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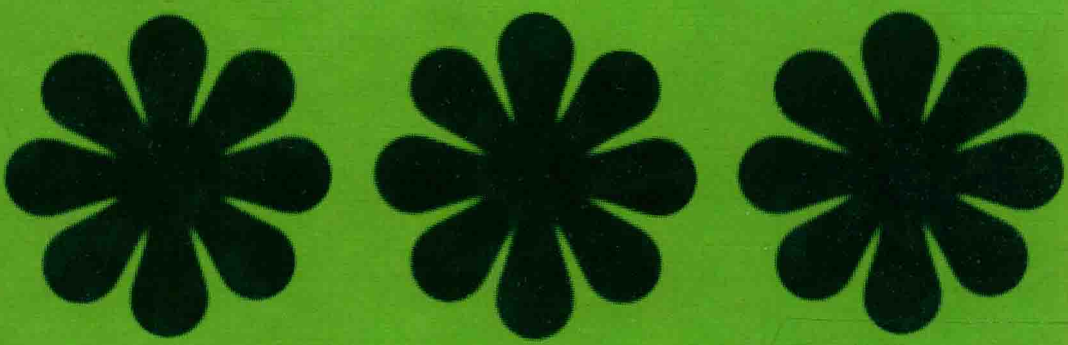

Plant Tissue Culture:

Applications and Limitations

S.S. Bhojwani (Editor)

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Applications and Limitations

edited by

S.S. Bhojwani

Department of Botany, University of Delhi, Delhi 110007, India

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INTRODUCTION

Plant tissue culture (PTC), an essential component of plant biotechnology, offers novel approaches to plant production, propagation, and preservation. During the past decade or so, major advances have been made in this field, and from being an art it has become an industrial technology. It is being used for large-scale multiplication of ornamentals and some fruit tree species. Feasibility of its applications in several other areas, such as the production of useful natural compounds (Chapter 11), generation of useful genetic variability (Chapter 13), and genetic transformation of crop plants (Chapter 15) has been demonstrated.

The publication of about 6000 papers and 100 books on PTC during the past five years (*see* Bhojwani et al., 1990, *Plant Tissue Culture, a Classified Bibliography 1985–1989*, Elsevier, Amsterdam) is a testimony to the popularity of this area of research. However, one ponders whether the progress in the field of PTC justifies this explosion of literature. In this context, Krikorian (1987) remarked that "the proponents of PTC and its followers have so effectively and so thoroughly carried out their communication and educational activities for quite a few years that many who were not directly involved in such work were led to believe that virtually all the problems had been solved and reduced as it were to the level of technology whatever that means. Part of the perception was naively generated by over-enthusiastic scientists simplifying their research activities for nonspecialized audience and part of it by ill-informed, albeit well meaning, bureaucrats seeking to justify their role as nominal advocates of the science."

Apparently, the real progress in the field of PTC is not commensurate with the number of papers published and the claims made therein. This is largely because many of the potentials of PTC projected from time to time could not be realised due to certain inherent problems. Over-emphasis of the positive results and exaggerated claims in the literature have overshadowed the real problems which must be addressed by the researchers if plant biotechnology has to find its rightful place. Therefore, at this critical stage in the progress of plant biotechnology it was considered appropriate to project a balanced and realistic picture of PTC.

Since most of the books published so far are largely concerned with the description of the various techniques of PTC, I had requested the contributors to this book to limit the scope of their chapters to brief description of the recent developments, a critical assessment of the proven and potential applications, highlighting the current problems limiting the application of PTC techniques, and projection of the trend of research in the twenty-first century. I feel that most of the chapters are in line with the theme of the book, i.e. applications and limitations.

Plant regeneration in tissue cultures *via* organogenesis (Chapter 2) or somatic embryogenesis (Chapters 3–5), which is a critical requirement to realise the full potential of the various techniques of plant biotechnology to crop improvement, has been applied so far without understanding these processes. This often makes

