Aust. J. Plant Physiol., 1990, **17**, 267–89

Current Applications of Tissue Culture in Plant Propagation and Improvement

M. K. Smith[A] *and R. A. Drew*[B]

[A] Queensland Department of Primary Industries, Maroochy Horticultural Research Station, P.O. Box 5083, S.C.M.C., Nambour, Qld 4560, Australia.
[B] Queensland Department of Primary Industries, Redlands Research Station, P.O. Box 327, Cleveland, Qld 4163, Australia.

Abstract

Plant tissue culture involves the culture of all types of plant cells, tissues and organs under aseptic conditions. This definition also extends to the culture of excised embryos and to protoplast culture. An overview of tissue culture techniques and their applications in plant propagation and genetic improvement of plants is presented. The areas under review include: (1) embyro culture, (2) meristem culture, (3) micropropagation, (4) somatic embryogenesis, (5) somaclonal variation, (6) *in vitro* selection, (7) anther culture and (8) protoplast culture. Problems and limitations of each of the techniques are also discussed. Examples are given of work that has been undertaken or that is currently in progress on the application of these techniques to the improvement of Queensland's subtropical horticultural industries. Key examples are: (1) embryo culture to facilitate incorporation of genes conferring disease-resistance from wild *Cucurbita* species into cultivated varieties, (2) meristem culture for virus elimination in strawberries (*Fragaria* × *ananassa*) and sweet potato (*Ipomoea batatas*), (3) micropropagation for rapid increase in new varieties of ginger (*Zingiber officinale*) and pineapple (*Ananas comosus*) to enable more rapid field evaluation and early release, (4) micropropagation of disease-free, genetically uniform planting material of superior female papaya (*Carica papaya*) selections and banana (*Musa* spp.) selections and (5) the use of somaclonal variation and gamma-irradiation for the genetic improvement of banana. Finally, future opportunities for the utilisation of tissue culture in plant propagation and improvement in Queensland's horticultural industries are summarised.

Introduction

Plant tissue culture has an important role to play in the manipulation of plants for improved agronomic performance. Plant tissue culture is an integral part of molecular approaches to plant improvement and acts as an intermediary whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells. The transformed plants that are regenerated in culture can then be evaluated by the geneticist and plant breeder (Fig. 1). Tissues of many plants species are difficult to culture and require optimisation of *in vitro* growing conditions. Therefore there continues to be an urgent need for extensive work in the field of basic tissue culture methods for many crop plants before any practical utilisation of molecular biology approaches in agriculture can be achieved.

Many papers in this issue deal with molecular approaches to study basic plant functions and for cultivar improvement. This review is intended to highlight those tissue culture techniques that are currently being applied to plant propagation and improvement. We wish to comment on the role of tissue culture and discuss the advantages and disadvantages of particular tissue culture techniques. Specific examples are given of work that has been

0310-7841/90/030267$03.00

undertaken, or that is currently in progress, on the application of these techniques to the improvement of Queensland's subtropical horticultural industries. While many of the techniques are well established, the examples provide tangible evidence of the role tissue culture has played and will continue to play in the development of agricultural industries.

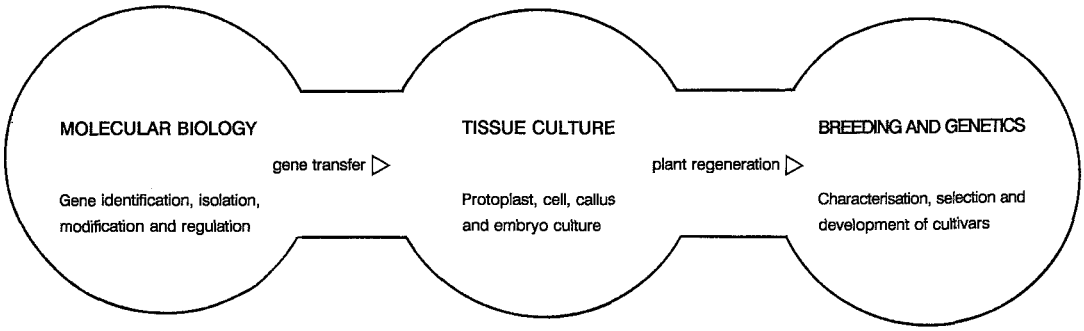

Fig. 1. The role of tissue culture for the genetic improvement of plants and its interaction with molecular biology and plant breeding.

