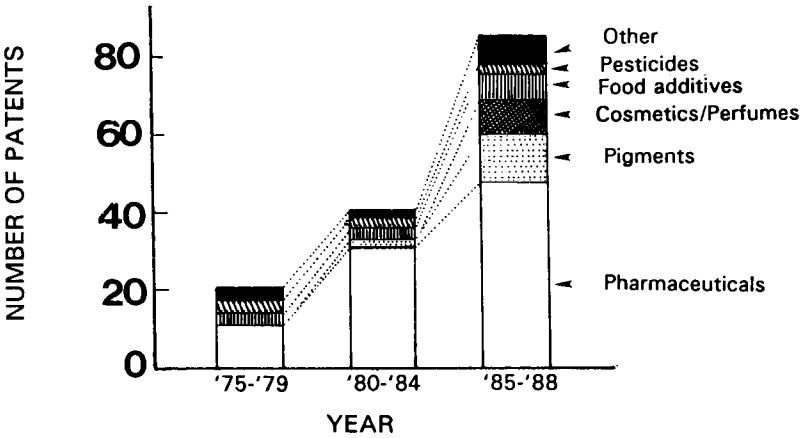

Fig. 17.1. Research trends in production of industrial compounds by plant tissue culture, based on patents granted in Japan (after Fujita, 1990).

ions to this field of research are Japan (Komamine et al., 1991) and Germany.

This chapter deals with the strategies used to improve metabolite production in cell cultures. The degree of success achieved and some of the constraints of this technology are also discussed.

17.2. STRATEGIES USED TO OPTIMIZE PRODUCT YIELD

17.2.1. Culture conditions

The productivity of cell lines is greatly influenced by the culture conditions, of which culture medium is the most important. In general, growth and production of secondary metabolites are inversely related, both, in whole plant and in cell cultures. Consequently, in cell cultures on media defined for optimum growth, production of secondary metabolites generally occurs in the late stationary phase when the medium gets depleted of some of its important constituents. Growth inhibition is often associated with cytodifferentiation and the induction of enzymes for secondary metabolism. In such cases, a 'dual culture system' is preferred. It involves biomass production in a medium optimum for cell proliferation ('growth medium') followed by transfer of healthy cells to a different medium ('production medium') which does not support good growth of the cells but is favourable for product yield. Zenk et al. (1977) were the first to use such a two stage culture system for the production of indole alkaloids by *Catharanthus roseus* cells. The same strategy was used by

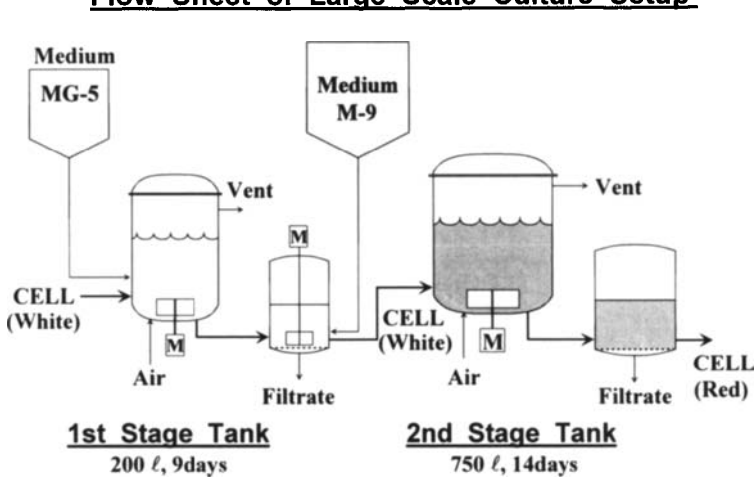

Fig. 17.2. Bioreactor system used to produce shikonin by two stage culture of *Lithospermum erythrorhizon* cells (courtesy Dr Y. Hara, Mitsui Petrochemical Co., Japan).

Fujita et al. (1981b, 1982) to establish a method for commercial production of shikonin by cell cultures of *Lithospermum erythrorhizon* (Fig. 17.2).

The most useful modification made in the growth medium for use in secondary metabolite production are: (a) reduction or elimination of 2,4-D or other phytohormones; (b) reduction of phosphate level; and (c) increase in sucrose level or alternation of carbohydrate (C)/nitrogen (N) ratio.

Fujita et al. (1981a) tested five basal media, viz. LS (Linsmaier and Skoog, 1965), W (White, 1954), B_5 (Gamborg et al., 1968), Bl (Blaydes, 1966) and NN (Nitsch and Nitsch, 1969) for growth and shikonin production in cell cultures of *L. erythrorhizon*. Of these, LS medium supported best biomass production, followed by B_5. The other three media did not favour much growth. However, shikonin derivatives were produced only in W medium. These experiments further revealed that the type of nitrogen in the medium mainly affected the production of shikonin derivatives. Stable production occurred when nitrate was present as the sole source of nitrogen, as in W medium. Even part substitution of nitrate by ammonium strongly inhibited shikonin production. Therefore, Fujita et al. (1982) devised a two-stage method in which the first stage involved biomass production in LS medium (growth medium) followed by the 2nd stage of shikonin production in W medium (production medium). By further modifications in LS and W media, MG-5 and M-9 media, re-

TABLE 17.4

Compositions of MG-5 and M-9 media used for biomass and shikonin production, respectively, in cell cultures of *Lithospermum erythrorhizon*[a]

Component	MG-5 medium ($mg\ l^{-1}$)	M-9 medium ($mg\ l^{-1}$)
NH_4NO_3	500	–
KNO_3	1900	80
$NaNO_3$	2480	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	694
KH_2PO_4	170	–
$NaH_2PO_4 \cdot 2H_2O$	–	19
KCl	–	65
$CaCl_2 \cdot 2H_2O$	150	–
$MgSO_4 \cdot 7H_2O$	120	750
$MgCl_2 \cdot 6H_2O$	203	–
Na_2SO_4	–	1480
$FeSO_4 \cdot 7H_2O$	27.8	–
$Na_2.EDTA \cdot 2H_2O$	37.3	–
$NaFe \cdot EDTA \cdot 3H_2O$	–	1.8
$MnSO_4 \cdot 4H_2O$	22.3	–
$ZnSO_4 \cdot 7H_2O$	8.6	3
H_3BO_3	1.9	4.5
$Na_2MoO_4 \cdot 2H_2O$	0.25	–
$CuSO_4 \cdot 5H_2O$	0.025	0.3
Sucrose	30000	30000
Inositol	100	–
Thiamine·HCl	0.4	–
3-Indoleacetic acid	–	1.75

[a]After Fujita (1988)

spectively, were developed (Table 17.4), which enhanced shikonin production 13-fold over that obtained using LS and W media (Fujita et al., 1981b, 1982; Yamada and Fujita, 1983; Fujita, 1990). The effect of nitrogen on alkaloid production is dependent on the carbon available to the cells which makes the C/N ratio an important factor to be taken into account (Moreno et al., 1995).

The optimum media for the production of different metabolites by a cell line are likely to be different. For ajmalicine production by *C. roseus* cell cultures, Schlatmann et al. (1992) analyzed, simultaneously, five variables, viz. phosphate, nitrate, ammonium, glucose and time. Glucose proved most critical, with an optimum concentration at 500 mM. For maximum yield, ammonium and phosphate should be absent and nitrate added at 12 mM. Under these conditions ajmalicine production was

10.36 μM g^{-1} DW. In contrast, the medium optimum for tryptamine had a lower concentrations of glucose (100 mM) and nitrate (near zero) and higher levels of phosphate (0.4 mM) and ammonium (8 mM).

In some cases it has been possible to combine growth and production steps in the same medium. Increased sucrose concentration (6%) in the medium improved both growth and production in batch cultures of *C. roseus* (Scragg et al., 1990). Smith et al. (1987a,b) developed a modified MS medium that promoted growth and alkaloid production by *C. roseus* cell cultures in a single stage. In cell cultures of *Coptis japonica* cell yield increases between 7 and 10 days whereas berberine content rises after the 10th day of culture initiation which suggests a dual culture system. However, 10-fold increase in copper concentration in LS medium enhanced berberine production by 20–30% without an adverse effect on biomass production (Morimoto et al., 1988).

Even in root cultures of *C. roseus*, alkaloid production requires two different media for optimum growth and product yield (Toivonen et al., 1989).

Plant growth regulators affect growth and differentiation and, thus, affect secondary metabolite production by cultured cells. In general an increase of auxin level, such as 2,4-D, which stimulates dedifferentiation and proliferation of cells, reduces the level of secondary metabolites. Therefore, generally auxins are added to the growth medium but omitted or used at a lower level in the production medium. Berberine production by cell cultures of *Thalictrum minus* was greatly influenced by the hormonal composition of the medium (Nakagawa et al., 1986). In the presence of 2,4-D the cells grew rapidly, producing little berberine. The alkaloid yield was remarkably increased by the combined presence of an auxin and a cytokinin. Of the various auxins tested (2,4-D, NAA, IAA and IBA), NAA was most effective. With 60 μM NAA and 10 μM BAP, berberine yield after 2 weeks was as high as 20 mg per 30 ml medium. However, the biomass production was low. Therefore, a dual culture system has been recommended for berberine production by cell cultures of *T. minus*, growth medium with 2,4-D and production medium with NAA and BAP.

GA_3 generally inhibits the secondary metabolite production (Hinderer et al., 1984; Ozeki and Komamaine, 1984; Yoshikawa et al., 1986). It had no effect on the production of indole alkaloid catharanthine by *C. roseus* cell cultures. Enhanced berberine production by cell cultures of *Coptis japonica* in response to GA_3 (10^{-8}–10^{-5} M) is a rare example of the promotive effect of this phytohormone on secondary metabolite production (Hara et al., 1988, 1994). This enhancement is ascribed to GA_3-induced inhibition of starch synthesis, decrease in nitrogen uptake and increased uptake and turnover of sucrose (Hara et al., 1994).

The pH of the medium is shown to enhance permeability of the cell membrane and, thus, helps in the release of intracellular alkaloids (Payne et al., 1988; Asada and Shuler, 1989; Jardin et al., 1991).

Light is an important regulatory factor in the production of alkaloids in plant cell cultures. The importance of light for the optimal expression of some pathways in cultured cells has been demonstrated, including for flavonoids (Hahlbrock and Grisebach, 1979), cardenolides (Ohlsson et al., 1983) and betacyanins (Berlin et al., 1986).

In *C. roseus*, light influences the ajmalicine/serpentine accumulation ratio (Moreno et al., 1995). Drapeau et al. (1987) observed higher accumulation of serpentine in the cultures under a 15 h photoperiod instead of 24 h illumination. Catharanthine production was completely repressed in the absence of light. Moreover, light affected not only production but also the site of product accumulation. In dark grown cultures of *C. roseus* 79% of the serpentine and 78% of ajmalicine were excreted into the medium but in light it dropped to 14 and 18%, respectively. In the calli grown under white light, ajmalicine accumulation preceded that of serpentine. However, the calli exposed to red or blue light had a constant ajmalicine content and their serpentine content was always lower than that observed under white light (Loyola-Vergas et al., 1992). A correlation between chlorophyll synthesis and serpentine accumulation in *C. roseus* cell cultures upon transfer from dark to light was observed by Loyola-Vargas et al. (1992).

The gaseous environment, mainly the availability of oxygen and carbon dioxide, also plays an important role in the production of secondary metabolites by cell cultures. Leckie et al. (1991) observed that by increasing the initial oxygen mass transfer coefficient in batch cultures of *C. roseus*, it was possible to shorten the time for which cells accumulate serpentine without altering the final yield. Apparently, the increased availability of dissolved oxygen (DO) stimulated the oxidative metabolism responsible for the conversion of ajmalicine into serpentine. Recently, it has been shown that the high density cultures of *C. roseus* cultivated at high DO concentration had a higher ajmalicine production than those cultivated at lower DO concentration (Schlatmann et al., 1994).

The type of closure can significantly affect the head-space gas composition of shake flask cultures. Depending on the permeability of the closure, gaseous compounds produced by the culture can accumulate in the head space of the flasks. The cultures of *C. roseus* with limited gas exchange accumulated ethylene and carbon dioxide and showed inhibition of ajmalicine production (Lee and Shuler, 1991). This inhibition was ascribed to high ethylene concentration in the cultures.

17.2.2. Selection of high yielding lines

The explants used to initiate tissue cultures are highly heterogeneous with regard to the metabolic productivity of its constituent cells. The heterogeneity is expected to increase under culture conditions which are known to induce genetic and epigenetic changes (see Chapter 9). Consequently, cells and cell clusters from a culture exhibit considerable variation for the accumulation of secondary metabolites (Zenk et al., 1977; Ellis, 1985).

The productivity of a heterogeneous culture would be an average of the productivity of its high and low yielding cells. Selection and cloning of high yielding cells from such cultures is, therefore, regarded as an effective method to improve in vitro production of secondary metabolites. It requires, firstly, initiation of cultures from selected high yielding genotypes. One should then screen a large number of individual cultures for the best producing variant. From established cultures of these lines, selection can be made for better yielding subclones. Selection of cell lines of *Papaver* helped improve the yields of sanguinarins considerably. Some of the lines yielded as high as 13% of DW compared to 1% by the mother lines (Songstad et al., 1990).

Since cultured cells are prone to spontaneous genetic changes it would be necessary to make periodic selections to maintain high productivity of the cultures. Selections should be made under conditions which are suitable for product formation.

Screening for high yielding lines for coloured compounds, such as shikonin, berberine and betanin, is very simple. The most coloured areas of cell clumps can be easily isolated and cultured separately. Cells with highly fluorescent compounds may be detected under UV light by naked eye or fluorescence microscope. Flow cytometry enables rapid analysis and subsequent sorting of a large amount of cells on the basis of their fluorescence (Aiken and Yeoman, 1986, Hara et al., 1989; Adamse, 1990). However, it requires single cells. For colourless compounds, specific reactions of squashed cells or immunological tests of extracts may help to find the best clones.

It is important that the selected lines are reasonably stable. Since metabolite synthesis by plant cells is highly influenced by the physiological state of the cells, the high yield shown by the selected lines may be due to altered gene expression. Berlin (1990) remarked that 'in the majority of the cells the different levels of a particular metabolite are most likely the consequence of differential and reversible gene expression'. Screening of *C. roseus* cells for high ajmalicine and serpentine production yielded lines with up to 10-fold increased productivity (Zenk et al., 1977; Deus and

Zenk, 1982; Deus-Neumann and Zenk, 1984). However, the lines were highly unstable and often reverted back to the level of unselected lines (Deus-Neumann and Zenk, 1984).

For the isolation of stable lines, repeated selections should be made, and only if the product of a cell line remains stable over several selection cycles, will a true variant be identified. Yamamoto et al. (1982) and Nozue et al. (1987) made about 30 consecutive clonings of cell aggregates with the highest anthocyanin content and established stable cell lines of *Euphorbia millii* and sweet-potato, respectively, that maintained increased anthocyanin content without further selection. Similarly, Sato and Yamada (1984) found it necessary to make recurrent selection of cell aggregates to obtain stable high berberine producing lines of *Coptis japonica*. The selected lines produced up to 13.2% berberine on a DW basis compared to 5% by the unselected lines.

High shikonin producing cell lines of *Lithospermum erythrorhizon* obtained by cell aggregate selection were unstable (Fujita, 1988), probably because of many chemotypes within the selected cell aggregates. In such cases single cell cloning would be more reliable. Fujita et al. (1985) made selections based on protoplast cloning and obtained high yielding lines of *L. erythrorhizon* (1.5-fold higher yield than the original line) which showed stability for shikonin production for up to 8 months.