a researcher reach a helpless dead-end like a computer operator whose partial understanding of the operation may lead him to a dead-lock position. Somatic embryogenesis, which is considered as the future method of commercial micropropagation (Chapters 4, 5), is beset with numerous problems. The formation of somatic embryos by a cell/tissue system is largely a chance happening. Moreover, a large proportion of the somatic embryos are structurally and/or physiologically abnormal and, therefore, incapable of germination. The involvement of a callus phase endangers the genetic fidelity of the plants produced through somatic embryogenesis (Chapter 12). The projected application of artificial seeds hinges on the solutions to these problems associated with somatic embryogenesis (Chapter 5). Unfortunately, the entire subject of spontaneous occurrence of in vitro variations, being utilized for crop improvement (Chapter 13), lacks scientific explanation and, consequently, reliable controls. This continues to be a serious impediment not only in clonal propagation of selected genotypes (Chapters 7, 8) but also in the production of industrial compounds by cultured cells (Chapter 11) and improvement of cultivars through cell fusion (Chapter 14) or genetic transformation (Chapter 15). The chapters on organogenic differentiation (Chapter 3), somatic embryogenesis (Chapter 4) and cytogenetics of cell cultures (Chapter 12) clearly suggest that the future of PTC applications lies in understanding the cellular basis of differentiation. Identification of genes concerned with different morphogenic expressions and their exploitation may also contribute to achieving reproducible regeneration in tissue cultures, including hitherto recalcitrant systems (Chapter 6). The feasibility of genetic engineering to modify the genome of higher plants by selective gene transfer has been well established but the availability of desirable genes continues to be a serious limitation (Chapter 15).

The achievements with tree tissue culture (Chapter 8) are still of academic nature and much remains to be achieved before these species can be considered for commercial micropropagation. Even the micropropagation of ornamentals, which forms a well established industry, requires further scientific and management inputs (Chapter 7).

Production of industrial compounds by plants depends on the nature and age of the tissue. Dedifferentiation of explant tissues in cultures acts against this basic requirement. The production of natural compounds by PTC is further complicated by the genetic instability of cell cultures. In the successful story of shikonin production (Chapter 11) this problem was offset by periodic selection of productive lines on the basis of their bright red colour. This may be possible in some other cases, such as berberine production (fluorescent yellow), but may not apply to most other situations.

Besides critical reviews on the subjects referred to above, this book includes an overview of the current status of PTC (Chapter 1), well established (Chapter 9) and emerging (Chapter 10) techniques of haploid production, genetic manipulation of plants by protoplast fusion (Chapter 14), transformation (Chapter 15) and embryo culture (Chapter 16), and in vitro conservation of germplasm (Chapter 17). The article on expectations of plant breeders (Chapter

18) is followed by the concluding chapter (19) projecting the possible trends of research in PTC in the twenty-first century.

It is hoped that the scientists in the field of plant biotechnology will find this book useful as it identifies the areas of the various PTC techniques which require immediate research inputs. For the teachers and students of plant sciences the book updates (with literature up to 1989 and sometimes 1990) the progress in the field of PTC.

I take this opportunity to appeal to the science administrators to relieve scientists of the pressure which often compels them to make tall claims extrapolated from very preliminary studies, and encourage more basic research in the bottleneck areas of PTC. On the part of the scientists, it would be desirable to change the current trend of literature proliferation (Bhojwani et al., 1990), which is caused by unnecessary, repetitive publication of papers, and the positive claims in research papers should be accompanied by information on the reproducibility of the results and a clear indication of the associated problems experienced, if any.

I would like to thank most sincerely all the contributors to this book for their co-operation in submitting camera-ready manuscripts of their chapters within the prescribed time limit. The help of several of my students – Prem, Arumugam, Madhu, Sanjay Saxena, Pradeep, Sanjay Mahendru, Anoop and Lokesh – in various ways during the preparation of this book for publication is gratefully acknowledged. I would also like to thank Messrs R.K. Gupta, Ashok Gulati and Dharam Pal for typing some portions of the book.

Finally, I dedicate this book to my wife – Shaku Bhojwani – for her patience and understanding throughout my involvement with writing books, since 1971.

SANT SARAN BHOJWANI
Delhi, India
15 July 1990

CONTENTS

Chapter 1

THE CURRENT STATUS OF PLANT TISSUE CULTURE

TREVOR A. THORPE
Plant Physiology Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4 (Canada)

1 INTRODUCTION

Today plant tissue culture or the aseptic culture of cells, tissues and organs, is an important tool in both basic and applied studies, as well as in commercial application. The technology owes its origin to the ideas of the German plant physiologist, Haberlandt, who in his famous address to the German Academy in 1902 suggested it should be possible to cultivate artificial embryos from vegetative cells (see Krikorian and Berquam 1969 for English translation). He introduced the concept of totipotency, viz, that all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant.