Review of Plant Tissue Culture Techniques and their Applications

Plant tissue culture can be defined as the culture of all types of plant cells, tissues and organs under aseptic conditions. This definition also extends to the culture of excised embryos and to protoplast culture. There are many articles that discuss in detail the basic procedure and methods involved in plant culture (Bhojwani and Razdan 1983; George and Sherrington 1984; Mantell *et al.* 1985; Pierik 1987).

The relations between various plant tissue culture techniques are shown in Fig. 2. There is appreciable overlap between different techniques so that success in one research area is very much dependent on success in other areas (i.e. a system for reliably regenerating plants from callus cultures would appear to be a pre-requisite if plants are to be obtained from protoplasts or if successful gene transfer at the cell level is to be exploited at the whole plant level). The tissue culture techniques and applications reviewed include: (1) embryo culture, (2) meristem culture, (3) micropropagation, (4) somatic embryogenesis, (5) somaclonal variation, (6) *in vitro* selection, (7) anther culture and (8) protoplast culture (Table 1).

(1) *Embryo Culture*

Embryo culture was one of the first tissue culture techniques to be applied to plant breeding. It involves the removal of the embryo from the seed and subsequent growth *in vitro* until the developing plant can be transplanted to soil and grown to maturity. Usually, late-stage embryos are used for embryo culture but attempts have been made to culture early-stage embryos (i.e. globular and heart-shaped stages) and even unfertilised ovules (Tilton and Russell 1984). Late-stage embryos can usually be cultured on simple nutrient media but, as more immature embryos are cultured, the hormone and growth factor requirements become more specific and success is less frequent.

The purpose of embryo culture, in most applications, is to recover plants (embryos) during attempts at wide hybridisation by sexual crosses between distantly related plants (Williams *et al.* 1982). The incompatibility, in many cases, is caused by breakdown of the endosperm which nourishes the developing embryo. By 'rescuing' an embryo and growing it on an appropriate medium, a plant can be grown to maturity (Collins and Grosser 1984). The primary reason for attempting wide crosses is the transfer of desired traits (e.g. disease