17.2.3. Elicitation

In nature, plant cells synthesize a number of compounds (phytoalexins) in response to chemical or microbial attacks. Increased production of secondary metabolites in media supporting poor growth of cells is also regarded as a stress response (DiCosma and Towers, 1984). Consequently, a number of biotic (fungal extracts) and abiotic (inorganic and organic chemicals, UV irradiation) factors have been tested as elicitor and shown to improve the production of secondary metabolites in plant cell and organ cultures (Eilert, 1987). Elicitor-induced products are frequently released into the medium (Brodelius, 1990).

The addition of conidia of *Verticillium dahliae* to cell cultures of *Gossypium arboreum* increased the yield of gossypol from 5–10 g l^{-1} to a remarkable level of 500 mg l^{-1} within 5 days (Heinstein, 1985). Similarly, a 26-fold increase of sanguinarine (2.9% of dry mass) was noted after addition of the homogenate of an isolate of *Botrytis* species (Eilert et al., 1985). Kurz et al. (1990) used solubilized chitin to elicit sanguinarine production by cell cultures of *Papaver somniferum*.

Several cell lines of *C. roseus* respond to elicitation by biotic and abiotic agents. Nef et al. (1991) studied the effect of elicitation with the ex-

tract of *Pythium vexans* under growth limiting conditions. At lower concentrations it increased ajmalicine production and induced de novo synthesis of catharanthine within the first 24 h but did not affect serpentine production. Asada and Shuler (1989) noted a synergistic effect of elicitation with a fungal homogenate, immobilization of cells with Ca-alginate and alkaloid adsorption with a neutral polycarboxylic ester (Amberlite XAD-7). The cultures subjected to a combination of the three treatments showed 45-fold higher ajmalicine in the medium than in the control.

Fungal elicitors may inhibit the growth of plant cells (Misawa, 1994). The amount of *Verticillium dahliae* required to stimulate metabolite synthesis by suspension cultures of *G. hirsutum* could be reduced by adding oxalate to the medium (Davis et al., 1992). A combination of the fungal elicitor and oxalate did not reduce the cell mass and, therefore, secondary metabolite synthesis was increased up to 10-fold. Dunlop and Curtis (1991) reported that a combination of phosphate limitation with elicitation by *Rhizoctonia solani* synergistically increased the production of sesquiterpene solativone by *Agrobacterium rhizogenes*-transformed hairy-root cultures of *Hyoscyamus muticus*. The increase was substantially higher than that with phosphate limitation or fungal elicitation alone.

Simple organic and inorganic molecules can also induce product accumulation in cultured cells. Smith et al. (1987a) observed enhanced accumulation of catharanthine in the cells of *C. roseus* in response to NaCl, KCl and sorbitol, individually. Addition of 1.7 g l^{-1} of NaCl to a 5-day-old culture increased product accumulation by about 90% of the control. Sorbitol (0.2 M) was not as effective as NaCl but KCl (3.3 g l^{-1}) caused an almost 200% increase in catharanthine accumulation.

Addition of vanadyl sulphate to cell suspension cultures of *C. roseus* resulted in the production of catharanthine, serpentine and tryptamine (Tallevi and DiCosmo, 1988). The effect was concentration dependent. At a lower concentration (25 mg l^{-1}) catharanthine and ajmalicine accumulated while at higher concentrations (50–75 mg l^{-1}) tryptamine accumulation occurred.

The production of dimeric alkaloids by shoot cultures of *C. roseus* can be induced by irradiation with near-ultraviolet light (Hirata et al., 1991, 1992).

The time of application of elicitor is critical for the yield of secondary metabolites by cultured cells. Most of the cultures respond to an elicitor only during the growth phase. For the production of sanguinarine by *P. somniferum* cell cultures, elicitation with the homogenate of *Botrytis* or solubilized chitin was optimum when applied 5.8 days after culture initiation. Elicitation at days 7 and 8.5 was 3 and 8 times less effective, re-

spectively (Kurz et al., 1990). Maximum increase in the production of benzophenanthridine alkaloids in suspension cultures of *Eschscholtzia californica* occurred when treated with an elicitor from yeast extract on the 6th day after culture which corresponds to the exponential phase of growth (Byun et al., 1992). The time of elicitor application may affect not only the quantity of the product but also the production pattern. For example, in 5-day-old cultures of *C. roseus* the homogenate of *Pythium* cultures stimulated *N*-acetyltryptamine formation and in 10-day-old cultures, it induced the accumulation of a whole spectrum of monoterpene indole alkaloids (Eilert et al., 1986). Elicitor treatment after a culture has already started to accumulate the inducible compound does not enhance or accelerate its production (Kombrink and Hahlbrock, 1985; Eilert et al., 1986).

17.2.4. Immobilization of cells

One of the major problems in commercialization of a cell culture based process for secondary metabolite production is the high production cost due to slow growth of plant cells, low product yield, genetic instability of the selected lines, low shear resistance of cells and intracellular accumulation of the product. Some of these problems can be reduced by immobilized cell culture. In this technique cells are confined within a reactor system, preventing their entry into the mobile phase which carries the substrate and the products. Brodelius et al. (1979) first reported the immobilization of *C. roseus* and *Daucus carota* cells by entrapping them in alginate beads. Since then the potential of this technique to improve secondary metabolite production has been examined by several research groups and some success has been achieved (Brodelius, 1985; Lindsey and Yeoman, 1985; Scragg, 1991).

Immobilization is only relevant where the production process involves two stages; in the first stage conditions are optimized for biomass production by suspension culture and in the second stage conditions are optimized for product formation by immobilized cells, showing little or no growth. The potential advantages of immobilized cell culture are: (1) it may enable prolonged use of biomass; (2) by immobilization of cells the cell density in a bioreactor can be increased 2–4 times that in suspension cultures (10–30 g l^{-1}, Brodelius, 1985) and this enables the use of small reactors, reducing the cost of medium, equipment installation and downstream processing; (3) the entrapped cells are protected against shear forces and, consequently, a simple bioreactor design may be used; (4) it separates the cells from the medium and, therefore, if the product is extracellular it can simplify downstream processing; (5) it uncouples

growth and product formation which allows product optimization without affecting growth; (6) the non-dividing immobilized cells are less prone to genetic changes and, therefore, provide a stable production rate; (7) it minimizes fluid viscosity, which in cell suspensions cause mixing and aeration problems; and (8) it promotes secondary metabolite secretion in some cases.

A wide range of bioreactors have been designed to culture immobilized cells (see Figs. 4.7–4.10). The best design to use depends on the method of immobilization. Entrapment of cells in gel or behind semi-permeable membranes is the most popular method for immobilization of plant cells. Some polymers used to entrap plant cells are alginate, agar, agarose and carrageenan. Of these, alginate has been most widely used because it can be polymerized at room temperature using Ca^{2+} (see also Sections 4.5.2 and 6.9.2). Polyurethane foam has also been used to immobilize a range of plant cells. Alternatively, plant cells can be entrapped by inclusion within membrane reactors (see Section 4.5.2). A semi-permeable membrane is introduced between the cells and the recirculating medium so that the cells can be packed at a very high density under very mild conditions. Some designs of membrane reactors are shown in Fig. 4.10.

Immobilization of cells on the surface of an inert support, such as fibreglass mats and unwoven short fibre polyester, has also been examined for in vitro production of secondary metabolites (Tyler et al., 1995). For surface immobilization of cells, a bioreactor (air lift or mechanically agitated design), provided with the support matrix, is inoculated with a plant cell suspension of suitable density and operated for an initial period as a suspension bioreactor. During this period virtually all cells spontaneously adhere to the surface of the support. Binding of the cells to the immobilizing support is regarded as a two-step process. In the first stage, cells are spontaneously attracted to the support surface due to van der Waal's force aided by entrapment of cells in pores or other irregularities on the surface of the support (Facchini et al., 1989; Facchini and Di-Cosmo, 1990). Subsequently, the cells appear to secrete a mucilaginous substance which firmly cements them to the support surface (Rhodes et al., 1985; Robins et al., 1986; Archambault et al., 1989). The immobilized cells grow as a more or less continuous layer or tissue-like structure on the surface of the support matrix.

Surface immobilization promotes the natural tendency of plant cells to aggregate which may improve the synthesis and accumulation of secondary metabolites. A special advantage of this method over the other methods of immobilization of cells is the absence of any physical restriction to mass transfer between the culture medium and the biomass surface. Since the surface immobilized cells grow on the surface of the sup-

port matrix, it should facilitate visual monitoring of the conditions, distribution and extent of the biomass and to routinely sample the biomass, if desired.

Some preliminary reports on phytochemical production by immobilized cells have been published. The accumulation of serpentine by *C. roseus* and anthraquinones by *Morinda citrifolia* were enhanced in the immobilized state when compared with cell suspensions (Brodelius and Nilsson, 1980). *Capsicum frutescens* cells immobilized on polyurethane foam produced 50 times more capsaicin than suspension cultures (Lindsey et al., 1983). Ishida (1988) immobilized the cells of *Dioscorea deltoidea* by passively entrapping them into polyurethane foam cubes and grew them in a medium containing 3% sucrose. This increased diosgenin production by 40% over the suspension cultures. Sanguinarine yield of *Papaver somniferum* cells immobilized on fabric of loosely woven polyester fibre arranged in a spiral configuration on a stainless steel support was twice as much as that of suspension cultures (Kurz et al., 1990). Immobilized cells can also serve as biocatalysts for biotransformation (see Section 17.2.6). The immobilized cells of *Digitalis lanata* maintained their capability for enzymatic conversion of β-methyldigitoxin to β-methyldigoxin for a period of 61 days (Alfermann et al., 1980).

An immobilized system which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form could dramatically reduce the cost of phytochemical production.

However, an immobilized plant cell system with commercial potential has not yet been developed (Misawa, 1994). Some of the limitations of an immobilized cell system are: (1) immobilization is normally limited to systems where production is decoupled from cell growth; (2) the initial biomass must be produced in suspension cultures; (3) secretion of products into the external medium is imperative; (4) when secretion occurs there may be a problem of extracellular degradation of product; and (5) when gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

17.2.5. 'Hairy root' culture

It is desirable to use morphologically undifferentiated plant cells for the production of secondary metabolites in ways similar to microorganisms but the genetic instability of these cells has been a serious limitation. Moreover, in many cases the production of a metabolite occurs only when the unorganized structure is induced to undergo organogenic or embryogenic differentiation. Undifferentiated calli of *Atropa bella-*

donna do not produce the tropane alkaloid hyoscyamin but the cultures gained the ability to synthesize this compound with the differentiation of roots (Raj Bhandary et al., 1969). Similarly, the calli of *Digitalis* produced the cardiac glycosides when it was induced to differentiate somatic embryos (Kuberski et al., 1984). The production of tropane alkaloids in 'hairy root' cultures of *Hyoscyamus muticus* declined dramatically when the roots were induced to form callus and reappeared with redifferentiation of roots (Flores et al., 1987). This should not be surprising because in the whole plant system the accumulation of most of the secondary metabolites appear to be developmentally regulated. Therefore, continuously growing plant organ cultures have been proposed as an alternative in vitro system for commercial production of secondary metabolites.

In nature, roots are the source of a variety of food products and other useful chemicals. The tropane alkaloids, atropine and hyoscyamine, steroidal precursors (e.g. solasidine) and *Catharanthus* alkaloids are some of the high value (>US$1000 kg^{-1}; Rhodes et al., 1987) compounds synthesized in roots. Therefore, root cultures are a potentially useful system for in vitro system for commercial production of metabolites.

In 1934, White established continuously growing root cultures of tomato in a medium containing minerals, sucrose and yeast extract. Subsequently, root cultures of many dicotyledonous and monocotyledonous species were established. Although, the ability of cultured roots to synthesise natural plant products was demonstrated fairly early (Dawson, 1942; Stienstra, 1954; Solt, 1957) not much attention was paid to apply this system for industrial production of natural plant products, probably because of their slow growth. In recent years this limitation has been overcome by using 'hairy roots'.

Stable integration of a portion of R_i (root inducing) plasmid into plant genome, following infection by the pathogenic soil-borne bacteria *Agrobacterium rhizogenes*, induces the 'hairy root' disease, in which proliferation of roots occurs at the site of infection. 'Hairy roots' can also be induced in most of the dicotyledonous plants by genetic transformation with *A. rhizogenes* or certain mutant strains of *A. tumefaciens*. These roots, which retain the ability to synthesize natural products as normal roots, can be used to establish continuously growing 'hairy root' cultures (Rhodes et al., 1987). The 'hairy root' cultures are characterized by a high degree of lateral branching, a profusion of root hairs and an absence of geotropism. Due to extensive branching, the 'hairy root' cultures show very high growth rate in the absence of a growth regulator. The increase in the number of branches is almost logarithmic during the early stage of growth (Flores and Filner, 1985; Flores, 1986; Flores et al., 1986) and, thus, the overall growth pattern is very similar to cell suspension cul-

tures. Starting with an initial inoculum of 2–4 mg (1–2 root tips), a typical 'hairy-root' clone of *Hyoscyamus muticus*, grown in batch cultures, showed 2500–5000-fold increase in 3 weeks (Flores et al., 1987), which is even faster than the fastest growing suspension culture (Noguchi et al., 1977). Another extremely important feature of the 'hairy root' system from the commercial point of view is their stable and high level production of secondary metabolites. Flores and Filner (1985) raised 20 clones of 'hairy root' cultures of *Hyoscyamus muticus*, which grew much faster than the normal roots and produced tropane alkaloids at the same level as the whole plant or normal root culture (Flores and Filner, 1985; Flores et al., 1987). Fast growth and alkaloid production in two selected clones were stable for over 40 months. Since then several groups have confirmed the potential of 'hairy root' cultures for industrial production of secondary metabolites.

Hamill et al. (1986) reported that 17-day-old cultures of 'hairy roots' of *Beta vulgaris* had twice as much betacyanin and three times as much betaxanthin as the seedling roots. On a mg g^{-1} DW basis, the concentrations of these betacyanins were equal to those reported for storage roots. The 'hairy roots' of *Valeriana officinalis* var. *sambucifolia* produced 44.3 mg of valepotriates per gram of tissue (DW). The productivity was 0.9 mg g^{-1} day^{-1} and the concentration was approximately 4 times higher than that of normal roots (Granicher et al., 1992). For the production of isoflavonoids by *Lupinus* sp. and harmane alkaloid and serotonin by *Peganum* species, 'hairy root' cultures were superior to suspension cultures (Berlin et al., 1990).

The ability to exploit plant root cultures as a commercial source of natural products depends on the development of suitable bioreactors. The unique structure of root cultures presents a tremendous challenge for aseptic engineering (Curtis, 1993). At about 200 g l^{-1} FW or 12 g l^{-1} DW, roots form an interlocked mass which becomes stationary within the vessel. In gyratory shaker flasks, root tissue can reach densities in excess of 350 g l^{-1} FW (20 g l^{-1} DW). The root matrix stagnates liquid flow and efficiently coalesces bubbles, which can result in severe mass-transfer limitations, despite the relatively slow rate of growth of roots (doubling time ca. 2 days).