Although Haberlandt was not successful in proving his ideas, his early studies, together with those of his students and contemporaries (see Gautheret, 1985), culminated in the successful and indefinite culture of excised roots of tomato (White, 1934). Further studies led to the independent demonstrations by Gautheret (1939), Nobécourt (1939) and White (1939) that cells in culture can be made to proliferate continuously and also undergo differentiation. These findings set the stage for the large increase in research undertaken during the 1940s, 1950s and 1960s. From this period, advances such as the eradication of viruses through meristem culture (Morel and Martin, 1952), the cultivation of single cells and suspension cultures (Miur et al., 1954), the auxin-cytokinin basis of organogensis (Skoog and Miller, 1957), somatic embryogenesis (Reinert, 1958), the large scale culture of cells (Tuleke and Nickell, 1959), large scale production of protoplasts (Cocking, 1960), anther culture (Guha and Maheswari, 1964), the regeneration of plants from single cells (Vasil and Hildebrandt, 1965), the uptake of DNA by plant cells (Ledoux, 1965), and the variability of cells in culture (Lutz, 1969) were made.

Research in and application of tissue culture technology can be divided conveniently into five broad areas, namely: (a) cell behavior, (b) plant modification and improvement, (c) pathogen-free plants and germplasm storage, (d) clonal propagation, and (e) product formation. The developments which have taken place during the last two decades in these areas will be

reviewed briefly in this chapter. In some areas the advances have been spectacular, while in others only modest progress has been made or the potential remains unrealized.

2 CELL BEHAVIOR

Under this general heading, studies on topics such as the cytology, nutrition, primary and secondary metabolism of cells in culture, as well as morphogenesis and pathology have been carried out.

2.1 Cytology

Studies on the structure and physiology of quiescent cells used as explants, changes in cell structure associated with the induction of division in these explants and the characteristics of developing callus have been carried out using light and electron microscopy (Yeoman and Street, 1977; Lindsey and Yeoman, 1985). These studies have shown, inter alia, that the transformation of the structure of quiescent cells largely reflects changes in the metabolism of the cells, which are detectable within 1 hr of excision. One early anatomical change is dedifferentiation, in which a wound cambium is established and the average cell size decreases. The wide variation of cell shape, form and structure, and patterns of cell division have also been revealed by examining cell suspensions. It is now clear that structural and biochemical heterogeneity exists at every stage of development of a cell culture (Lindsey and Yeoman, 1985). Current research with plant cells and protoplasts is making a significant contribution to the field of plant cell biology (Fowke, 1986, 1987). Examples of this progress include the discovery and exploration of the coated vesicle-mediated endocytotic pathway, the isolation of the larger membrane-bound cell organelles for studies of their structure and function, and the examination of the distribution and role of the plant cytoskeleton, particularly the distribution of microtubules and their role in cell wall formation and cell shaping. Nuclear cytology has also shown that endoreduplication, endomitosis and nuclear fragmentation are common features of cultured cells (D'Amato, 1978; Nagl et al., 1985). These chromosomal changes lead to polyploidy and aneuploidy, and often to loss of morphogenic capacity.

2.2 Nutrition

Nutrition was the earliest aspect of plant tissue culture investigated (Street, 1969). The development of a high salt medium (Murashige and Skoog, 1962) and derivatives therefrom (see Gamborg et al., 1976) has allowed for the culture of a variety of plant species. In contrast to animal cell culture

work, plants can generally be cultured on defined media, containing 5 classes of substances, namely inorganic macro- and micro-nutrients, a carbon/energy source, a reduced form of nitrogen, some B-vitamins, and phytohormones to achieve growth (Aitchison et al., 1977; King and Street 1977; Ozias-Akins and Vasil, 1985) and organized development (Phillips, 1980; Thorpe, 1980; Evans et al., 1981; Christianson, 1987). Only rarely is it necessary to add such complex addenda as coconut water.

In most cases, a comparative study using different mineral salt formulations at different dilutions has been sufficient to select a basal medium, which can then be used to optimize other factors like phytohormones, and culture environmental conditions. Progress is also being made with photoautotrophic cell cultures, which require no exogenous carbon/energy source for growth and differentiation (Yamada et al., 1978). These cultures are able to develop good photosynthetic rates (Hüsemann, 1985; Neumann and Bender, 1987), but often at elevated CO_2 levels. Unfortunately to date, such cultures can only be developed in C_3 plants. Finally, there are still some recalcitrant species, such as explants from mature conifers and some small-seeded legumes, which cannot yet be manipulated easily in culture.