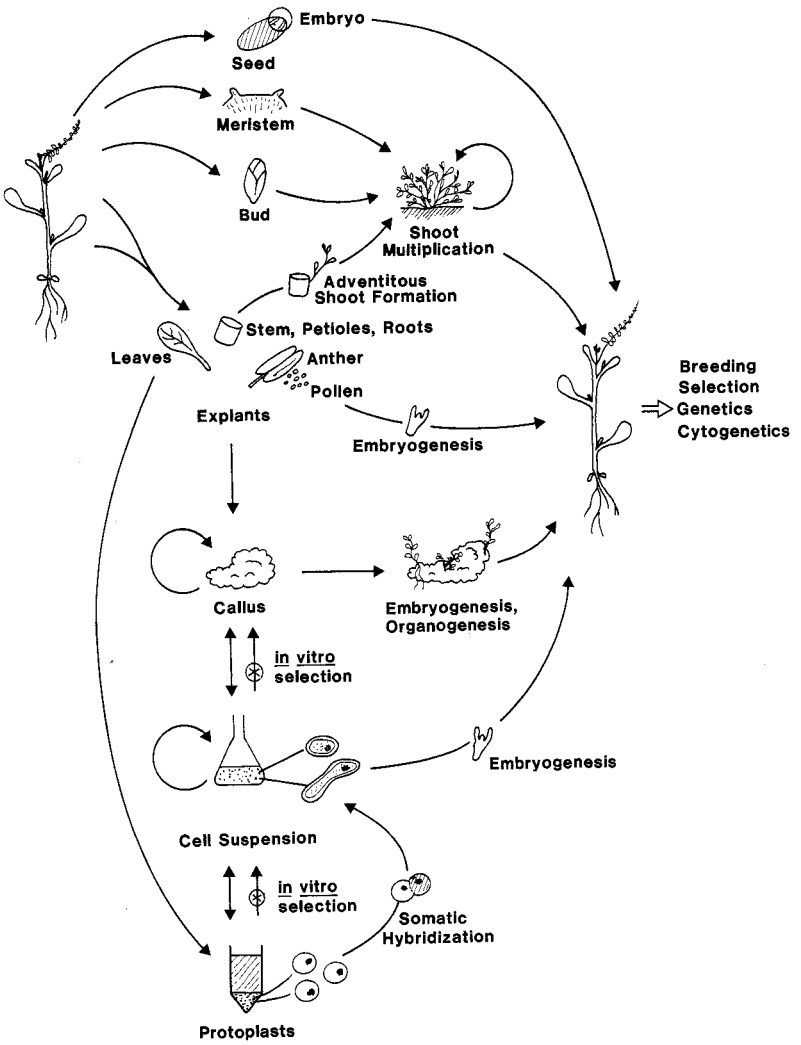

Fig. 2. Relationships between various plant tissue culture techniques.

resistance, stress tolerance) from distantly related species to cultivated varieties. Embryo culture has been applied successfully with interspecific crosses in cotton, tomato, barley, rice, cabbage, melons, beans and jute (Raghavan 1976). It has also been successful in some intergeneric crosses such as barley × rye, wheat × rye and wheat × *Elymus* (Raghavan 1976).

Embryo culture is also used to break dormancy in seeds, thereby shortening the breeding cycle by months or even years (Randolph 1945). It can also be used when important seed lots have lost viability during storage and have poor germination (Biggs *et al.* 1986).

The culture of unfertilised ovules and ovaries with subsequent fertilisation *in vitro* is another approach to the problem of wide hybridisation (Tilton and Russell 1984), although it is technically much more difficult than embryo culture. Success was first demonstrated with opium poppy (Kanta *et al.* 1962). With further understanding of the process of fertilisation in plants, and of the factors that influence incompatibility, these techniques may have greater application in the future.

Table 1. Summary of plant tissue culture techniques and their applications

Technique	Application	Comments and references
1 Embryo culture	To 'rescue' embryos during attempts at wide hydridisation by sexual crosses between distantly-related plants and culture them to maturity. To break dormancy in seeds.	a. Relatively easy to culture. b. Immediate application. c. Chances for success good but difficulty increases with more immature embryos. Hormone and growth factor requirements are more specific with early-stage embryos. d. Raghaven 1976; Williams *et al.* 1982; Collins and Grosser 1984.
2 Meristem culture	Elimination of diseases (particularly viral diseases) from plant propagating material.	a. Immediate application if plants amenable to tissue culture. b. Heat treatment/meristem culture does not ensure that the material has been freed of virus. Quarantine and virus indexing are still recommended to verify that the material is in fact disease-free. c. Quak 1977.
3 Micropropagation	Rapid propagation of a superior plant while maintaining the genotype. To maintain stock under controlled conditions. Germplasm storage.	a. Immediate application; however, the ease with which plants can be micropropagated varies from species to species and even certain genotypes within a species can prove to be more recalcitrant than others. In general, herbaceous species are more amenable to tissue culture techniques than woody perennials. b. Somaclonal variation can be a problem with some micropropagation techniques. Field evaluation of plants is required to verify trueness-to-type and to check for any genetic variants. c. Hussey 1978, 1983; Withers 1989.
4 Somatic embryogenesis	To rapidly increase desirable plants while maintaining the genotype of the original plant.	a. Involves regeneration from callus and cell suspensions and as such is more difficult to achieve than micropropagation. More research and development is needed to successfully develop the technique and a time frame that may involve years of trial and error with a recalcitrant species. b. Orbital shakers, centrifuges and microscopes are needed when working with cell suspensions. c. Because regeneration is from undifferentiated cells the chances of somaclonal variation increase. d. Narayanaswamy 1977.
5 Somaclonal variation	To induce desirable, heritable changes in regenerated plants.	a. Involves regeneration from callus and cell suspensions therefore the constraints and limitations are the same as those above.