A large number of reactor configurations have been tried for 'hairy root' culture (Curtis, 1993). The standard tank reactor is not suitable for mass cultivation of roots because the contact of impeller not only damages the root tip but also induces callus-formation (Hilton et al., 1988). A modified stirred tank, in which the impeller is isolated by placing a stainless steel mesh around the root mass has been used for root culture of *Datura stramonium* (Hilton and Rhodes, 1990). The cage also provides

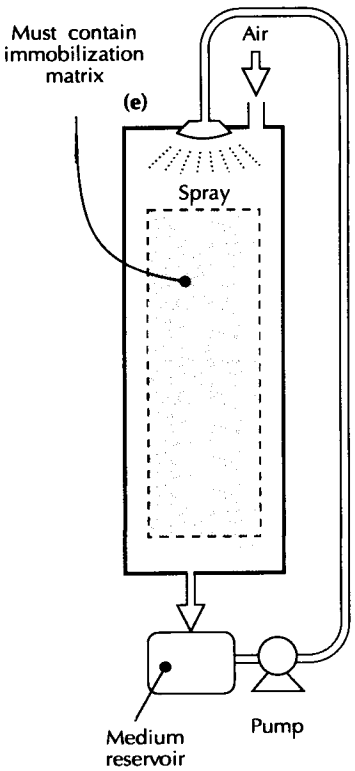

Fig. 17.3. Trickle-bed reactor used for hairy root culture (after Curtis, 1993).

a support matrix for the roots to grow. McKelvey et al. (1993) compared the suitability of gas-dispersed sparged reactor and liquid dispersed trickle and inclined reactors with gyratory-shake flask control for the culture of transformed roots of *Hyoscyamus muticus*. Of these, only the trickle-bed reactor (Fig. 17.3), in which the medium is sprayed over the roots and permitted to drain down through the root tissue, provided a growth rate comparable to the control. Recently, Yukimune et al. (1994) have described a circulation culture system for high density root culture of *Duboisia myoporoides*. It consists of a vessel for root culture, an aeration tank, a medium reservoir and a peristaltic pump, and the pure oxygen-saturated medium is circulated continuously through the culture vessel. In this system roots could be cultured at a density 20× (120 g DW dm^3) the amount that can be used in ordinary flask cultures without a decline in scopolamine content. It is important to note that most of these studies are based on small-scale culture and can be considered only as feasibility reports.

17.2.6. Biotransformation

A possible reason why de novo synthesis of many secondary metabolites in tissue cultures is lacking or unsatisfactorily low is the absence or poor expression of one or more corresponding biosynthetic enzyme(s) (Berlin, 1988). For example, the lack of serotonin biosynthesis by *Peganum harmala* cell cultures could be clearly traced to the lack of tryptophan decarboxylase (Berlin and Sasse, 1988). These non-producing cell cultures were able to transform large amounts of externally supplied tryptamine to serotonin (Sasse et al., 1987). Thus, even non-producing cultures may be used for specific biotransformation or as a source of enzymes of a seemingly non-expressed pathway.

The chemical conversion of an exogenously supplied substance by living cell cultures, permeabilized cells or entrapped enzymes derived from cell cultures is referred to as biotransformation (Yeoman et al., 1990). It may be a single step (mediated by single enzyme) or a multistep (mediated by two or more enzymes) process. The substrate used for biotransformation may be natural or synthetic, and the product can be novel or compounds already known to occur in plants. Generally, single step biotransformations are more efficient; the yield decreases with increase in the number of steps between precursor and product. The versatility of plant cell cultures to effect biotransformations, which are often regio- and stereospecific, is considerable, e.g. epoxidations, esterifications, glycosilations, hydrolyses, isomerizations, methylations, demethylations and dehydrogenations (Pras, 1992). Some promising examples of biotransformation are given below.

Arbutin, a skin depigmentation agent, is produced by biotransformation of hydroquinone using *Catharanthus roseus* cells (Yokoyama et al., 1990). Addition of the inexpensive precursor into the liquid culture medium of this cell line produced arbutin efficiently. Scientists at the Shiseido Co. in Japan have been studying this process for commercial production of arbutin. A high producing line was cultivated in a 5-l jar fermentor and fed with hydroquinone at 6 mM in the beginning and after it was completely consumed, the precursor was continuously fed at the rate of 1.4 mM h^{-1}. Such cultures produced 9.2 g l^{-1} of arbutin (45% of cell DW) within 3–4 days after administration of hydroquinone. As the cost of production of arbutin by the plant cell culture process is comparable to the chemical process (Yokoyama, 1991), it is likely to be commercialized (Misawa, 1994).

The dimeric indole alkaloids vinblastine and vincristine are high value drugs in cancer therapy. They are currently produced commercially by extraction from *C. roseus* plants but the process is not efficient because of

the low yields (0.0005% on a DW basis; Misawa, 1994). In order to produce these anticancerous drugs more efficiently, many scientists have tried to apply plant tissue culture technology (Moreno et al., 1995). However, the suspension cultures of *C. roseus* do not produce either of these alkaloids due to their inability to produce vindoline, a precursor of these alkaloids. However, these cells are able to metabolize vindoline (Hamada et al., 1990). The vinblastine molecule is derived from two monomeric alkaloids, catharanthine and vindoline. The concentration of vindoline in intact plants of *C. roseus* is approximately 0.2% which is much higher than that of catharanthine or vinblastine. Misawa et al. (1988) produced the dimeric alkaloid 3,4-anhydrovinblastine (AVLB) by coupling the monomeric alkaloids catharanthine (produced by cell cultures) and vindoline (commercially available) with the aid of cell-free enzyme extract of *C. roseus*. The coupling of the two monomeric alkaloids to AVLB and leurosine has also been achieved with an immobilized system of enzyme extracted from suspension cultures of *C. roseus* (Kutney, 1988). This coupling reaction was catalyzed more efficiently by ferric ions in the absence of the enzyme (Misawa, 1994). The chemical coupling not only produced AVLB but also vinblastine, with yields of 52.8 and 12.3%, respectively, after 3 h of incubation at 30°C. Scientists at the Mitsui Petrochemical, Japan have further improved the production of catharanthine by *C. roseus* cells (Fujita et al., 1990) and chemical coupling of catharanthine and vindoline. This novel and efficient semi-synthetic method of vinblastine production is likely to be applied commercially (Misawa, 1994).

Some of the cardiac glycosides produced by *Digitalis* species have been employed in the treatment of heart diseases. For commercial production, *Digitalis* plants are being cultivated in several countries, including Argentina, Hungary and the Netherlands. Although, a number of studies have reported the production of the cardiac glycosides in cell cultures of *D. lanata* and *D. purpurea* (Rucker, 1988), generally the yields were low. Moreover, during successive transfers of the cells the amount of cardenolides often decreased and disappeared completely. In contrast to this de novo synthesis, the biotransformation process with *Digitalis* cells seems to be more promising commercially. Graves and Smith (1967) reported that *D. lanata* and *D. purpurea* callus rapidly transformed progesterone to pregnane. Leaf and root cultures of *D. lanata* and shoot forming callus of *D. purpurea* accumulated an increased amount of digoxin and/or digitoxin when progesterone was added to the cultures (Hagimori et al., 1980; Lui and Staba, 1981). Biotransformation of digitoxin to digoxin using *D. lanata* cells, which are unable to produce cardiac glycosides de novo, is the most famous example of biotransformation (Alfermann et al., 1985). It is of commercial interest since digoxin has a higher demand as a

TABLE 17.5

Biotransformation of β-methyldigitoxin to β-methyldigoxin by *D. lanata* cells in a 20 l bioreactor over a period of 17 days[a]

	g (%)
β-Methyldigitoxin added	17.24 (100)
Unconverted β-methyldigitoxin	2.0 (11.8)
β-Methyldigoxin formed	14.36 (81.7)
By-product	0.28 (1.4)
Yield	(94.90)

[a]After Misawa (1994).

drug for heart diseases than digitoxin. The results of a typical experiment using a 20 l bioreactor is shown in Table 17.5. About 600–700 mg l^{-1} of β-methyldigoxin was obtained using a 200 l bioreactor. The process was studied for commercialization by Boehringer Mannheim Co. in Germany but so far it has not been industrialized (Misawa, 1994).

17.2.7. Permeabilization of cells

Generally, secondary metabolites accumulate intracellularly; only a few lines release the product into the surrounding medium. Although these products can be extracted from the cells using destructive methods, it would be more desirable if the metabolites can be sequestered from the cells without undue loss of their viability and synthetic ability, so that each culture can be used for repeated cycles of secondary metabolite production and harvest (Hunter and Kilby, 1990). It would help in reducing the cost of in vitro production of industrial chemicals which is currently very high.

Permeabilization of cells to allow controlled release of products into the medium has been attempted by various techniques, such as dimethylsulphoxide (DMSO) application (Parr et al., 1986), using media of low pH (Kilby, 1987), sonication with continuous ultrasound (Kilby and Hunter, 1990, 1991) and electric treatment (Hunter and Kilby, 1988). The essential feature of such permeabilization is that it should be reversible. Parr et al. (1986) observed that permeabilization of *Cinchona ledgeriana* cells by DMSO was irreversible.

Hunter and Kilby (1988) demonstrated that secretion of vacuole-held betanin in the cells *C. roseus* can be induced by giving electric (400–720 V cm^{-1} DC) or ultrasound (1.02 MH_2) treatments. The treated cells retained the capacity to grow and produce the pigment (Kilby and Hunter, 1990,

1991). Sonication of cells for >50 s and incubation allowed pigment harvest from the cells every 7th day during a 21-day culture period (Kilby and Hunter, 1990). The sonication-induced pigment release is mediated by cavitation events generated in the supporting medium. The free radicals induced by cavitation are intimately associated with the process of product release. However, sonication induced the release of only 5–10% of the extractable pigment (Kilby and Hunter, 1990).

Jardin et al. (1991) were able to achieve 100% release of alkaloids produced by immobilized cells of *C. roseus* by periodic addition of acid or base solution without apparent lysis or decrease in culture viability.

17.2.8. Removal of secreted products

The secondary products released into the medium naturally or by induced permeabilization of cells may suffer enzymatic or non-enzymatic degradation. Therefore, it is important that the secreted metabolites are removed from the medium. It would also eliminate any feedback inhibition regulating the biosynthetic pathway. A number of investigations have been carried out, using 'two phase culture', to trap excreted products into the 'second' phase, which may be liquid or solid.

Amberlite XAD-7 (10–20 g l^{-1}) was used to adsorb indole alkaloids (ajmalicine and serpentine; Payne et al., 1988) and anthraquinones (Robins and Rhodes, 1986) from the culture medium of *C. roseus* and *Cinchona ledgeriana*, respectively. The removal of anthraquinones from the medium enhanced its production by *Cinchona* cell cultures from 1.2 to 17.4 mg l^{-1} day^{-1} which was more than with any chemical or elicitor treatment. The amount of XAD-7 and the time of its application were critical. The best time to add the adsorbant was 7 days after culture initiation. Activated charcoal (Knoop and Beiderbeck, 1983) and reverse-phase silica (Becker and Herold, 1983) are other solid phases employed to increase the production of secondary metabolites. In the latter case the production of valepotriates by cell cultures of *Valeriana wallichii* increased up to 4-fold, and around 95% of the total product was recovered from the silica phase.

Various liquid 'second phases' have also been tested for the extraction of secondary metabolites from the media. A dimethyl siloxane polymer, a silicon-based antifoam fluid was used by Byun et al. (1990; cited in Brodelius, 1990) to accumulate benzophenanthridine alkaloids produced by elicited cells of *Eschscholtzia californica*. Addition of 23% (v/v) of the polymer, which did not affect the growth of cells, increased alkaloid production by 3-fold; combined with elicitation the gain was 17.5-fold.

17.3. COMMERCIAL ASPECTS

17.3.1. Scale-up

Commercial production of secondary metabolites by de novo synthesis or by biotransformation of externally fed substrates requires the successful cultivation of plant cells/organs on a large scale. Technological aspects of cell suspension and immobilized cell cultures on laboratory and large scale are discussed in Section 4.5.2 (see also Section 17.2.4). Suspension cultures of *Catharanthus* have been grown up to 5000 l (Schiel and Berlin, 1987) and tobacco cells have been scaled up to 20 000 l (Hashimoto et al., 1982) using conventional fermentors. The first commercial process, the production of shikonin, was also performed in stirred tanks (Fujita et al., 1982). A special reactor designed for hairy root culture is described in Section 17.2.5.

In many cases a set of two reactors is required for optimum production of the desired chemical. In the first reactor cell suspensions or hairy roots are grown under optimum conditions for rapid biomass production and the second reactor provides optimum conditions, generally growth limiting, for product formation, as in the case of shikonin production by cell cultures of *L. erythrorhizon* (see Fig. 17.2).

17.3.2. Economic outlook

The economic aspect of producing metabolites from plant cells grown in bioreactors has been discussed in many papers (Sahai and Knuth, 1985; Drapeau et al., 1986, 1987; Scragg, 1986; Panda et al., 1989; Lambie, 1990; Moreno et al., 1995). At this stage, when the technology for in vitro production of phytochemicals is not sufficiently advanced only speculative appraisal of the commercial feasibility of the process can be given. This laboratory technique is likely to be of commercial value only for the production of very costly compounds or cheaper chemicals for which market demand is very high. Any process using plant cell culture is not going to be economically favourable if the desired product can be manufactured by chemical or microbial fermentation methods.

The interest in cell cultures of *C. roseus* was stimulated by the high value anticancerous alkaloids, vinblastine and vincristine, produced by this plant but none of these compounds are produced by cell cultures of this plant. However, these cell cultures produced the monomeric alkaloid ajmalicine which is also of pharmaceutical interest. The annual market demand for this alkaloid is ca. 3600 kg, with a market price of US$2000 kg^{-1} (Moreno et al., 1991). The estimated production cost of

ajmalicine from the roots of field grown plants is US$619 kg^{-1} (Verpoorte et al., 1991). Considering a yield of 0.6% on DW basis and a production volume of 800 kg per year, Drapeau et al. (1987) calculated the production cost of an amount of biomass containing 1 kg ajmalicine at US$3215. The authors suggested that the specific rate of biosynthesis in cell cultures (0.2 mg kg^{-1} day^{-1}) would have to be increased 40 times for the bitechnological process to be economically competitive with the extraction from plants. Based on yearly production of 3000 kg and a yield of 0.9% on DW basis, Van Gulik et al. (1988) worked out the cost of production of the biomass containing 1 kg of ajmalicine at US$1500. Considering the specific growth rate twice as high and the alkaloid yield 10 times higher, the estimated cost was US$430 per amount of biomass containing 1 kg of ajmalicine, which is still high to compete with the traditional method (Moreno et al., 1995).

In the case of shikonin, which has been commercially produced by plant cell cultures of *Lithospermum erythrorhizon*, the natural plant roots contain 1–2% of shikonin and it takes 3–4 years after seed formation before industrially useful roots can be harvested. In contrast, the cultured cells produce 15–20% of shikonin and the cells are ready for harvest after 23 days (Fujita, 1988, 1990). Plant cell culture is, therefore, the technique of choice for the production of shikonin. The extinction of *L. erythrorhizon* from Japan and its declining population in other native countries is another consideration for commercialization of the in vitro process for shikonin production.

The prime factor determining the economic feasibility of plant cell culture is the specific biosynthesis rate of the target compound, which is very low in most of the cases studied. Until stable, high yielding lines are developed, cell culture technology may be suitable for the production of only those compounds that are highly valued or that are extracted from endangered or rare species or that are costly to cultivate as whole plant.

The cost of production of secondary metabolites can be reduced by increasing the rate of biosynthesis of the specific compound either by developing high yielding cell lines or by manipulating the natural biosynthetic machinery of the cells through the environmental conditions that regulate the biosynthetic pathways. Mutant cell lines that can excrete metabolites into the extracellular medium and produce more than one useful metabolite will be of considerable economic importance. Exploiting inexpensive nutrients such as molasses and corn steep liquor can also substantially enhance the economics of plant cell culture via reduction in costs. Judicious selection of reactor in which high cell density can be achieved, such as those that use immobilization or a cell recycle system is another way to reduce the production cost. The attractiveness of the

plant cell culture process will also increase as the natural plant products become more popular. Finally, better utilization of spent plant cell biomass (the biomass left after extraction of the desired product), which is a rich source of polysaccharides, for cultivation of industrially important microorganisms or for production of soluble sugars will also favourably enhance the overall economics of the process (Smith et al., 1985).