2.3 Metabolism

(i) Primary metabolism. In vitro cultures, particularly cell suspension cultures, have proven superior to tissue slices in the study of many aspects of metabolism (Neumann et al., 1985; Dougall, 1987). As indicated earlier, large amounts of viable organelles, such as nuclei (Saxena et al., 1985) and vacuoles (Marty et al., 1980; Leonard and Rayder, 1985) can be obtained from protoplasts. Studies with such vacuoles have demonstrated the presence of hydrolytic enzymes which may have a lysosomal function. Vacuoles have also been used to study transport, storage and turnover of metabolites (Leonard and Rayder, 1985). In primary metabolism, such studies as the regulation of inorganic nitrogen and sulfur assimilation (Filner, 1978), carbohydrate metabolism (Fowler, 1978), and photosynthetic carbon metabolism (Bender et al., 1985; Herzbeck and Hüsemann, 1985) clearly show the importance of the cell culture approach for elucidating pathway activity. In addition, the application of newer approaches such as NMR is allowing for a dynamic examination of primary metabolism in cultured tissues (e.g., Thorpe et al., 1989).

(ii) Secondary metabolism. The use of cell and tissue cultures in studies of secondary metabolism has achieved great significance during the 1970s and 1980s. Much of this activity is related to the potential for producing commercial products (see later). However, since an understanding of cell

biochemistry is fundamental to all work on natural product synthesis, much useful basic information has been obtained (Barz et al., 1977; Neumann et al., 1985; Constabel and Vasil, 1987, 1988). One important finding is that there is a turnover and degradation in secondary metabolites (Barz and Nicolas, 1978; Barz and Koster, 1981, Eltayeb and Roddick, 1985). It seems as though compounds which appear first with many kinds of callus are those which are widespread in seed plants (e.g., phenolics and flavonoids), occur in cells without special structures, and which are biologically less complex (Constabel, 1987). Another important finding is that physical or chemical stresses can play important roles in the accumulation of phytochemicals in vitro. Recent evidence also suggests that the regulation of secondary metabolism is linked with induction of morphological differentiation in some cases (Fowler, 1985), thus stimulating an interest in the use of organ cultures. Cell cultures have also proven useful for the biotransformation of synthetic precursors. Some of the most elegant work done during this period involves the study of the regulation of phenylpropanoid (flavonoid and coumarin) metabolism, using parsely cell cultures (Hahlbrock and Grisebach, 1979; Hahlbrock and Scheel, 1989). The enzymes involved can be divided into two groups according to their coordinated regulation within each induction group, and the changes in enzyme protein synthesis have been shown to depend on regulation of mRNA transcription.

2.4 Morphogenesis

The origin of form (morphogenesis) is an area of research with which tissue culture has long been associated, and one in which tissue culture has made significant contributions both in terms of fundamental knowledge and application. Three aspects - xylogenesis, organogenesis and somatic embryogenesis - will be discussed.

(i) Xylogenesis. In vitro tracheary element formation has long been used to study the process of cytodifferentiation. A variety of experimental systems such as internodes, storage tissue, callus, suspension cultures, single cell cultures and protoplasts have been used (Roberts, 1976, Phillips, 1980, Fukuda and Komamine, 1985). Cytological and cytochemical changes during the process have been well described, and the role of microtubules clearly indicated. As well the importance of phytohormones, particularly auxin and cytokinin, in xylogenesis has been shown. It has also been determined that RNA and protein synthesis precede the process, and many biochemical changes associated with polysaccharide and lignin synthesis have been reported. One of the most significant advances was the optimization of the Zinnia mesophyll single cell system by Fukuda and Komamine (1980), which has allowed for some

definitive studies on the process (see Fukuda and Komamine, 1985). The timing and nature of the sequential events occurring during tracheary element cytodifferentiation have been elaborated. The process can be divided into four phases over a period of 96 hrs, but more importantly, does not require a prior cell division, a presumed requirement for differentiation. The induction mechanism of cytodifferentiation remains unclear, but work is progressing on the fundamental aspects of the topic.