		b. Not all changes are desirable; in fact most are deleterious or of no agronomic use. c. Not recommended where suitable genetic diversity is already present in a species; better application in vegetatively propagated material with a limited gene base. d. Screening the many thousands of plants for those with useful characters is expensive and time-consuming. If a selection pressure can be applied at the cellular level then better use can be made of somaclonal variation. See *in vitro* selection. e. Larkin and Scowcroft 1981; Evans 1989.
6 *In vitro* selection	To induce desirable, heritable changes in regenerated plants by subjecting a population of cells to a selection pressure.	a. Involves regeneration from callus and cell suspensions. Constraints and limitations as above. b. Important to have a reproducible system for the regeneration of large numbers of plants from unstressed cells as the selecting agent may lower the ability to regenerate plants. c. Important that tolerance to the stress operates at both the cellular and whole plant level so that there is a greater chance of recovering desirable plants. Unfortunately, many of the agriculturally important traits are multi-genic and depend on the structural and physiological integrity of the whole plant. d. Tomes and Swanson 1982; Chaleff 1983.
7 Anther culture	To produce homozygous, pure-breeding lines of plants for hybrid production and genetic studies. To improve the efficiency of *in vitro* selection.	a. Involves regeneration from callus, cell suspension and pollen. Constraints and limitations as above. b. Important to increase the frequency of regeneration and to be able to distinguish between plants regenerated from haploid somatic tissue found in the anther. c. The use of colchicine may be needed to double the chromosome number of haploid plants. d. Collins and Genovesi 1982; Hu and Zeng 1984.
8 Protoplast culture	To incorporate potentially useful genes from one plant species to another by fusion of protoplasts and regeneration from the hybrid cell line. Somatic hybridization. To transfer specific genes into protoplasts and regenerate transgenic plants.	a. Protoplasts are cells from which the cell wall has been removed either by mechanical and/or enzymatic methods. b. Orbital shakers, centrifuges and microscopes are needed. c. Regeneration of plants from protoplasts is generally very difficult to accomplish and a long lead time is often needed to develop the techniques with a particular species. d. Bhojwani *et al.* 1977; Binding 1986.

(2) *Meristem Culture*

Meristem culture was pioneered by Morel (1960) and usually involves the removal of the meristem with two or three leaf primordia and subsequent culture on a nutrient medium. The meristem is a dome of actively dividing cells, about 0·1 mm in diameter and 0·25 mm long. Endogenous contaminants do not easily invade or rapidly multiply in the meristem, often resulting in the formation of a disease-free plant. When combined with micropropagation techniques, large numbers of disease-free plants may be produced from meristematic explants.

Meristem culture has been used successfully in the elimination of viruses from plants (e.g. garlic, taro, strawberry, potato, sugarcane) (Quak 1977) and is now used routinely for the eradication of many viral diseases from plant material.

(3) *Micropropagation*

Micropropagation can be considered as an extension of the more traditional methods of plant propagation. Its aim is the rapid, clonal multiplication of superior genotypes of disease-free and pest-free plants.

Buds from a desired plant are placed on an appropriate culture medium under specific growth conditions to enhance production of axillary shoots. Subculture of the buds and shoots is repeated until many plants are produced all having the genetic characteristics of the original plant (Hussey 1978, 1983).

One of the principal applications of micropropagation is the mass propagation of superior plants. In many instances conventional propagation is a slow process during which disease and pest problems can limit production. Micropropagation offers the potential to produce thousands, or even millions, of plants per annum but is usually limited by the numbers that can be handled. In many cases the rapid increase serves as a valuable initial boost for the establishment of large populations prior to multiplication by conventional means (Damiano *et al.* 1983).

Micropropagation also provides a means of germplasm storage for maintenance of disease-free stock (Wilkins and Dodds 1983; Withers 1989). Space requirements are small and plants can be grown in a controlled environment free from the vagaries of weather, diseases and pests which constantly threaten field genebanks. Conditions can also be created in which the growth rate is slowed to minimise subculture requirements. When new stock is required, subculture can be resumed under optimal growth conditions. An extension of this approach has been the cryopreservation of meristems whereby germplasm may be stored indefinitely in liquid nitrogen (Kartha 1985).