17.3.3. Successful cases

At least two products (shikonin and ginseng cells) from plant cell cultures have been commercialized so far and some other compounds are close to commercialization.

Shikonin was the first tissue culture produced chemical to be commercialized. It is a non-alkaloid compound with antibacterial, anti-inflammatory, granulation and other medicinal properties and has been used as an ingredient of medicines for treatment of burns, wounds or haemorrhoids. It is also a valuable vegetable dye which assumes a wide spectrum of colours from red and purple to blue. Traditionally, shikonin is obtained from reddish purple roots of *L. erythrorhizon*, a member of the family Boraginaceae. It takes 3–4 years of plant growth to yield industrially useful roots and the shikonin content of the roots is very low (1–2%). Due to uncontrolled mass collection of the plant from nature, this species has almost become extinct in Japan and is likely to meet the same fate soon in other countries (China and Korea) where it grows wild. Therefore, scientists at the Mitsui Petrochemical Co. Japan, developed a commercial tissue culture process for the production of shikonin using cell cultures of *L. erythrorhizon*, established by Tabata et al. (1974). The research group at the Mitsui Petrochemicals, selected high yielding lines, and defined culture conditions which enhanced yields to 15% of the biomass. A two stage process was used for commercial production of shikonin. In the first stage a 200 l reactor was used to produce biomass and in the second stage a 750 l reactor was used for shikonin production (Fujita, 1988, 1990). In the spring of 1984 'Lady 80 biolipstick', containing plant tissue culture (PTC) derived shikonin was released for sale, as the first example of commercialization of a PTC produced metabolite. Later in the same year a biotechnological soap 'Nautre Murasaki (Purple)' was introduced in the market.

The roots of *Panax ginseng* (ginseng), a perennial herb have been widely used as a tonic and precious medicine since ancient times, particularly in oriental countries, including China and Korea. The cultivation of ginseng, largely restricted to areas of 30–48° north latitude, require 4–7 years and it is impossible to plant consecutively for 20–50 years but the

demand for the plant has increased dramatically in the world and its price has soared (Misawa, 1994). Therefore, many scientists have tried to produce ginseng cells through tissue culture.

Furuya et al. (1971, 1983) have studied *P. ginseng* callus cultures since 1970, and Meiji Seika Kaisha of Japan investigated large scale cultivation of the cell lines established by Furuya using various fermentors (Furuya et al., 1973). The concentrations of crude saponins in the callus (21.1%), in the crown gall (19.3%) and the redifferentiated roots (27.4%) were much higher than those in the natural roots (4.1%). The saponins were found to contain ginsenosides Rb and Rg which have sedative and stimulative activities, respectively. Although, Meiji Seika Kaisha abandoned further development of their ginseng project, another Japanese company, Nitto Denko, worked out scale-up cultivation of ginseng cells, and in 1988 it obtained Government approval to market the cultured ginseng cell mass as a food additive. The product has been used as an additive to wine, tonic, drinks, soups, herbal liqueurs and others (Misawa, 1994).

Taxol, a diterpine amide, is an anticancerous drug, currently manufactured by extraction from the bark of *Taxus brevifolia* trees growing in the wild. The demand for taxol is likely to increase but its supply is limited. In the US, Phyton Catalytic Inc. and ESCAgenetics announced in 1992 that they have established a culture process to manufacture taxol (Misawa, 1994).

17.4. CONCLUDING REMARKS

There is a continued commercial demand for a wide range of plant secondary metabolites, particularly in the food and pharmaceutical industries. Successful scaling up of synthesis of these compounds by cultured cells/roots should reduce or eliminate the need to cultivate the source plants under variable climatic conditions or, alternatively, need to conduct complex and expensive organic synthesis. It would also help in conserving the endangered plant species which have been collected on a very large scale from the wild for commercial purpose.

For more than 30 years many researchers have investigated plant cell culture for the production of useful chemicals but only two products, shikonin and ginseng cells, are so far being produced on a commercial scale. This is because cell culture has not become a cost effective technology. Slow growth of plant cell cultures, low and unstable yield of metabolites are some of the reasons limiting industrial application of this technology. Several methods have been tried to improve product yield in cell cultures and considerable progress has been made. Of the various

strategies tried, biotransformation of externally supplied precursors or other substrates is regarded as most promising (Misawa, 1994; Moreno et al., 1995). Further improvement in the productivity of plant cells requires a better understanding of the regulation of specific secondary metabolism so that it can be manipulated as desired.

Plant cell cultures have proved to be useful sources of enzymes which catalyze specific biosynthetic steps (Philippson, 1990). Techniques are now available to clone these enzymes and express them in microorganisms, thus offering the prospect of ready availability of plant enzymes or the use of fast growing microorganisms for the production of plant secondary metabolites. Transfer of the thaumatin gene to a microbial system has opened up the possibility for commercial production of thaumatin, which is 3000 times sweeter than sucrose (see Venkataraman and Ravishankar, 1991).

Demonstration of the safety of the products produced by tissue cultures, particularly those used as pharmaceuticals, is an essential component of the process of their commercialization (Fujita, 1990).

Chapter 18

Germplasm Storage

18.1. INTRODUCTION

The primitive cultivars and wild relatives of crop plants constitute a pool of genetic diversity which is invaluable for future breeding programmes. However, the existence of these plants has been endangered by the continual introduction of new cultivars. This danger of the erosion of genetic resources was recognized in 1974, when the Consultative Group on International Agricultural Research established the International Board for Plant Genetic Resources with the proposal to develop a global network of genetic resource centres. This was to ensure that the genetic resources of cultivated plants are collected, conserved, evaluated and made available to plant breeders anywhere in the world (Frankel and Hawkes, 1975; Henshaw, 1979).

The plant genetic resources of a country include, besides the primitive relatives of the present day crop plants, a large number of wild species which affect human life in more than one way. Some of these species, such as several medicinal plants, are extremely valuable commercially but have never been cultivated. Overexploitation of some of these plants from the wild has reduced their number to such an extent that they are threatened with extinction. Many other man-made and natural causes are resulting in the erosion of our genetic resources. Conservation of plant diversity is, therefore, of utmost importance to ensure protection of a healthy environment and meeting basic human needs of food, healthcare, clothing and fuel (Fay, 1992).

Conventionally, germplasms are maintained in the form of seeds because they occupy a relatively small space and can be stored at low temperature for many years. They can be easily dried and packed for transmission to the introduction centres and gene banks. The limitations of this method, however, are: (a) some crops such as cacao, coconut, and mango, produce large recalcitrant (short-lived) seeds which lack a dormancy mechanism and cannot bear subjection to desiccation or exposure to low temperature; (b) the seeds may be destroyed by pathogen and pest attacks; (c) discrete clones cannot be maintained in the form of seeds except for apomictic species, and (d) it is not applicable to vegetatively propagated crops such as *Dioscorea*, *Ipomoea* and potato. Moreover, the

cost of maintaining a large proportion of the available genotypes of the crop plants and endangered species in nurseries or fields is extremely high, and there is a risk of the material being lost as a result of environmental hazards.

The possibility of regenerating whole plants from somatic and gametic cells, and minute shoot apices prompted workers to investigate the feasibility of in vitro storage of genotypes. The potential advantages of this method are: (a) relatively little space is needed for the preservation of large numbers of clonally multiplied plants (as 'vegetative seeds'). According to the estimate of Morel (1975), 800 cultivars of grapes, each replicated six times, could be maintained in cultures in 2 m^2 of laboratory space. It would require about 1 ha in the field. Similarly, nearly 6000 accessions of cassava can be stored in a 5 × 6 × 3 m room (Roca et al., 1984). (b) The plants are maintained free from pests, pathogens, virus and other natural hazards. (c) The plants are not exposed to the threat of changing government policies and urban development. (d) Under special storage conditions the plants do not require frequent splitting and pruning. (e) The material could serve as an excellent form of nucleus stock to propagate large numbers of plants rapidly, when required. (f) Being free from known viruses and pathogens the clonal plant material could be sent from country to country, thus minimizing the obstructions imposed by quarantine systems on the movement of live plants across national boundaries.

In cultures, plants can be maintained by serial subculture at 4–8 week intervals for virtually unlimited periods. However, the storage of germplasm by serial subculture risks the loss of plant material by microbial contamination due to human error and, also, is uneconomical. Moreover, in long-term callus and suspension cultures the regeneration potential, biosynthetic properties and genetic make-up of the cells suffer. A basic requirement for the practical feasibility of a plant tissue culture method in the preservation of genetic resources, therefore, is to reduce the frequency of subcultures to a bare minimum. There are two options for in vitro storage of plant materials: (1) long-term preservation at the super low temperature of liquid nitrogen (cryopreservation); and (2) short or medium-term storage under growth limiting conditions. Withers and Williams (1986) have referred to them as 'in vitro base genebank' and 'in vitro active genebank', respectively.

18.2. LONG-TERM STORAGE

Since 1975 several papers have reviewed the technology of freeze preservation of plant cells and organs and its potential application in long-term storage of plant germplasm (Kartha, 1985, 1987; Bajaj, 1990; Grout,

1990, 1995; Engelmann, 1991; Withers, 1991; Benson, 1994). In this technique the plant material is frozen and maintained at the temperature of liquid nitrogen (LN), which is around –196°C. At this temperature the cells stay in an almost completely inactive state. In theory, if fully satisfactory procedures were available for freezing and thawing plant material with a fairly high rate of recovery of viable cells/organs capable of regrowth under normal conditions it should be possible to store them in LN indefinitely. Of course, a constant supply of LN would be essential to maintain the material at a safe temperature. This method has been very successfully used to preserve microbes and animal cells (Withers, 1990). The storage of sperms in LN for artificial insemination is a standard practice. More recently, attention has been paid to developing cryopreservation methods for embryos, including those of humans, and tissues and organs for transplantation.

Initially, LN-freeze preservation of cell suspensions was considered as a potential method for plant germplasm storage (Bajaj and Reinert, 1977; Withers and Street, 1977a; Bajaj, 1979b). While preservation of cell lines remains useful with respect to in vitro production of secondary metabolites, cultured cells are not the ideal system for germplasm storage. Therefore, the emphasis of research in this area has been largely on freeze-preservation of organized structures, such as shoot apices, embryos and young plantlets. Some of the reasons for this shift from cell cultures to organized structures are as follows:

(a) Genetic instability of cells in long-term callus and suspension cultures is a very common phenomenon and there is no effective measure known so far to control it (see Chapter 9). Moreover, most callus cultures are initiated from non-meristematic cells of the plant body which in many angiosperms exhibit polysomaty. Thus, the cultured cells may exhibit genetic heterogeneity from the very beginning. In contrast, plants raised from shoot apices have generally proved to be true-to-type.

(b) Cultured cells of several important crop plants do not exhibit totipotency, and in those plants where the cells initially form organs/embryos and whole plants this potentiality is often lost after some time in culture. On the other hand, excised shoot apices possess a high regeneration ability which is retained in prolonged cultures. Shoot apices are also widely used for raising virus-free plants (see Chapter 15) and for their rapid clonal multiplication (see Chapter 16).

(c) Haploidy, which is highly unstable in callus and suspension cultures, can be maintained through shoot-tip culture and axillary bud proliferation.

(d) Cells of shoot tip and young embryos are small and meristematic. They appear to be better suited than larger cells to survive LN freezing and thawing.

During the last 15 years or so shoot tips of several crop plants have been successfully frozen in LN and viable plants recovered from them (Tables 18.1 and 18.2). Somatic embryos, pollen embryos, and young plantlets have also been reported to withstand freezing at –196°C. The viability of the thawed shoots has been fairly high in some cases.

Broadly speaking, freeze preservation involves four steps (Fig. 18.1): (a) freezing, (b) storage, (c) thawing, and (d) reculture. The main consideration in freeze preservation is the protection of cells against cryogenic injuries. The two potential sources of cell damage are the formation of large ice crystals inside the cell, leading to the rupture of organelles and the cell itself, and the intracellular concentration of solutes increasing to toxic levels. Cells may also suffer the loss of vital solutes through leakage during the freezing process. Besides freezing and thawing processes, the factors known to affect the viability of cells frozen to cryogenic temperature are the nature of the plant material, pre-freezing treatments, and cryoprotectants.

18.2.1. Nature of the plant material

The morphological and physiological conditions of the plant material, prior to freezing, considerably influence its ability to survive freezing at –196°C. In general, small, richly cytoplasmic, meristematic cells survive better than the larger, highly vacuolated cells (Nag and Street, 1975; Withers, 1980). Therefore, cell suspensions should be frequently subcultured and frozen in the late lag phase or exponential phase when the majority of cells are in the preferred condition. In comparatively larger specimens, such as shoot apices, embryos and plantlets, highly vacuolated cells are severely damaged, and regrowth occurs from the meristematic cell component. Young, globular embryos survive better than the older ones.

18.2.2. Pre-freezing treatments

(i) *Pre-culture.* A brief culture of shoot apices before freezing has proved beneficial in several cases. Freshly harvested shoot apices of *Solanum etuberosum* did not survive supercooling unless they were conditioned by culturing for at least 48 h, in light (Towill, 1981). Grout and Henshaw (1978) recorded a similar requirement for some species of potato. To ensure a consistently high frequency of survival of shoot apices

after freezing in LN, it proved necessary to pre-culture them for 48 h in the presence of 5% dimethyl sulphoxide (DMSO). Seibert and Wetherbee (1977) have shown that growing plants at 4°C for 3 days before taking shoot apices for freeze preservation increased the survival of the specimens from 30 to 60%. Similarly, Yamada et al. (1991a) observed a significant increase in the survival of white clover shoot tips after freezing in LN if precultured at 4°C for 2 days, in B_5 medium supplemented with 5% each of DMSO and glucose.

(ii) *Desiccation*. One of the most important factor for successful cryopreservation is the exclusion of freezable water from the cells before freezing. Amorphous freezing cryoprotectants are used for this purpose. Desiccation of cells prior to freezing not only excludes the need for cryoprotectants but also simplifies the whole cryopreservation process. Desiccated cells survive direct immersion in LN and, therefore, an expensive programmable freezing apparatus is not required.

Desiccated somatic embryos of carrot, with 3% water, could be frozen directly in LN (Kaimori and Kameya, 1987). Similarly, melon somatic embryos desiccated (11.8% water content) under a controlled humidity environment, after ABA treatment, showed as good recovery after direct immersion and 1 h storage in LN as the desiccated unfrozen embryos (Shimonishi et al. 1991). Ca-alginate encapsulated shoot tips of kiwifruit dehydrated by storage with silica gel, after successive preculture on a medium containing increasing concentrations of sucrose (1 day each on 0.1, 0.4 and 0.7 M sucrose), showed very high (85%) survival after direct immersion in LN (Suzuki et al., 1994).

In *Poncirus trifoliata* (Radhamani and Chandel, 1992) and *Camellia sinensis* (Chaudhury et al., 1991), where whole seeds or zygotic embryos could not be cryopreserved, excised embryonal axis survived direct immersion in liquid nitrogen after desiccation.

There is a dehydration optimum which may vary with the species and tissues (Sun, 1958; Withers, 1979; Abdelnour-Esquivel et al., 1992; Radhamani and Chandel, 1992; Suzuki et al., 1994). For the encapsulated shoot apices of kiwifruit desiccation to 29% water content was optimum, beyond which the survival significantly declined (Fig. 18.2) due to desiccation injury (Suzuki et al., 1994). The optimum water content for cryopreservation of embryonal axes of tea is 13% (Chaudhury et al., 1991).