(ii) Organogenesis. The classical findings of Skoog and Miller (1957) continue to be the guiding principles on in vitro organogenesis. A major question, first posed by Torrey (1966), was whether the exogenous phytohormones act directly on target cells to induce organogenesis or only indirectly by setting up conditions which allow some intrinsic cellular program to be initiated. Transformation of cells with appropriately modified T-DNA will allow for either shoot or root formation in a manner identical to the effects of the exogenous auxin and cytokinin (Schell et al., 1982; Schell, 1987). Furthermore, the exogenous phytohormones can reverse the T-DNA-induced morphogenesis, suggesting that indeed the phytohormones play a direct role in organized development.

In vitro organogenesis has been achieved in over 1000 plant species through empirical selection of the explant, the medium composition and control of the physical environment (Thorpe, 1980; Brown and Thorpe, 1986). Although progress is being made, the determinative events are not yet entirely known (Hicks, 1980; Christianson, 1987). In addition to traditional bulky explants such as cotyledons, hypocotyls and callus (Thorpe, 1980), thin (superficial) cell layers (Tran Thanh Van and Trinh, 1978; Tran Thanh Van, 1980) have been used. In all systems examined, the organogenic process begins with changes in a single or a small group of parenchyma cells, which then divide to produce a globular mass of cells or meristemoid, which is plastic and can give rise to either a shoot or root primordium (Hicks, 1980; Thorpe, 1980). These events can occur directly in the explant or indirectly after some callus formation. These structural changes are themselves a manifestation of preceding physiological, biochemical, biophysical and molecular events which reflect selective gene activity in those cells (Thorpe, 1980; Brown and Thorpe, 1986; Thompson and Thorpe, 1990). However, less information is available on these aspects. Work in my laboratory has indicated the importance of RNA and protein synthesis, and of carbohydrate and nitrogen metabolism during de novo organogenesis - a process which has high requirements for energy and reducing power (Thorpe, 1980, 1983, 1988a; Brown and Thorpe, 1986; Thompson and Thorpe, 1990). Carbohydrate also plays an osmotic role.

(iii) Embryogenesis. In contrast to organogenesis, which produces a unipolar shoot or root primordium, somatic embryogenesis gives rise to a bipolar structure with a root/shoot axis. Asexual embryogenesis has been reported in over 130 species, including cereals, grasses, legumes and conifers, hitherto very recalcitrant groups (Thorpe, 1988b). The process can be divided into two major phases: the induction of cells with embryogenic competence, and their subsequent development into embryos. Empirical manipulation of the explant, medium and culture environment has led to success in a process which is very plastic, and may be structurally and/or cellularly different from zygotic embryogenesis (Ammirato, 1983, 1987).

Carrot tissues have proven to be very useful in the study of somatic embryogenesis, but to date most of the physiological and biochemical studies have dealt with embryo development rather than the whole process (Thorpe, 1988b). However, the recent development of a method for selecting single cells, which form cell clusters and then somatic embryos in a relatively synchronous fashion (Normura and Komamine, 1985), is allowing for an in-depth examination of the entire process. The process up to the globular stage, which can be divided into 4 phases over a 12 day period, begins with a quantal cell division in the presence of auxin (Normura and Komamine 1986a, b). This is followed by the induction of polarities in DNA and RNA synthesis and Ca^{2+} and mRNA localizations. In the absence of auxin, the embryogenic cell clusters show active turnover of RNA and protein, and the synthesis of new species of mRNA and protein during the globular stage. Other studies at the biochemical and molecular biological levels (e.g., Sung and Okimoto, 1983; Choi et al., 1987; Thomas and Wilde, 1987) are also generating fundamental information on somatic embryogenesis.

2.5 Pathology

Cell culture is also making a positive contribution to the study of plant-microbe interaction and pathology. The use of in vitro culture to study tumorigenesis has long been established (Butcher, 1977). The recent explosion of research on Agrobacteria, although aimed mainly at plant improvement, is providing fundamental information in this area (Schell, 1987). Also, much progress is being made in understanding the biochemistry of virus multiplication (Rottier, 1978; Zaitlin and Hull, 1987) and phytotoxin action (Earle, 1978). Studies on disease resistance in plants, particularly as affected by phytoalexins, have also been carried out (Miller and Maxwell, 1983). Although it is not yet possible to readily grow pathogenic fungi in axenic culture (Dewan and Sivasithamparam, 1989), progress is being made through the use of dual cultures. These dual cultures of obligate biotrophs

and the callus tissues of their hosts have proven useful for the maintenance of supplies of aseptic inoculum, the cloning of isolates, the safe international transport of isolates, and for studies of host-parasite interaction (Ingram, 1980). Pathogenic fungi (Buczacki, 1980; Ingram, 1980), ecto- (Mason, 1980) and endo-mycorrhizae (Hepper and Mosse, 1980; Rhoades, 1983) and nematodes (Riedel et al., 1983) have been cultured in this way.