(4) *Somatic Embryogenesis*

Somatic embryogenesis refers to the development of embryo-like structures from cells of somatic (non-sexual) origin. It is sometimes regarded as an advanced micropropagation technique but there are several advantages of recovery of plants from cells via somatic embryogenesis compared with micropropagation. Somatic embryos can be produced from cells growing in suspension, thereby making possible batch culture techniques which can be scaled-up with minimum handling costs. The multiplication rate in some plants (e.g. carrots, tobacco, potato, celery) (Narayanaswamy 1977) is very high and in the case of carrots, celery and tomato the embryos have been encapsulated and treated as artificial seeds (Ng 1986; Redenbaugh *et al.* 1987). Plant Genetics, Inc. has patented a process of producing synthetic seeds by encapsulating embryos in a biodegradable polymer (Gebhart 1985) and several of the large timber and paper companies in the USA are exploring the potential of somatic embryogenesis with conifers (Karnosky 1981).

Although callus production is relatively easy for most plant species, regeneration from unorganised cells, such as callus and cell suspensions, is usually a more difficult process. In addition, there are some important considerations involved with the use of somatic

embryogenesis for the rapid clonal multiplication of an important plant. There are numerous reports that show that regeneration from callus and cell suspensions may lead to genetic variation in regenerated plants. This variation from tissue cultured plants has been termed somaclonal variation (Larkin and Scowcroft 1981, see below). It is important, therefore, that more research be conducted to understand the molecular basis of the genetic changes produced during the artificial culture of cells and some caution must be exercised in the use of somatic embryogenesis as a cloning technique.

Somatic embryogenesis is preferred to organogenesis as a method for regenerating plants from cell culture, especially in conjunction with *in vitro* selection and anther culture. The main reason is that the whole plant develops from the somatic embryo and only requires growth to maturity. When organogenesis occurs, shoots or roots develop from callus and induction of the complementary structures frequently requires different culture requirements and media formulations. This can sometimes be achieved only with difficulty or not at all.

(5) *Somaclonal Variation*

The variation that occurs in a population of plants is of paramount importance in plant breeding and all of our cultivated crops were founded by exploiting the variation that exists in populations of plants to create suitable varieties and hybrids. The use of plant tissue culture to produce somaclonal variants is one means of generating variation that may be needed, and lacking, in a breeding program. This is particularly true in a species that is traditionally propagated asexually or for which only few cultivars are available.

Deliberate attempts to induce changes in plants by mutagens (Micke 1987) and changes in the ploidy by the use of colchicine (Burnham 1962) have been underway for the last 50 years. Utilising somaclonal variation follows on from mutation breeding approaches and involves the ability to change one or a few characters of an otherwise outstanding cultivar without altering the remaining, and often unique, part of the genotype.

Although most changes are deleterious or of no commercial value, there are some instances where somaclonal variation has produced agriculturally useful changes in the progeny [e.g. sugarcane—increases in cane and sugar yield, and resistance to eye-spot disease (Heinz *et al.* 1977); potato—improvement of tuber shape, colour and uniformity, and late blight resistance (Shepherd *et al.* 1980); tomato—increased solids, resistance to *Fusarium* race 2 (Evans 1989)]. Despite these successes we are unaware of any commercial cultivar releases from these programs at this time.

Stability of the altered phenotype is an important consideration. For instance, eye-spot disease resistance in sugarcane induced through somaclonal variation was lost after 10 years of asexual propagation (Maretzki 1987), indicating epigenetic variation. Stable genetic changes can also be induced through somaclonal variation and be inherited in a Mendelian fashion, as indicated from work with tomato (Evans and Bravo 1986). Studies such as these indicate that once new variants are identified they need to be rigorously field tested in replicated plots to ascertain genetic stability.

One of the limitations of mutation breeding is the number of individuals that must be examined before those with desirable characteristics are identified. If a selection pressure can be applied to a large population of somatic cells to uncover a desired phenotype (i.e. *in vitro* selection, see below) then the number of plants that eventually require field evaluation would be decreased and more direction built into a program.

(6) *In vitro Selection*

In vitro selection is a useful tool in identifying plants which are resistant or tolerant to stresses produced by phytotoxins from pathogens, herbicides, cold temperature, and aluminium, manganese and salt toxicity (Tomes and Swanson 1982; Chaleff 1983). *In vitro* selection usually involves subjecting a population of cells to a suitable selection pressure,

recovering any variant lines which have developed resistance or tolerance to the stress, and then regenerating plants from the selected cells. This approach presumes that tolerance operating at the unorganised cellular level can act, to some degree of effectiveness, in the whole plant. If the tolerance has a genetic basis then the trait can be transferred to other plants.