(iii) *Vitrification*. Vitrification is a physical process by which a concentrated aqueous solution cooled to low or ultralow temperature directly solidifies into an amorphous 'glassy' state, without crystallization. The significance of this phenomenon in cryopreservation of plant materials is

TABLE 18.1

Some examples of cryopreservation of shoot tips/embryos/plantlets at −196°C by slow or pre-freezing method under sterile conditions[a]

Species	Organ	Storage period	Survival (%)	Reference
Arachis hypogaea	Shoot tip	3 weeks	23–32	Bajaj (1979a)
Beta vulgaris	Shoot meristem	?	7–58	Braun (1988)
Cicer arietinum	Shoot tip	3 weeks	27–36	Bajaj (1979a)
Daucus carota	Somatic embryos, plantlets	60 days	ca. 100	Withers and Street (1977a), Withers (1979)
Dianthus caryophyllus	Shoot tip	2 months	60	Seibert (1976), Seibert and Wetherbee (1977)
	Shoot tip	10 min	100	Uemura and Sakai (1980)
	Apical and axillary bud	7 days	98	Dereuddre et al. (1988)
Fragaria × *Ananassa*	Shoot tip	5 min	60–80	Sakai et al. (1978)
	Shoot tip	1–8 weeks	56–95	Kartha et al. (1980)
Lycopersicon esculentum	Shoot tip	1 day	30–45	Grout et al. (1978)
Manihot utilissima	Shoot tip	?	21	Bajaj (1977b)
Mentha arvensis	Shoot tip	15–60 min	50	Towill (1988)

M. hybrid 312	Shoot tip	15–60 min	75	Towill (1988)
M. piperita cv. Mitchan	Shoot tip	15–60 min	70	Towill (1988)
M. piperita cv. Citrata	Shoot tip	15–60 min	58	Towill (1988)
M. piperita	Shoot tip	15–60 min	47	Towill (1988)
M. spicata	Shoot tip	15–60 min	29	Towill (1988)
Nicotiana tabacum	Pollen embryos (mature globular)	?	2–31	Bajaj (1978)
Phoenix dactylifera	Shoot meristem	24 h	30	Bagniol and Engelmann (1992)
Pisum sativum	Shoot tip	26 weeks	61	Kartha et al. (1979)
Prunus sp.	Shoot tip	1–5 days	69–74	Brison et al. (1995)
Pyrus communis	Shoot tip	?	55–80	Niino et al. (1991)
P. pyrifolia	Shoot tip	?	80–85	Niino et al. (1991)
Solanum etuberosum	Shoot tip	5 min	42–76	Towill (1981)
S. gonicalyx	Shoot tip	3 weeks	62.6	Grout and Henshaw (1978, 1980)
S. tuberosum	Shoot tip	?	18	Bajaj (1977a)
Trifolium repens	Apical meristem	10 months	67–76	Yamada et al. (1991a)
Zea mays	Zygotic embryos	?	?	Withers (1978)

?, not known.

[a]In addition, shoot buds of apple, pear, gooseberry, raspberry and currants have been freeze-preserved for various periods by Sakai and Nishiyama (1978).

TABLE 18.2

Some examples of cryopreservation of shoot tips and embryos by ultrarapid cooling method after desiccation or vitrification treatment under sterile conditions

Species	Organ[a]	Storage period	Survival (%)	Reference
Desiccation				
Actinidia deliciosa	Encapsulated shoot tip	1 h	83	Suzuki et al. (1994)
Asparagus officinalis	Axillary bud	1 h	63	Uragami et al. (1990)
Camellia sinensis	Embryonal axes of ZE	17 h	95	Chaudhury et al. (1991)
Cocos nucifera	Mature ZE	24 h	73–93	Assy-Bah and Engelmann (1992b)
	Immature ZE	24 h	43	Assy-Bah and Engelmann (1992a)
Coffea arabica	ZE	1 h	96	Abdelnour-Esquivel et al. (1992)
C. canephora	ZE	1 h	42	Abdelnour-Esquivel et al. (1992)
	Torpedo shape SE	24 h	64	Tessereau et al. (1994)
C. arabica × *C. robusta*	ZE	1 h	84	Abdelnour-Esquivel et al. (1992)

Cucumis melo	SE	24 h	65	Shimonishi et al. (1991)
Daucus carota	Torpedo shape SE	24 h	80	Tessereau et al. (1994)
	Encapsulated SE	1 h	77	Zecouteux et al. (1991)
Juglans regia	Embryonal axes of ZE	?	75	de Boucaud et al. (1991)
Poncirus trifoliata	Embryonal axes of ZE	8 months	68	Radhamani and Chandel (1992)
Vitrification				
Asparagus officinalis	SE	30 min	55	Uragami et al. (1989)
Ipomoea batatas	Shoot tip	60 min	95	Towill and Jarret (1992)
Malus domestica	Shoot tip	?	40–70	Niino et al. (1992c)
Mentha sp	Shoot tip	60 min	56	Towill (1990)
Morus bombycis	Shoot tip	1 day	40–70	Niino et al. (1992b)
Pyrus communis	Shoot tip	?	50–72	Niino et al. (1992c)
P. pyrifolia	Shoot tip	?	40–70	Niino et al. (1992c)
Trifolium alexandricum	Apical meristem	30 min	50	Yamada et al. (1991b)
T. pratense	Apical meristem	30 min	55	Yamada et al. (1991b)
T. repens	Apical meristem	30 min	83	Yamada et al. (1991b)

?, not known.
[a]SE, somatic embryo; ZE, zygotic embryo.

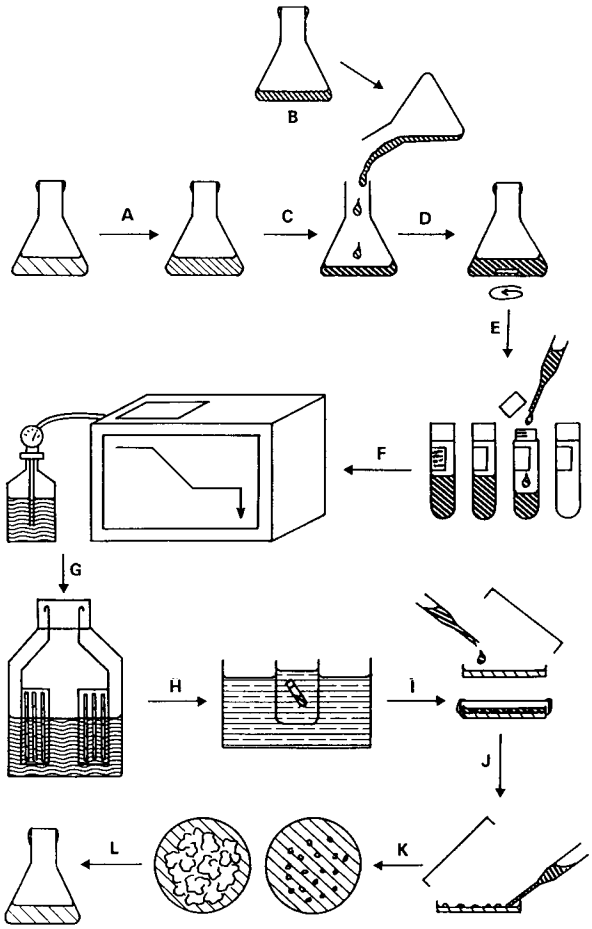

Fig. 18.1. Steps involved in cryopreservation of cultured plant cells. (A) Cultured cells are transferred to pregrowth medium. (B) Cryoprotectant solution is prepared and chilled. (C) To the cells cooled on ice cryoprotectant solution is added. (D) Cells are incubated in cryoprotectant with continuous stirring. (E) The cells suspended in cryoprotectant solution are transferred to ampoules. (F) The ampoules are cooled slowly to the transfer temperature and (G) stored in a LN cooled refrigerator. (H) Frozen cells are thawed in a water bath at 35–40°C. (I) The cells and cryoprotectant solution are transferred to recovery medium in a petri plate. (J) Excess cryoprotectant solution is removed. (K) Cells are left on recovery medium. (L) Cells are transferred to liquid medium for further growth (after Withers, 1990).

that the cells applied with a highly concentrated solution of osmotically active compounds, are protected from internal damage from ice crystal formation during freezing. This pretreatment also causes dehydration of the cells. The substances generally used for vitrification are the commonly employed cryoprotectants.

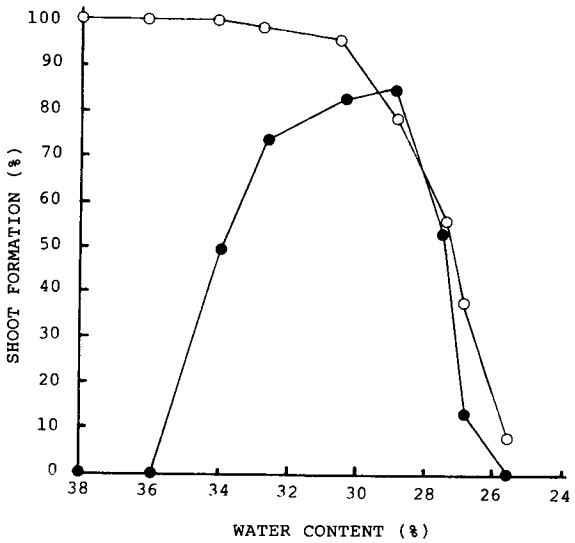

Fig. 18.2. Effect of pre-freezing desiccation of Ca-alginate encapsulated shoot tips of kiwifruit to various levels of water content on their survival after (●) and before (○) exposure to LN (after Suzuki et al., 1994).

Sakai et al. (1990, 1991) developed a simple cryopreservation method for nucellar cells of navel orange through vitrification. The cells were treated, at 25°C, for 3 min, with a highly concentrated cryoprotective solution followed by direct plunging into LN. Yamada et al. (1991b) also found this one step approach to cryopreservation very effective for white clover shoot tips (see Appendix 18.II.2). The cryoprotective mixture (designated as PVS2) used in these studies comprised (w/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in B_5 medium containing 0.4 M sucrose. About 80% of the meristems treated with PVS2 survived direct immersion and 30 min storage in LN.

18.2.3. Cryoprotectants

As a result of dehydration of cells before or during freezing, the intracellular concentration of the solutes increases before freezing of the protoplasm occurs. Cryoprotectants are substances added to the freezing mixtures to protect cells from this toxic solution effect. According to Withers and Street (1977a) cryoprotectants also prevent the formation of large ice crystals inside the cells. The cryoprotectants are commonly dissolved in the culture medium.

For plants, DMSO has proved to be the most effective cryoprotectant. The other compound sometimes used successfully, either alone or in combination with DMSO, is glycerol (Nag and Street, 1975; Bajaj, 1978). However, it was completely ineffective for pea shoot tips (Kartha et al., 1979), and also a poor substitute of DMSO for shoot tips of carnation (Uemura and Sakai, 1980) and strawberry (Kartha et al., 1980). Withers and King (1979) reported that for cell suspensions of maize and some other species proline was a far superior cryoprotectant than DMSO or a combination of DMSO and glycerol. It is worth noting that the cell suspensions grown in a nutrient medium supplemented with 10% proline for 3–4 days survived freezing even without further cryoprotection (Withers and King, 1979). Plant cells are known to accumulate free proline under water and salt stresses, and it is regarded as a natural protective agent of enzyme and cellular structures under these conditions (Huang and Cavalieri, 1979).

The optimal concentration of DMSO for cultured cells ranges from 5% to 8%. However, some of the shoot apices and plantlets may tolerate even higher levels (5–20%) (Sakai et al., 1978; Withers, 1979; Grout and Henshaw, 1980). The optimal concentration of proline was 10% (Withers and King, 1979).

To protect cells against osmotic shocks, it is suggested that cryoprotectants should be added gradually over a period of time (30–60 min). When using glycerol it may be necessary to prolong this period because of its low permeability (Withers and Street, 1977a). During DMSO treatment the material is maintained at around 0°C in order to minimize the toxic effects of the cryoprotectant. High grade reagents should be used as cryoprotectants. DMSO should be stored at room temperature (>19°C) and dispensed under a fume hood because of its unpleasant and penetrating odour. Use of a fresh stock solution of cryoprotectants is recommended (Withers, 1990).

Addition of 'cryoseeds' (0.15–5 mm xygon coated acrylic polymer beads produced by Cell Systems Ltd, Cambridge, UK) to the cryoprotective solution enhanced the survival rate of cryopreserved cells of *Aegilops squarrosa* by 15% (Sugawara and Ichi-ishi, 1992). The cryoseeds increased the ice nucleation temperature from –13.8 to 5.9°C.

18.2.4. Freezing

The sensitivity of plant cells to low temperature varies considerably with the species. Therefore, no single method can be considered as being universally applicable. In practice, the material suspended in the culture medium and treated with a suitable cryoprotectant is transferred to ster-

ile, polypropylene ampoules with a screw cap and frozen by one of the following methods.

(i) *Slow cooling method.* This involves freezing the material at a cooling rate of 0.5–4°C min^{-1} from 0 to –100°C, and then transferring it to LN. This method has proved especially successful with cells from suspension cultures (Kartha, 1987).

(ii) *Rapid cooling method.* The successful development of whole plants from isolated shoot apices subjected to –196°C for 2 months was first demonstrated by Seibert (1976). He had frozen the shoot apices of *Dianthus caryophyllus* by pouring LN directly on the freezing vial and dipping the vial into an open flask filled with LN. In this procedure cooling between –10 and –70°C occurred at the rate of >1000°C min^{-1}. Later, Seibert and Wetherbee (1977) reported that the best cooling rate for this material was 50°C min^{-1}. Uemura and Sakai (1980) observed that direct plunging of *Dianthus* shoot tips in liquid nitrogen gave no survival but if the shoot apices were gradually frozen to –15°C and then cooled rapidly at the rate of >1000°C min^{-1}, by immersing in LN, all remained alive. Shoot apices of strawberry (Sakai et al., 1978) and *Solanum gonicalyx* (Grout and Henshaw, 1980) did not survive slow (1°C min^{-1}) or intermediate (60°C min^{-1}) cooling but withstood ultrarapid cooling (1000°C min^{-1}) to –196°C.

In combination with desiccation or vitrification pre-treatments (Section 18.2.2) ultra-rapid cooling is proving to be the most attractive method for cryopreservation of plant materials (Appendix 18.II.1 and 18.II.2). Shoot tips, somatic embryos and embryonal axes from zygotic embryos of a number of plant species could be freeze-preserved by direct immersion in liquid nitrogen after desiccation to an appropriate level or treatment with a concentrated solution of osmotically active compounds. The survival rates of the tissues cryopreserved by this simple, one step freezing method has been fairly high (Table 18.2). Even a cryoprotectant is not required when the desiccation pre-treatment is applied.

(iii) *Pre-freezing method.* This method, in which the material is first cooled gradually (ca. 1°C min^{-1}) or step-wise (5°C min^{-1}) to an optimum intermediate temperature (–30 to –50°C), held at that temperature for about 30 min, and then rapidly cooled by plunging into LN, proved very favourable for freeze preservation of shoot apices (Sakai et al., 1978; Kartha et al., 1979, 1980; Towill, 1981) and buds (Sakai and Nishiyama, 1978). For strawberry, ultrarapid cooling gave 40–60% survival which could be improved to 60–80% by following this method (Sakai et al.,

1978). The pre-freezing method has also been successfully applied to cells from suspension cultures (Appendix 18.II.3).

The initial slow freezing reduces the amount of intracellular freezable water by dehydrating the cells. Early in the freezing process ice is first formed outside the cells, and the unfrozen protoplasm of the cells loses water due to the vapour pressure deficit between the supercooled protoplasm and the external ice. This initial cooling, thus, acts as another pretreatment for dehydration of the cells.

Different types of freezing units have been used to freeze plant materials. To achieve a greater control on the rates of freezing, particularly in the slow cooling and pre-freezing methods, and to handle large numbers of specimens at one time, the use of a programmable freezing unit has been recommended.

With further refinements in the techniques and introduction of desiccation and/or vitrification pretreatments it may be possible to do away with an expensive freezing machine.