3 PLANT MODIFICATION AND IMPROVEMENT

In vitro methods are being used increasingly as an adjunct to traditional breeding methods for the modification and improvement of plants. These methods help breeders in two ways (Murashige, 1978). First, some techniques, like embryo, ovule and ovary cultures, aid in attaining traditional breeding objectives. Second, parasexual approaches involving protoplasts, can lead to the production of genotypes, that are not attainable by traditional methods. Cultures of isolated cells, anthers and microspores are useful to both.

3.1 In vitro fertilization and embryo culture

The technique of controlled in vitro pollination on the stigma, placenta or ovule can be used in several ways (Yeung et al., 1981; Zenkteler, 1984). These include the production of interspecific and intergeneric hybrids, overcoming sexual self-incompatability and the induction of haploid plants. To date, with the exception of maize (Gengenbach, 1977, 1984), not much work has been done with important agricultural crops. The potential of this approach appears great, but at present it is perhaps the most underexploited in vitro technology for plant improvement.

Embryo, ovary and ovule culture have been used in overcoming embryo inviability, monoploid production in barley, and in overcoming seed dormancy and related problems (Raghavan, 1980; Yeung et al., 1981). Embryo abortion is a common problem in breeding programs, due to failure of endosperm development. By aseptically culturing the embryo (or in some cases the ovary), this problem can be overcome. Interspecific and intergeneric hybrids of many important crops have been obtained by this embryo rescue approach (see Collins and Grosser, 1984). One novel use of embryo culture has been in the production of monoploids and doubled monoploids of barley. Using Hordeum vulgare as the female and H. bulbosum as the pollen parent, fertilization and initial embryo development is achieved (Kasha and Kao, 1970). However, the H. bulbosum chromosomes are all eliminated, giving rise to haploid H. vulgare embryos, which can then be doubled. This approach to barley breeding, which is being used worldwide, also works with hexaploid wheat.

The causes of seed dormancy are varied, but excision of the embryo is

often enough to allow germination, as in the case of the immature embryos of orchids (Yeung et al., 1981), which led to the first commercial application of tissue culture technology. Embryo culture has also proven useful in reducing the breeding cycles of new varieties, in cases where long dormancy and/or slow growth of the seedlings result in long breeding seasons. Finally, the technique has been used to test seed viability.

3.2 Haploidy

Natural haploidy has been known for a long time, but it was the studies on in vitro induction of androgenesis or the direct production of haploid embryos from anthers in the early 1960s, that led to renewed interest in this phenomenon. The interest in haploids stems largely from their considerable potential for plant breeding. Haploids may be used to facilitate the detection of mutations and recovery of unique recombinants, since there is no masking of recessive alleles (Bajaj, 1983; Sangwan and Sangwan-Norreel, 1987). Furthermore, doubling of the chromosome number of haploids offers a method for the rapid production of homozygous plants, which in turn can be used for producing inbred lines for hybrid production.

The requirements for successful androgenesis or gynogenesis must be determined empirically (Bajaj, 1983; Evans et al., 1984; San and Gelebart, 1986; Keller et al., 1987). Direct regeneration, rather than via a haploid callus is the usual route, but only a low percentage of the explants respond positively. At present, much more success has been achieved with androgenesis (Keller et al., 1987) than gynogenesis (San and Gelebart, 1986), perhaps due to the earlier success with the former. Nevertheless, the latter has allowed for haploid production in certain genera for which anther culture is not an effective technique. Androgenesis has been reported in some 171 species, of which many are important crop plants (Hu and Zeng, 1984), and gynogenesis in 15 species (San and Gelebart, 1986). Androgenic haploids have been integrated into breeding programs as homozygous diploid lines. In China, this method has led to the development of new varieties of rice, tobacco and winter wheat, and new breeding lines of maize and sugar cane (Hu and Zeng, 1984).

At present, there are some problems in the use of haploids. For example, regenerated plantlets cannot be obtained from some economically important plants (Hu and Zeng, 1984). In addition, the induction frequency of pollen-derived plants in some important crops like soybean and maize is low and some pollen-derived plants show genetic and chromosomal instability. Finally, there is a high degree of albinism in cereals (Bhojwani and Razdan, 1983).