While straight-forward in theory there are a number of technical obstacles that must be overcome in producing new plants through *in vitro* selection. In the first instance it is important that there is a reproducible system for regenerating large numbers of plants from unstressed cells, as the selecting agent frequently reduces the ability of the cell to regenerate plants. Tolerance to the stress should operate at both the cellular and whole plant level so that there is a greater chance of recovering desirable plants. Unfortunately, many of the agriculturally important traits are multi-genic and depend on the structural and physiological integrity of the whole plant. Traditional breeding and selection, at the whole plant level, has been successful in incorporating many of these traits into cultivated varieties. *In vitro* selection will complement these traditional methods and should be of most use where no natural resistance is found within a species, or where conventional breeding practices are difficult within a species.

(7) *Anther Culture*

Much of the rapid progress in the field of microbial genetics can be attributed to the haploid nature of many micro-organisms, whereas in higher plants (which are diploid or polyploid) similar investigations have been encumbered by the problems of dominance and segregation. Natural haploids of higher plants are rare and restricted to a few species (e.g. tobacco, cotton, maize, rice) (Kimber and Riley 1963). Geneticists and plant breeders have therefore sought dependable methods for the production of haploid plants for the purpose of obtaining mutants at a much higher frequency than from diploids, and also for producing homozygous breeding lines after chromosome doubling.

Anther culture is one means of producing haploid plants (Maheshwari *et al.* 1980). The technique involves the removal of anthers and/or immature pollen from a plant and placing them on a suitable culture medium which will induce regeneration from the haploid tissue of the microspores or pollen. The culture of unfertilised ovules is another method of obtaining haploids (Zhu and Wu 1979). The chromosomes of the resultant plants are then doubled to the normal diploid state using colchicine. Chromosome doubling can also occur spontaneously.

Haploid cells are useful in *in vitro* selection schemes and have been used successfully to produce plants resistant to various metabolic inhibitors, environmental stresses, herbicides and phytopathotoxins (Chaleff 1983; Mazur and Falco 1989). Also, apart from fundamental genetic studies with homozygous and isogenic lines, homozygous plants are important in the production of F_1 hybrids which are of increasing importance in agriculture. Anther culture offers the possibility of obtaining homozygous plants in a matter of months rather than the years of inbreeding required by traditional breeding methods (Collins and Genovesi 1982).

Although the potential uses of haploids are apparent, the use of anther culture for the routine production of haploids has been limited. Most progress has been with the Solanaceous species (Hu and Zeng 1984) but with many other agriculturally important plants, such as cereals and legumes, anther culture has been more difficult. Even when production of haploids is possible, it is usually at a low frequency (0·1–1·0% of the anthers cultured) and requires a considerable investment in time and labour to generate the numbers needed for critical evaluation in a breeding program. Another difficulty lies in distinguishing plants that regenerate from diploid somatic tissue from spontaneously doubled haploids, although the use of pollen cultures circumvents this problem. In spite of these difficulties, progress is being made and the routine production of haploids from a wider range of species is increasing each year.

(8) *Protoplast Culture*

Protoplasts are cells from which the cell wall has been removed by mechanical and/or enzymatic methods. Because such cells lack a cell wall they are amenable to a number of techniques that are not possible with plant cell cultures (Carlson 1973; Bhojwani *et al.* 1977, Shepherd *et al.* 1983). Protoplasts have also been used in recombinant DNA research for manipulation of plant genes (Caplan *et al.* 1983). The techniques for gene transfer to protoplasts and a review of protoplasts in somatic hybridisation studies are presented elsewhere in this issue (Larkin *et al.* 1990; Rose *et al.* 1990). We only wish to say that regeneration of plants from protoplasts is generally more difficult to accomplish than from cell and callus cultures. This difficulty has limited the widespread application of protoplasts in somatic hybridisation and gene transfer studies. This has been particularly true with cereals, which have been one of the most recalcitrant group of crop plants to culture because of problems with developing a reproducible method of establishing cultures capable of regeneration (Ozias-Akins and Lorz 1984; Morrish *et al.* 1987). Nevertheless, successful regeneration of whole plants from protoplasts of an increasing number of plant species is possible (e.g. potatoes, carrots, tobacco, petunia) (Binding 1986) and the list of such plants can be expected to grow.