18.2.5. Storage

Maintaining the frozen material at the correct temperature is as important as proper freezing itself. Temperatures above –130°C may allow ice-crystal growth inside the cells and, consequently, reduce their viability (Henshaw, 1975; Withers and Street, 1977a). Long-term storage of the material frozen at –196°C, therefore, requires a LN refrigerator. A refrigerator holding about 4000 ampoules of 2 ml each is estimated to consume 20–25 l of LN per week (Withers and Street, 1977a). In theory, as long as a regular supply of LN is ensured it should be possible to maintain the frozen material with little further care. However, actual demonstration of long-term storage of shoot tips thus frozen is awaited. There is some indication in the literature that a drop in the viability of frozen shoot tips may occur with the passage of time (Kartha et al., 1980).

When storing large numbers of germplasms it is vital to follow an efficient recording system. This would not only facilitate checking what has been stored and for how long, but also reduce the time the other samples are exposed to ambient temperature when trying to remove a particular sample.

18.2.6. Thawing

Rapid thawing of the material frozen at –196°C is achieved by plunging it into water at 37–40°C which gives a thawing rate of 500–750°C min^{-1} (Sakai et al., 1978). After about 90 s the material is transferred to

an ice bath and maintained there until recultured or its viability is tested. Slow thawing at room temperature has generally proved fatal. Rapid thawing seems to protect the cells from the damaging effects of ice-crystal formation, which may occur during slow warming. The principle involved is the same as in the rapid cooling method of freezing plant material. However, if the water content of the cells has been reduced to an optimal level prior to freezing, the thawing rate is less critical.

18.2.7. Reculture

Routinely, before culturing, the thawed material is washed several times to remove the cryoprotectant which may otherwise be toxic to the cells. A gradual dilution of the cryoprotectant is desirable in order to avoid any deplasmolysis injury to the cells. Withers (1980) has, however, suggested that there is no need for thorough washing of the thawed material. For carrot suspension cultures the dilution of DMSO was found to be adequate (Withers and Street, 1977b) and for carrot somatic embryos and young plantlets even dilution proved unnecessary (Withers, 1979). Indeed, the frozen and thawed cells of *Zea mays* showed a faster recovery and higher survival rate if they were recultured in the presence of the surrounding medium (Withers and King, 1979). Yamada et al. (1991a) found that planting the thawed shoot tips of white clover, without washing, onto a filter paper disc placed on the regeneration medium increased normal regeneration compared to when the thawed shoot tips were washed before reculture. The deleterious effect of post-thaw washing may be due to the loss of some vital solutes leached out by the cells during storage, and/or triggering of rapid deplasmolysis.

The plant material frozen at –196°C may exhibit some special requirement(s) for better survival when recultured. Shoot tips from frozen seedlings of tomato directly developed into plantlets only if the medium was supplemented with GA_3. In its absence the apices callused, followed by the differentiation of adventitious shoots. GA_3 was not required by the control (non-frozen) shoot apices to directly develop into plantlets (Grout et al., 1978). GA_3 (10 mg l^{-1}) in the recovery culture medium also improved the survival rate of cryopreserved zygotic embryos of coffee (Abdelnour-Esquivel et al., 1992). The overall survival of frozen and thawed plantlets of carrot was greatly enhanced by activated charcoal (Withers, 1979).

18.2.8. Survival of freeze-preserved cells and organs

The viability of freeze-preserved cells may be tested simply by using specific staining techniques, such as fluorescein diacetate, Evan's blue,

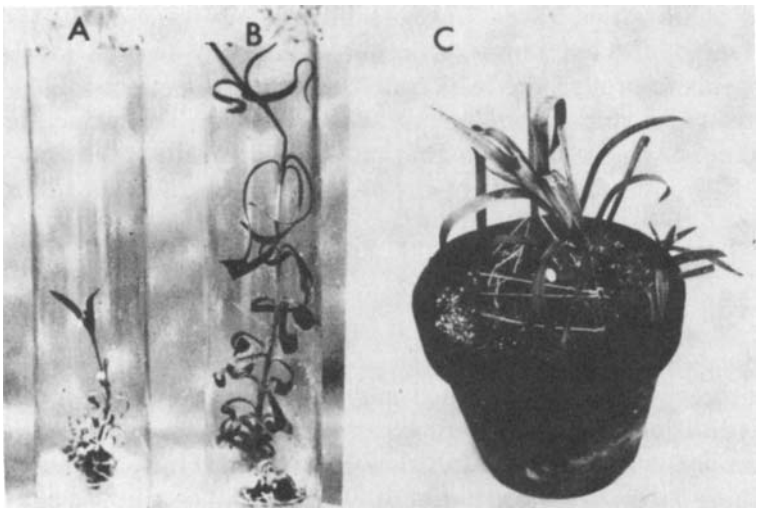

Fig. 18.3. Young plants regenerated from shoot tips of carnation frozen ultrarapidly to the temperature of LN, (A) 50 days, (B) 80 days and (C) 5 months after culture (after Uemura and Sakai, 1980; courtesy of A. Sakai, Japan).

and 2,3,5-triphenyltetrazolium chloride. Some of these are described in Chapter 4. However, staining methods do not provide a correct picture of viable cells capable of regrowth. In a population of cells that initially appear to be viable, only a minor proportion recovers completely and proceeds to divide (Withers and Street, 1977a). The best test for survival, therefore, is the entry of cells into the division phase and regrowth of shoot apices/embryos in culture (see Fig. 18.3A–C). The viability is then synonymous with the survival and may be represented as: (no. of cells or organs growing/no. of cells or organs thawed) × 100.

Ultrastructural studies of plant organs grown in vitro after freezing at –196°C have revealed that even those organs which regenerated into whole plants suffer cellular injuries to varying extents during cooling and thawing. The injury may cause damage to the complete embryonic tissue (Withers and Street, 1977a; Haskins and Kartha, 1980) or it may be only localized in the meristem (Grout and Henshaw, 1980). In the former case the regeneration of the whole plant occurs due to the differentiation of secondary shoot meristems (see Fig. 18.4A,B) or embryos from cells subjacent to the original meristem/embryo.

18.3. SHORT OR MEDIUM-TERM STORAGE

Some of the drawbacks of cryopreservation are: (1) it is technically demanding and generally requires special instruments (see Appendix 18.I)

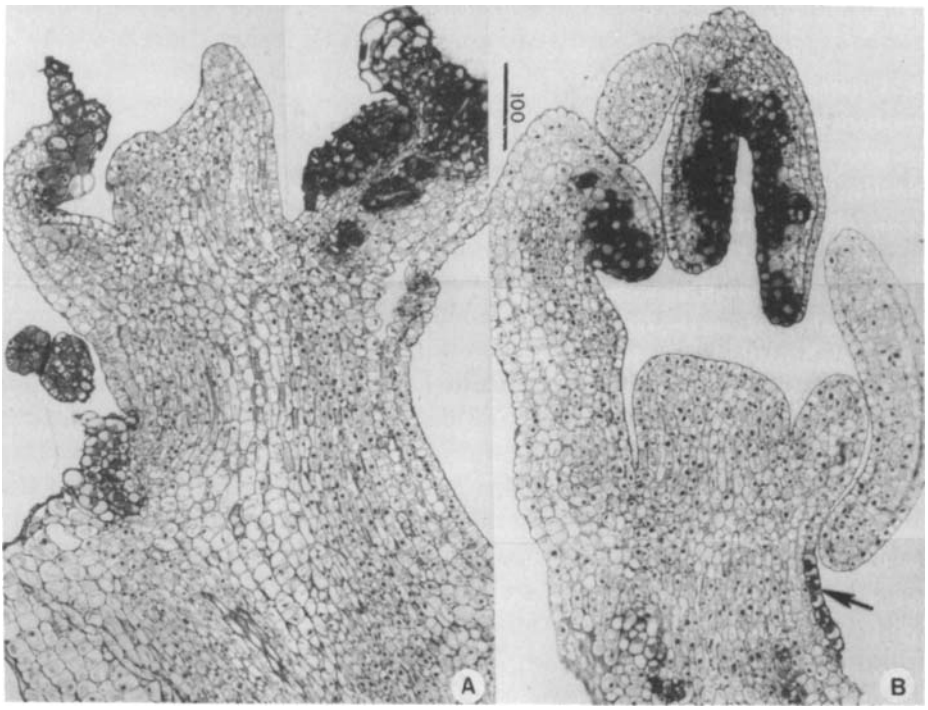

Fig. 18.4. (A,B) Photomicrographs of longitudinal, near median, semi-thin sections through the 'dome' area of pea shoot tips following freeze-preservation, thawing, and reculture, for 2 and 5 days, respectively. Dark areas (also arrow marked) represent the sites of renewed cell activities. Note, no cell in the dome areas has survived. Bar = 100 μm (after Haskins and Kartha, 1980; courtesy of R.H. Haskins, Canada).

for cooling and storage; (2) it requires preculture with cryoprotectants; and (3) there is always the possibility of severe damage due to ice crystal formation. For these reasons cryopreservation has not become a practical method for germplasm storage so far. In contrast, short- or medium-term storage methods are simple, less injurious and allow periodic check of viability of the stored materials. This involves maintaining the cultures under growth limiting conditions to increase the interval between subcultures. Plant species, such as coffee, which have an inherently slow growth can be maintained for several months under standard culture room conditions without a subculture. The storage time of coffee meristems and plantlets derived from them could be increased to 2–2.5 years by growing them on a medium with reduced nutrients and lacking sucrose (Kartha et al., 1981). Of the several methods tried for short or medium-term storage of germplasms, lowering the culture temperature (cold

storage) has been most popular so far and is being used for routine maintenance of germplasm of a range of crop species (Withers, 1991).

18.3.1. Cold storage

Germplasm storage at non-freezing low temperatures has been very successful. At lower temperatures the ageing of the plant tissues is slowed down but not completely stopped, as in freeze preservation. Consequently, subculture of the plant material is necessary although very infrequently. Some examples where shoots/plants have been cold stored for various durations are listed in Table 18.3.

Mullin and Schlegel (1976) maintained virus-free strawberry plants for 6 years at 4°C. A few drops of liquid medium were added to the cultures every 3 months. Preil and Hoffmann (1985) stored about 700 breeding lines of chrysanthemum at 2–3°C in diffuse light (10–15 lx). Some of the lines survived 5 years at the growth limiting conditions. The authors observed that aeration of the cultures during storage was essential. Under poor gas exchange conditions the shoots became vitrified. Cold storage in the dark also induces vitrification of shoots and, thus, reduces survival (Williams and Taji, 1987).

Rooted shoots of *Beta vulgaris* could be kept alive for 12 months at 5°C in diffuse light. However, non-rooted shoots and plantlets in the dark did not survive the cold treatment (Miedema, 1982). Therefore, the cultures should be cold-stored in diffuse light. The presence of a root system may increase the survival capacity of shoots as observed by Kartha et al. (1981) with *Coffea arabica*. Microtubers instead of shoot cultures can be successfully employed for the storage of potato germplasm (Kwiatekowski et al., 1988).

Cold storage has been applied with most promising results to shoot/plantlet cultures and less successfully to callus cultures. Although slow growth can retard the loss of totipotency of cultured cells, there is evidence for the loss of their growth potential and the capacity to synthesize secondary metabolites in callus cultures stored for relatively brief periods (Hiraoka and Kodama, 1984; Seitz, 1987).

The storage temperature depends on the sensitivity of the species. Whereas for temperate species it ranges from 5 to 9°C, for tropical species it is often much higher (Table 18.3).

Shoot tip cultures of kiwifruit can be maintained at 8°C for at least 52 weeks with 100% survival (Monette, 1986b, 1987). The shoots retrieved after 52 weeks of storage appeared normal with respect to growth and proliferation. Similarly, the cultures of *Colocasia esculenta*, another tropical species, tolerated 3 years of storage at 9°C (Zandvoort and

Staritsky, 1986). On the contrary, oil palm plantlets and somatic embryos are not able to tolerate even a short exposure to temperatures lower than 18°C (Corbineau et al., 1990). Banana cultivars stored below 15°C suffered damage and/or died within 3 months. At 15°C some of the genotypes survived for up to 17 months with a viability of 92% but in others viability was reduced to 50% within 13 months (Withers and Williams, 1986). According to Ko et al. (1991) the best condition for the storage of banana shoots is to place them on cotton saturated with a 3% solution of ribose and wrapped in cheese cloth and incubate at 17°C. Cavendish banana stored under these conditions for 24 months showed 67% survival, and most of the plants appeared normal. Cassava plantlets have to be stored at temperatures higher than 20°C (Roca et al., 1984).

The cold storage method of maintaining cultured plants holds great promise in the nursery industry employing micropropagation techniques (Preil and Hoffmann, 1985). During the periods of low demand for a particular genotype or species for which potential markets exist in the future, the cultures may be simply shelved in a refrigerator and the time, and consequently money, required to maintain them by serial subcultures or restarting fresh cultures saved. This also applies to research material.

One of the anticipated problems in cold storage of germplasm may be the gradual habituation of some materials to slow growth conditions (Withers, 1991).

18.3.2. Modification of gaseous environment

Growth reduction can also be achieved by lowering the oxygen level available to the tissue system. Several methods have been tried to decrease the concentration of available oxygen. The simplest is to cover the tissue with mineral oil. This technique was first tried by Caplin (1959) with carrot callus. Recently, Augereau et al. (1986) and Moriguchi et al. (1988) successfully applied it for medium-term conservation of *Catharanthus* and grape calli, respectively. None of the grape calli survived for 360 days at 15°C. However, 95% of the calli immersed in silicon during storage at 15°C remained viable. Embryogenic cultures of white spruce could be maintained in viable state for a year at ambient temperature without a subculture by sealing the culture vial with serum cap and stretch-n-seal (Joy et al., 1991).

Another method involves lowering the oxygen partial pressure using a controlled atmosphere or decreasing the atmospheric pressure of the culture vessel. Tobacco and chrysanthemum plantlets could be stored for 6 weeks under 1.3% oxygen without impairing their further development

TABLE 18.3

Some examples of cold storage of shoots/plantlets

Species	Storage conditions	Storage period	Viability (%)	Reference
Actinidia deliciosa	8°C, dark	52 weeks	100	Monette (1986b, 1987)
Allium sativum	4°C, light	16 months	72	El-Gizawy and Ford-Lloyd (1987)
Beta vulgaris	5°C, light	12 months	42–100	Miedema (1982)
Cheiranthera volubilis	10°C, dark	12 months	100	Williams and Taji (1987)
Chrysanthemum morifolium	2°C, light	2–5 years	?	Preil and Hoffmann (1985)
Coffea arabica	20°C	6 months	95	Bertrand-Desbrunais et al. (1992)
C. canephora	20°C	6 months	95	Bertrand-Desbrunais et al. (1992)
Colocasia esculenta	9°C, dark	102 months[a]		Bessembinder et al. (1993)
Fragaria × *Ananassa*	4°C, dark	72 months	100	Mullin and Schlegel (1976)
F. virginiana	4°C, dark	72 months	100	Mullin and Schlegel (1976)
F. vesca	4°C, dark	72 months	100	Mullin and Schlegel (1976)
Grevillea biternata	10°C, dark	12 months	100	Williams and Taji (1987)
Ipomoea batatas	16–18°C, light	12–18 months	?	Love et al. (1987)
Malus domestica	1–4°C, dark	12 months	100	Lundergan and Janick (1979)

Manihot esculenta	20°C, light	15–18 months	90	Roca et al. (1982)
Medicago sativa	?	15–18 months	95	Cheyne and Dale (1980)
Musa sp.	17°C, light	21 months	30	Ko et al. (1991)
	15°C, light	17 months	0.9–1.6	Banerjee and de Langhe (1985)
Picrorhiza kurroa	5°C, dark	10 months	70	Upadhyay et al. (1989)
Populus alba × *P. grandidenta*	4°C, dark	5 years	25	Son et al. (1991)
Prostanthera calycina	10°C, dark	36 months	100	Williams and Taji (1987)
P. eurybiodes	10°C, dark	36 months	100	Williams and Taji (1987)
Prunus cerasus	3°C, dark	300 days	100	Marino et al. (1985)
P. communis	2°C, dark	14 months	95	Zimmerman (1978)
P. domestica	3°C, dark	300 days	100	Marino et al. (1985)
P. persica × *P. amygdalis*	3°C, dark	300 days	100	Marino et al. (1985)
Pyrus communis	4°C, dark	18 months	94	Wanas et al. (1986)
Rauwolfia serpentina	15°C, light	15 months	66.6	Sharma and Chandel (1992)
Rhagodia spinescens	10°C, dark	12 months	100	Williams and Taji (1987)
Rubus sp.	2°C	14 months	90–100	Zimmerman (1978)
Saussurea lappa	5°C, dark	12 months	80	Arora and Bhojwani (1989)
Solanum tuberosum	10°C, light	24 months	?	Mix (1982, 1985)
Trifolium repens	5°C, dark	11 months	90–100	Bhojwani (1981)
Xanthosoma brasiliensis	13°C, dark	3 years	86	Zandvoort et al. (1994)
X. robustum	13°C, dark	3 years	74	Zandvoort et al. (1994)
X. sagittifolium	13°C, dark	3 years	100	Zandvoort et al. (1994)

?, information not available.