Examples of Tissue Culture Applications in Queensland's Horticultural Industries

(1) *Embryo Culture for Cucurbit Improvement*

In Queensland the potyvirus, papaya ringspot virus type W (PRV-W), formerly called watermelon mosaic virus type 1 (Purcifull *et al.* 1984), causes a serious viral disease of commercial cucurbit varieties. Production losses for cucurbits throughout the State are estimated to be $8·5 million annually in a total production of 135 000 t valued at $56 million. In order to reduce losses caused through deformed fruit and reduced yield it is desirable to incorporate genetic resistance to this disease into commercially important cultivars.

Most accessions of cultivated *Cucurbita* spp. lack resistance or tolerance to PRV-W (Provvidenti *et al.* 1978). By contrast the wild species, *C. ecuadorensis* Culter & Whitaker, is highly resistant to isolates of PRV-W in Australia as well as in the USA and France (Provvidenti *et al.* 1978; Herrington *et al.* 1988*b*).

As part of a cucurbit virus resistance breeding program (Herrington *et al.* 1988*a*, 1989), interspecific hybrids were produced between three species by hand pollination followed by embryo culture (Wall and York 1960). The three species include *C. maxima* Duch., cv. 'Queensland Blue', *C. moschata* Duch., cv. 'Butternut' and *C. ecuadorensis*.

Currently, embryos are removed from the seed and placed on a hormone-free Murashige and Skoog (1962) basal medium supplemented with 2% sucrose and solidified with 0·8% Difco Bacto-agar. The cultures are incubated at 25°C with a 16 h photoperiod and cool-white fluorescent tubes provide a photon flux density at the culture surface of 80 μmol m^{-2} s^{-1}. The use of embryo culture has been particularly useful in the cross *C. moschata* × *C. ecuadorensis* and subsequent backcross generations where the production of seed with well developed embryos is low to absent. Embryo culture has made it possible to obtain plants that otherwise would not have been recovered.

Progress has been made in the selection of PRV-W tolerant *C. moschata* and *C. maxima* phenotypes but a high level of resistance is lacking. Sufficient genetic variation exists in the breeding populations, however, to warrant continued selection for yield and reduced severity of symptoms on the fruit.

(2) *Meristem Culture for Virus Elimination*

Planting material for strawberry, *Fragaria* × *ananassa*, production in Queensland is administered by a State-regulated scheme and approximately 1·5 million runners are produced annually. The Department of Primary Industries is responsible for the maintenance

of disease-free mother stock and for inspection of runners during production and prior to sale. Meristem culture, combined with heat treatment, has played an important role in obtaining virus-free mother plants (Drew *et al.* 1986).

Strawberry plants of cv. 'Redlands Crimson' were heat treated for 30 days at 38°C with continuous light before transfer to a glasshouse where runner production was enhanced. Shoot tips were removed from the runners and surface sterilised in 1% sodium hypochlorite solution for 10 min followed by two rinses in sterile water. Meristems with 2–4 leaf primordia were aseptically removed and cultured on a modified Adams (1972) medium, containing 0·44 μM benzylaminopurine and 4·92 μM indole-3-butyric acid, and incubated at 25°C with an 18 h photoperiod. Yield of 6 clones of 'Redlands Crimson', free of mild yellow edge virus (MYEV), and one virus infected clone were monitored for a fruiting season. Removal of MYEV had no effect on fruit quality but uninfected clones had a 13% yield advantage of marketable fruit over the whole season. This increase occurred during the two fruiting flushes and was a result of more vigorous plants having the capacity to produce more fruit. Commercial plantings of clones free of MYEV over five seasons have been characterised by high early yields. George and Sherrington (1984) have reported other studies that show that yield, as well as fruit quality grades, in strawberries have been found to be markedly improved by using virus-free planting material rather than standard stock.