[a]After 29 months of continuous storage, 3 yearly transfers to fresh medium were made.

(Bridgen and Staby, 1981). Engelmann (1990) employed this technique for the storage of oil palm somatic embryos. After 4 months in an atmosphere of 1% oxygen, reproliferation of somatic embryos could be obtained very rapidly from whole cultures whereas the control embryos cultivated under standard conditions were severely damaged. This method is particularly attractive for tropical plants due to their cold sensitivity (Engelmann, 1991).

Several attempts have been made for storing organized structures using this technique (Chatti-Dridi, 1988; Jouve et al., 1991). Indeed growth reduction is achieved by it but it also induces hyperhydration of the material during storage. Regrowth of such shoots is very slow, and partial or complete necrosis of the explant is often observed. Mannonen et al. (1990) observed that the preservation of cell lines of *Panax ginseng* and *Catharanthus roseus* under mineral oil was as good as at the super low temperature of LN so far as their growth potential was concerned but the biosynthetic capacity of the lines stored under mineral oil was lost as in routinely subcultured lines. In contrast, the cryopreserved cells retained their capacity to synthesize catharanthine (*C. roseus*) and ginsenosides (*P. ginseng*).

18.3.3. Desiccation

Partial desiccation of callus and somatic embryos has also been tried for short-term storage of germplasm. Nitsche (1978) reported that carrot callus dried on filter paper in the air stream of a laminar flow chamber overnight, after pretreatment with ABA in the presence of a high concentration of sucrose, could be stored at –80, –20 or –15°C for 1 year in a viable state (see also Nitsche, 1980). The carrot calli desiccated to 3% water content retained their growth and morphogenic potential even after immersion in LN and storage in it for 30 min without a cryoprotectant (Kaimori and Kameya, 1987). Fresh calli given similar exposure to liquid nitrogen did not show any growth. Calli of several varieties of japonica and indica rice could be preserved in desiccated form for 3 months without loss of totipotency by treating them with 10^{-5} M ABA and 9% sucrose followed by slow drying in a medium (Shin et al., 1991).

Techniques have been developed that allow for the desiccation of somatic embryos in a way that reflects the physiology and development of zygotic embryos of the orthodox seeds (Ammirato, 1989). Plant regeneration from dried somatic embryos has been reported for several species, including orchardgrass (Gray, 1987), alfalfa (Senaratna et al., 1989) and asparagus (Uragami et al., 1990). McKersie et al. (1990) could dehydrate *Medicago sativa* somatic embryos down to 15% water and store them for

8 months at room temperature. ABA treatment increased the desiccation tolerance. In asparagus, desiccation tolerance of axillary buds was considerably enhanced by their pre-culture on a medium containing 0.7 M sucrose for 2 days (Uragami et al., 1990).

18.4. CONCLUDING REMARKS

The proven, invaluable applications of freeze preservation of animal tissues and semen induced optimism to apply this method for long-term storage of plant germplasm. During the past 25 years considerable progress has been made in the area but cryopreservation of plant cells and meristems is still far from being a routine laboratory method. The challenge is to devise a cost effective protocol that allows in vitro plant material to be recovered from the cryogen at high viability and in a structurally and functionally unaltered state. The ability of desiccated or vitrified plant meristems and somatic embryos to withstand direct immersion in LN, without a cryoprotectant, should help evolve simple, single step freezing protocols, which do not require a sophisticated programmable freezing apparatus.

A major problem with cryopreservation of plant cells is the sudden arrest of metabolic activities at all levels. The implication is that cell division and DNA replication are interrupted before completion. This can have negative consequences at the cellular level and could cause genotypic changes (DeVerna et al., 1992; cited in Anonymous, 1993). There is also a small risk of injury to genetic material during long-term cryopreservation resulting from external ionizing radiation. Any such injury will be preserved in the stored tissues and accumulate due to the nonfunctioning of cellular repair mechanism at the cryogenic temperature (Grout, 1995).

Short- and medium-term in vitro storage of germplasm at low temperature appears a reality. It is now routinely employed in many laboratories and international/regional centres, such as CIAT, CIP and CATIE (Engelmann, 1991). However, the optimum storage temperature and period should be carefully determined for each system, not only with respect to the maximum viability but also the stability and quality of the stored material. Son et al. (1991) observed high frequency of phenotypic variation in the poplar plants stored at 4°C for 5 years; 12.8% plants showed abnormal pigmentation, 0.25% plants were albino and one plant was rosette. Controlled desiccation of embryos, naked or encapsulated, and shoot tips appears an attractive alternative for short- or medium-term conservation of germplasm, especially of the tropical species which are sensitive to low temperature exposure.

APPENDIX 18.I: REQUIREMENTS FOR CRYOPRESERVATION WORK

1. Standard tissue culture facilities.
2. Programmable freezing apparatus (e.g. Cryomed, USA; Planner, UK; L'Air Liquide, France)
3. Pre-sterilized polypropylene ampoules (2 ml capacity) and a small rack to hold them during filling.
4. LN cooled refrigerator, either one that stores ampoules attached to metal canes held in cannisters or one in which ampoules are placed in stacks or drawers.
5. One or two Dewar flasks of 25 l capacity for storage and transport of LN.
6. A thermostated water bath for thawing.
7. Plugged Pasteur pipettes and graduated pipettes with wide enough mouth to allow unimpeded passage of cell aggregates.
8. Insulated gloves to touch heat conducting materials in contact with LN.

APPENDIX 18.II: PROTOCOLS FOR CRYOPRESERVATION

18.II.1. Cryopreservation of zygotic embryos of *Coffea arabica* by ultrarapid cooling method involving desiccation (after Abdelnour-esquivel et al., 1992)

(a) Dissect out embryos from yellow fruits, about 4 days before they turn red.
(b) Transfer the excised embryos on small pieces of filter paper and leave them exposed under the sterile air of the laminar flow cabinet for 30 min to reduce the water content from 50% to about 16%.
(c) Transfer the embryos to 2 ml cryovials and plunge them in LN.
(d) After storage in LN for about 1 h thaw the embryos by plunging the cryotubes into a water bath at 40°C for 60–90 s.
(e) Transfer the thawed embryos to culture medium containing minerals and vitamins of MS medium, 3% sucrose, 0.2% gelrite and 1 mg l^{-1} BAP and place under low light (2 μE m^{-2} s^{-1}, 12 h photoperiod) for 2 days and then transfer them under normal light (44 μE m^{-2} s^{-1}).

18.II.2. Cryopreservation of shoot tips of *Trifolium repens* by ultrarapid cooling method involving vitrification (after Yamada et al., 1991b)

1. Pre-culture the apical meristem from 2–3 weeks old aseptic seedlings (raised on B_5 medium) on semi-solid B_5 medium containing 1.2 M sorbitol, at 4°C, under continuous light (15 μE m^{-2} s^{-1}), for 2 days.
2. Transfer 20 meristems to a 1.8 ml plastic cryotube and add 1 ml of the highly concentrated cryoprotective solution (PVS 2) containing (w/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in B_5 medium with 0.4 M sucrose, and pH adjusted to 5.8.
3. Incubate the meristem in PVS2 at 25°C for 5 min or at 0°C for 15 min with three changes of the cryoprotective solution, and finally suspend them in 0.5 ml of the solution.
4. Plunge the tube in LN.
5. After storage in LN for about 30 min thaw the tip by dropping the tubes into water bath at 25°C.
6. Drain out the PVS2 from the cryotubes and replace it with liquid B_5 medium containing 1.2 M sucrose. Give two changes of the medium.
7. Transfer the meristems onto a sterilized filter paper disc placed on semi-solid B_5 medium containing 0.5 mg l^{-1} BAP, in petri dishes.
8. After 1 day, transfer the tips to a fresh filter paper disc placed on the same medium as in step 7 and incubate at 25°C under a 14 h photoperiod provided by cool fluorescent tubes (30 μE m^{-2} s^{-1}).

18.II.3. CRYO-PRESERVATION OF CELLS FROM SUSPENSION CULTURES OF *ZEA MAYS* BY PRE-FREEZING METHOD (AFTER WITHERS AND KING, 1979)[1]

(a) Subculture the actively growing suspension culture into fresh medium supplemented with 10% proline.
(b) After 3–4 days wash the cells in proline-free medium and suspend them in fresh medium.
(c) Add chilled culture medium containing 20% proline to an equal volume of the chilled cell suspension. The proline-containing medium should be added gradually, in four aliquots, over a period of 1 h.

[1] This protocol is very similar to the pre-freezing method for shoot apices, except that the cryoprotectant commonly used for shoot apices is DMSO (ca. 10%).

(d) After maintaining the mixture on ice for 1 h transfer the cells to sterile polypropylene ampoules with screw caps (1 ml into each 2 ml ampoules).
(e) Transfer the ampoules to controlled freezing apparatus and cool to –30°C at a rate of about 1°C min^{-1}.
(f) After holding at –30°C for 30–40 min transfer the ampoules to the LN container or refrigerator.
(g) To thaw, take out the ampoules from the storage container and agitate them in water at 40°C for 1–2 min.
(h) Spread the cells on agar medium along with the surrounding liquid medium for regrowth.

Glossary of Terms Commonly used in Plant Tissue Culture

Adventitious	Adjective used to describe roots, shoots, or other organs developing from abnormal positions, e.g. shoots from roots, leaves or callus, and embryos from any cell other than the zygote.
Aflatoxin	A potent toxin produced by certain fungi that commonly grow on damp or poorly dried seeds, grains and nuts.
Agar	A polysaccharide derived from certain algae, and commonly used as a gelling agent in tissue culture media.
Alkaloid	A colourless, crystalline, bitter organic substance, such as caffeine, quinine, and strychine, that has alkaline properties. Mostly found in plants, and can have toxic effects on humans.
Androgenesis	Development of plants from male gametophytes.
Aneuploidy	The situation which exists when the nucleus of a cell does not contain an exact multiple of the haploid number of chromosomes; one or more chromosomes being present in greater or lesser number than the rest.
Anther	The terminal portion of a stamen which contains pollen in pollen sacs.
Anther culture	Culture of excised anthers, to obtain haploids.
Apex	Tip; plural: apices
Apical	Pertaining to tip or apex.
Apical dominance	The phenomenon of suppression of growth of an axillary bud in the presence of the terminal bud on the branch.
Apical meristem	The meristem located at the apices of main and lateral shoots.
Aseptic culture	Raising cultures from a tissue or an organ after freeing it of bacteria, fungi, and other micro-organisms, in an environment free of these microorganisms.
Asexual	Not involving sex.

Asexual propagation — Plant multiplication by using a vegetative part or a portion of it.

Atomic weight (AW) — The relative weight of an atom based on comparison with standard, such as hydrogen (AW 1) and oxygen (AW 16).

Autoclave — A vessel for sterilizing with steam under pressure.

Autotrophic — Capable of making its own food from inorganic substances.

Auxins — A class of plant growth hormones which cause cell elongation, apical dominance, root initiation, etc. Indoleacetic acid (IAA), naphthaleneacetic acid (NAA), indolebutyric acid (IBA), and, 2,4-dichlorophenoxyacetic acid (2,4-D) are some of the auxins commonly used in tissue culture.

Axenic culture — A culture without foreign or undesired life forms. An axenic culture may include the purposeful cocultivation of different types of cells, tissues or organism.

Axil — The angle formed by a lateral organ (such as leaf) and the stem.

Axillary shoot — Shoot that develops from the bud located in the axil of a leaf.

Backbulb — An old pseudobulb, often without leaves, but still alive and bearing one or more buds behind the actively growing portion of a sympodial orchid.

Batch culture — A suspension culture in which cells grow in a finite volume of nutrient medium and follow a sigmoid pattern of growth.

Bio-conversion — The transformation or breakdown of substances from one form to another or to its constituents through the activities of micro- organisms or enzymes.

Bioreactor — A vessel used for large scale aseptic culture of cells, embryos or shoots under controlled growth conditions.

Biosynthesis — Formation of chemicals by living cells.

Biotechnology — Application of techniques to modify the fundamental processes of growth and reproduction in plants, animals or microorganisms, to enhance productivity or produce new products.

Browning — Used here to describe the occasional purplish/darkening of media by oxidized products of phenols leached out from explants, which may be phytotoxic.

Callus — A tissue arising from disorganized proliferation of cells either in cultures or in nature. Plural: calluses/calli.

Carbohydrates	Organic compounds, composed of carbon, hydrogen and oxygen such as sugars, starch, and cellulose.
Casein hydrolysate	A mixture rich in amino acids obtained by digestion (acid hydrolysis) of the milk protein, often used as a helpful supplement in tissue culture media.
Catalyst	A substance that affects the rate of a reaction without otherwise being involved in that reaction.
Cell culture	Culture of single cells or small groups of similar cells.
Cell generation time	The interval between consecutive divisions of a cell.
Chemically defined medium	A nutritive medium for culturing cells in which each component is chemically defined.
Chemostat	An open, continuous culture in which growth rate and cell density are held constant by a fixed rate of input of growth-limiting nutrient/s.
Chimeras	Plants or tissues with cells of more than one genetic make up.
Chlorosis	Reduced development or absence of green pigments leading to yellowing or whitening of green tissues in plant due to deficiency of light, magnesium, iron, or other factors.
Chromosome	Nucleo-protein bodies (generally thread-like) found in the nucleus, and carry genes arranged in a linear order. Each species has a characteristic chromosome number.
Clonal multiplication/ propagation	Asexual plant multiplication starting from a single individual.
Clone	A population of cells derived from a single cell by mitotic divisions. It is also commonly used to denote a population of plants derived from a single individual through vegetative propagation.
Cloning	Asexual multiplication starting from a single cell or an organism. In molecular biotechnology it refers to the replication of a small DNA molecule or a gene as in cloning a vector.
Closed continuous culture	A continuous culture in which inflow of fresh medium is balanced by outflow of corresponding volumes of spent medium. Cells are separated mechanically from outflowing medium and added back to the culture.
Coconut milk/water	The liquid endosperm from immature coconuts, used as a supplement in the culture medium.
Complementation	The ability of two different genetic defects to compensate for one another.

Contaminants — In the present context, refers to microorganisms, which may inhibit the growth of cells or tissues in culture.

Continuous culture — A suspension culture continuously supplied with nutrients by the inflow of fresh medium. The culture volume is normally constant (see also closed continuous culture and open continuous culture).

Cryopreservation — Preservation and storage of cells, tissues and organs at temperatures around -196°C or by immersion into liquid nitrogen.

Culture — Growing cells, tissues, plant organs, or whole plants in nutrient medium, under aseptic conditions, e.g. cell culture, embryo culture, shoot-tip culture, anther culture, etc.

Culture medium — See 'Nutrient medium'.

Cybrid — A cell or plant with nucleus of one parent and extranuclear genes of another or both parents, from the fusion of protoplasts.

Cytokinins — A class of plant growth hormones which are adenine (e.g. kinetin, benzylaminopurine, 2-isopentenyladenine, zeatin) or urea (e.g. thidiazuron) derivates, and cause cell division, cell differentiation, shoot differentiation, breaking apical dominance, etc.

Cytoplasmic hybrid — See 'Cybrid'.

Cytoplasmic inheritance — Inheritance attributable to extranuclear genes; for example genes in cytoplasmic organelles such as mitochondria and chloroplasts.

Cytoplast — An enucleated protoplast.

Dedifferentiation — Reversal of organized structures into an undifferentiated state.

Deionize — To remove ions from water by the use of a deionizer.

Differentiation — The development of different physiological and/or morphological characteristics. For example, the origin of various types of cells, tissues, and organs during the development of a multicellular organism from a single-celled zygote. In tissue culture the term is used to describe the formation of different cell types, roots, shoots, embryos, or any other organ in callus or cell cultures.

Diploid — Having two copies of each chromosome characteristic for the species.

Disease-free — A plant certified through specific tests as being free of specified pathogens.

Dominant gene / character	One that is expressed in a diploid cell or organism irrespective of its allele present on the homologous chromosome.
Electroporation	Creation, by means of an electrical current, of transient pores in the plasmalemma to allow exogenous material, especially DNA, or genes to enter the cell from the external medium.
ELISA serological method	A method used to test for the presence of viruses in plant tissues. The acronym ELISA stands for Enzyme-linked Immunosorbent Assay.
Embryo	An organized structure formed following a predetermined mode of development inside the female gametophyte with or without fertilization.
Embryo culture	Culture of embryos excised from immature or mature seeds.
Embryogenesis	The process of embryo initiation and development either in the seed or in tissue cultures.
Embryoid	Non-zygotic embryo formed in tissue cultures.
Endosperm	The nutritive tissue or liquid surrounding the developing embryo in a seed.
Enzyme	Any one of a number of specialized proteins produced in living cells that functions like organic catalyst.
Epigenetic variation	Phenotypic variability which does not result from an alteration in DNA sequence.
Etiolated	A state of growth induced by low level or absence of light and characterized as being pale or white and elongated.
Euploidy	The situation which exists when the nucleus of a cell contains an exact multiple of the haploid number of chromosomes.
Explant	A plant organ or piece of tissue used to initiate a culture.
Exudate	Matter discharged or diffused from a cultured tissue.
Feeder layer	A layer of cells, usually inactivated chemically or by irradiation used to nurture cell cultures of a different origin. The nurse cells are in contact with the medium and the cultured cells or tissues are planted on them either directly or separated by a porous matrix such as filter paper or membranous filters.
Gamete	A sex cell derived from a male or female gametophyte, with one-half the number of chromosomes present in the somatic cells of the plant.

Gametoclonal variation	Variation in phenotype, either genetic or epigenetic, expressed by gametoclones.
Gametoclone	Plants regenerated from a gametic cell.
Gene	One of a set of units of heredity, having a specific effect on the characteristics of an organism. Genes are composed of DNA and are arranged linearly on the chromosomes.
Genetic engineering	A laboratory technique for genetic modification of plants by introducing one or a few selected genes into cells to change their inherited characteristics.
Genotype	The genetic make-up of an individual as determined by the set of genes carried in the chromosomes.
Germplasm	Total variability of genetic material of a particular species. It can be preserved in the form of whole plants, seeds or regenerable tissue cultures.
Gibberellins	A class of plant growth hormones, which influence cell enlargement.
Growth regulators	Organic compounds other than nutrients that, in small amounts, influence growth, differentiation and multiplication, such as auxins, cytokinins, ethylene and gibberellin.
Habituation	Ability of cells to grow in the absence of phytohormones after prolonged cultivation in the presence of hormones.
Haploid	Having single copy per cell of each chromosome characteristic of the species (= n).
Hardening-off	The process of gradual acclimatization of tissue culture raised plants to greenhouse or field conditions such as low humidity, high light intensity and autotrophic mode of nutrition.
HEPA filter	'High efficiency particulate air' filters, an essential component of laminar air flow hoods used for aseptic manipulation of tissue cultures.
Heterokaryon	A cell in which two or more nuclei of unlike genetic make-up are present, usually derived as a result of cell fusion.
Heterotrophic	Organisms requiring external sources of organic nutrients.
Heterozygous	Having different alleles at one or more corresponding loci in homologous chromosomes. Self-fertilization of a heterozygous individual would give a heterogeneous population.

Homokaryon	A cell possessing two or more genetically identical nuclei in a common cytoplasm, usually derived as a result of cell fusion.
Homozygous	Diploid or polyploid individuals having identical alleles on the homologous chromosomes. Self-fertilization of homozygous individuals would give a homogeneous population.
Hormones	Natural or synthetic chemicals that strongly affect growth, development or metabolism at very low concentrations, i.e., cytokinins, auxins, and gibberellins.
Hybrid	An organism resulting from a cross between genetically unlike parents.
Hybridization	Any process by which hybrids are created.
Hyperhydration	An abnormality shown by some shoots developed in culture, in which the shoots appear as overly succulent, crisp, water-logged, or glassy tissues. Earlier termed 'Vitrification'
Hypocotyl	The portion of a seedling stem below the cotyledon/s and above the roots.
In vitro	Literally 'in glass'; now applied to any process carried out in sterile cultures.
In vivo	Literally 'in life', applied to any process occurring in a whole organism.
Indexing	Testing of plants for pathogens or contaminants.
Induction	To cause initiation of a process or a structure.
Inoculum	Aliquot of a suspension culture used for its subculture.
Juvenile	A phase in the sexual cycle of a plant characterized by differences in appearance from the adult and which lacks the ability to respond to flower-inducing stimuli.
Karyoplast	A nucleus, surrounded by a thin layer of cytoplasm and a plasma membrane, obtained by enucleation of a cell.
Karyotype	The characteristic number, size and shape of the chromosomes during metaphase of a somatic cell of an organism.
Medium	See 'Nutrient medium'.
Meiosis	A type of cell division involved in the production of gametes; results in the formation of four cells each with half the normal number of chromosomes contained in somatic cells.

Meristem	A localized group of actively dividing cells, from which permanent tissue systems (root, shoot, leaf, flower) are derived. The main categories of meristems are: apical meristems (in root and shoot tips), lateral meristems (vascular and cork cambiums), and intercalary meristems (in the nodal region and at the base of certain leaves).
Meristem culture	In the present context, in vitro culture of a shiny, dome-like structure measuring less than 0.1 mm in length excised from the shoot apex.
Meristemoid	A localized group of meristematic cells that arise in the callus and may give rise to roots and/or shoots.
Micrografting	Grafting of a shoot tip onto the young stem of an in vitro growing seedling.
Microinjection	Insertion of genes or DNA into cells or embryos using a very thin ($< 10\mu$m) glass needle.
Micropropagation	Asexual or vegetative propagation of plants in vitro.
Microspore	A uninucleate, haploid cell which develops into a pollen grain (male gametophyte).
Microtubers	Potato tubers produced in vitro, regardless of their size and whether they are derived from shoot or stolons.
Mitosis	A type of cell division resulting in two daughter cells, each with the same number of chromosomes as in the parent cell.
Mole	Molecular weight of a compound expressed in grams.
Molecular genetics	Study of the nature and biochemistry of genetic material.
Molecular weight	The sum of atomic weights in a molecule.
Monopodial	A type of plant development in which the terminal bud of the stem continues its vegetative growth indefinitely. Lateral branches may or may not develop. Among orchids, this growth habit is shown by *Angraecum, Phalaenopsis, Renanthera* and *Vanda*.
Morphogenesis	The anatomical and physiological events involved in the growth and development of an organism resulting in the formation of its characteristic organs and structures, or in regeneration.
Mutagen	An agent that causes mutations.
Mutant	An organism with one or more mutations, making its genetic function or structure different from that of a corresponding wild- type organism.

Mutation — The occurrence of a heritable variation in an individual due to a change in genes or chromosomes.

Mycorrhiza — Root fungus; an association of fungi with the roots of trees and other plants, increasing the capacity of the plant to absorb nutrients from the soil.

Nitrogen fixation — The conversion of atmospheric nitrogen (N_2) into ammonia and amino acids; in the present context by nitrogen-fixing organisms.

Node — A region on the stem from where a leaf bearing an axillary bud arises.

Nucellus — A tissue in the ovule encasing the embryo sac which in turn encloses the embryo.

Nucleic acids — An acid molecule composed by sub-units containing a sugar, a phosphate group and one of the four bases, viz. adenine, thymine/uracil, cytosine and guanine, found especially in the nucleus of all living cells. The two types are RNA and DNA.

Nurse culture — See 'Feeder layer'.

Nutrient medium — A combination of nutrients and water, usually including several salts, a carbohydrate (e.g. sucrose), and vitamins. Such a medium, liquid or gelled, is often referred to as a basal medium and may be supplemented with growth hormones and, occasionally, with other defined and undefined substances; plural: Nutrient media.

Open continuous culture — A continuous culture in which inflow of fresh medium is balanced by outflow of a corresponding volume of culture. Cells are constantly washed out with the outflowing liquid. In a steady-state the rate of cell washout equals the rate of formation of new cells in the system.

Organ culture — Aseptic culture of organized structures, e.g. root tip, shoot tip, shoot segments, embryo, etc.

Organogenesis — Differentiation of organs from cultured cells or tissue.

Passage — The transfer of cells, tissues or organs, with or without dilution/dividing, from spent culture medium to fresh medium (see also subculture).

Pathogen — Disease causing microorganism.

Pathogen free — Free from specific pathogens based on specific tests for the designated pathogens.

pH — A measurement of the degree of acidity or alkalinity on a scale of 1–14.

Photoperiod	The length of time plants are exposed to light in an alternating light-dark interval sequence.
Plagiotropic	Horizontal growth as opposed to vertical growth.
Plantlet	A small rooted shoot or germinated embryo.
Plating efficiency	The percentage of the plated cells/protoplasts that divide and form colonies.
Ploidy	The number of full chromosome sets per nucleus.
Pollen	Male gametophyte, which is haploid and bears the male gametes (sperms).
Polyembryony	Development of more than one embryo in a seed, or multiple embryo development in cultures.
Polyploidy	Containing three or more sets of chromosomes per nucleus ($3n$ = triploid, $4n$ = tetraploid, $5n$ = pentaploid, etc.)
Polysaccharide	A group of carbohydrates composed of many units of various sugars.
Population density	The number of cells per unit area or volume of a culture vessel. Also the number of cells per unit volume of medium in a suspension culture.
Primary culture	A culture started from cells, tissues or organs excised directly from organisms.
Primordia	Plural of primordium; plant organs in the earliest stages of differentiation.
Proliferation	Rapid multiplication of new units (cells, embryos, shoots etc.).
Propagule	A small bit of plant that is being used for propagation.
Proteins	A large group of complex organic substances containing amino acids and diverse elements.
Protocorm	In orchids, seed contains an unorganized embryo comprising only a few hundred cells. During seed germination the embryo first forms a tuberous structure called a protocorm, from which develops a complete plant. In cultures, vegetative parts of several orchids form round, glistening, protocorm-like structures which may be multiplied indefinitely or induced to regenerate a whole plant.
Protoplast fusion	Technique in which related or unrelated protoplasts are fused to form a homokaryon or a heterokaryon.
Protoplasts	Single cells with their walls stripped off.
Pseudobulb	A thickened portion of the stem of many orchids.

Recessive gene / character	One that is expressed in a diploid cell or organism only when homologous chromosomes carry the same alleles for a phenotype.
Recombinant DNA (rDNA)	The hybrid DNA produced in vitro by joining together pieces of DNA from different organisms.
Regeneration	In tissue cultures, a morphogenetic response that results in the production of new organs, embryos or whole plants from cultured explants or calli derived from them.
Secondary products	Products of plant metabolism that are not primarily related to growth and reproduction; some of these are used as pharmaceuticals, flavourings, dyes, pesticides, etc.
Senescence	The process of ageing of organ or plant which is part of normal development sequence in plants, a genetically determined phenomenon.
Shoot tip/shoot apex	Terminal 0.1–1.0 mm portion of a shoot comprising the meristem (0.05–0.1 mm) together with primordial and developing leaves and adjacent stem tissue.
Somaclonal variation	Heritable differences observed among plants propagated through tissue culture of a single mother plant.
Somatic	Referring to vegetative or non-sexual part or process.
Somatic embryogenesis	In plant tissue culture, the process of embryo initiation and development from vegetative or non-gametic cells.
Somatic hybrid	The plant resulting from somatic hybridization.
Somatic hybridization	The creation of hybrids by fusion of protoplasts derived from genetically different cells.
Somatic tissue	All the tissues that form the body of a plant.
Stock plants	The plants from which cuttings or explants are taken for multiplication.
Stock solutions	Concentrated solutions from which portions are used to make media.
Subculture	With respect to plant tissue culture, this is the process by which the tissue or explant is first subdivided, then transferred into fresh culture medium (see also 'passage').
Substrain	A cell line derived from a strain by isolating a single cell or a group of cells having properties or markers not shared by all cells of the parent strain.
Suspension culture	A type of culture in which cells or cell aggregates are cultured in liquid medium.

Symbiosis — Two dissimilar organisms living together usually to mutual advantage.

Sympodial — A type of plant development in which the terminal bud of the stem stops growing due to either its abortion or its transformation into a flower or an inflorescence, and the uppermost lateral bud takes over the further axial growth of the stem. Among orchids this growth habit is shown by *Cattleya*, *Cymbidium*, *Dendrobium* and *Odontoglossum*, etc.

Synchronous culture — A culture in which a majority of cells are in the same phase of the cycle or the embryos are in the same stage of development.

Synthetic seeds — Somatic embryos encapsulated in hydrated or desiccated coating, which protects the embryo from mechanical damage during handling and allows their germination like sexual seeds. The capsule may also serve as an artificial endosperm.

Totipotency — Potentiality or property of a cell to produce a whole organism.

Transformation — See 'Genetic engineering'.

Transgenic plants — Plants which have been genetically engineered to modify certain characteristics by introducing one or a few selected genes.

Turbidostat — An open continuous culture into which fresh medium flows in response to an increase in the turbidity of the culture. A preselected biomass density is uniformly maintained by wash-out of excess cells.

Variant — A cell line or plant exhibiting a stable phenotypic change whether genetic or epigenetic in origin.

Vector — Vehicle for the transmission of genetic information from one cell or organism to another. The vectors usually selected are plasmids, although viruses or other bacteria may be used.

Viroid — Infectious agents smaller than viruses and composed only of RNA, with no coat protein, and causing plant diseases.

Virus — Any of a large group of submicroscopic agents infecting plants, animals, and bacteria and unable to reproduce outside the tissues of the host. A fully formed virus consists of nucleic acid (DNA or RNA) surrounded by a protein coat.

Virus-free — A plant certified through specified tests as being free of specified viruses.

Vitrification — A physical process by which a concentrated aqueous solution cooled to low or superlow temperature directly solidifies into an amorphous glassy state without crystallization; the term was earlier used to refer to hyperhydration.

Wild-type — The most frequently encountered phenotype in natural breeding populations.

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SUBJECT AND PLANT INDEX

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