Meristem culture has also been used to remove virus from 12 imported cultivars of sweet potato, *Ipomoea batatas* (Drew 1980*a*) and demonstrated the importance of this technique for quarantine purposes. The ease of producing plants from meristem culture varied with cultivars with a yellow fleshed cv. 'Rajo Blanc' being the most difficult. A range of mineral combinations and hormone balances were tested for shoot and root growth *in vitro*. Optimum shoot growth from meristematic shoot tips was achieved with Murashige and Skoog (1962) medium containing 2·8 μM indole acetic acid and 4·4 μM benzylaminopurine or 9·3 μM kinetin. Best root initiation occurred when shoots were transferred to Murashige and Skoog medium containing 2·7 μM napthalene acetic acid with 4·6 μM kinetin.

(3) *Micropropagation for Rapid Increase of New Varieties for Early Release*

Micropropagation is of importance in the mass propagation of important new varieties; however, in many instances micropropagated plants may not be the preferred form of planting material. Pineapple and ginger are two crops traditionally propagated by vegetative means and the hardiness of conventional forms of planting material (slips and tops for pineapple, rhizomes for ginger) means that field establishment is much easier than with micropropagated material. Micropropagated plants are prone to desiccation and over-wetting of leaves can cause soft rots to develop. Both of these problems are related to poor cuticular development on leaves grown *in vitro* and micropropagated plants require special care during glasshouse establishment and hardening-off, which in turn adds to the already high cost of this material. Micropropagation therefore has found an important niche in the rapid initial propagation of material for establishment of field nursery areas. These nurseries then provide conventional propagation material for field production blocks.

(i) *Ginger (Zingiber officinale Rosc.)*

Ginger is grown in a small farming area in south-eastern Queensland. From a total area of approximately 150 ha, 5600 t of rhizomes are processed annually for an estimated value of $13·5 million.

The Buderim Ginger Growers Co-Operative obtained rhizomes from three overseas sources and plants were established *in vitro* and multiplied for early field evaluation. Sections of rhizomes bearing the growing points, 10 mm^3, were removed and surface sterilised in 3% sodium hypochlorite and rinsed three times in sterile water. Bleached material was pared and the explant embedded on Murashige and Skoog (1962) basal medium supplemented with 3% sucrose and 20 μM benzylaminopurine and solidified with 0·8% Difco Bacto-agar.

The cultures were incubated at 28°C with a 16 h photoperiod. Cool-white fluorescent tubes provided a photon flux density at the culture surface of 80 μmol m^{-2} s^{-1}. This medium was suitable for the multiplication of all three lines and multiplication rates of 4–5 per month were obtained. Micropropagation enabled the industry to make an assessment of the new material within a year from being released from Quarantine rather than the 3–4 years required by conventional propagation.

(ii) *Pineapple (Ananas comosus (L.) Merr.)*

Smooth Cayenne pineapples are grown for both processing and fresh markets. The main production areas (6500 ha) are in central and southern Queensland, where two crops are harvested over three years from each planting. About 105 000 t of pineapples are processed annually for a value of $35–40 million.

Fig. 3. A planting of 8100 plants of Hawaiian pineapple variety 'Champaga' (F180) produced *in vitro* by a commercial laboratory.

Micropropagation techniques have been developed for pineapple (Drew 1980*b*) and are now being used by commercial tissue culture laboratories for rapid release of new varieties (Fig. 3). Currently, multiplication is from axillary buds removed from slips or suckers and placed on differing media to promote establishment of the explant, single shoot growth, shoot proliferation and root initiation. Multiplication rates as high as 30 to 50 per month can be achieved *in vitro* on media containing 10 μM benzylaminopurine with 10 μM kinetin (Drew 1980*b*) compared with 4–5 per year with conventional propagation. However, the use of high levels of cytokinins in multiplication medium has been implicated in the induction of somaclonal variants (George and Sherrington 1984). Plantings from commercial laboratories using a multiplication rate of 4 per month have contained a maximum of 5% variants (predominantly rough-leaved forms) from 10 000 plants. This represents an acceptable level for nursery establishment considering the advantages in rapid release of a new variety.

Pineapple plants, in the first generation after culture, have also been characterised by large numbers of slips and suckers. This has been an advantage for further increases in planting material in the nursery blocks but is a disadvantage in a fruiting crop.

(4) *Micropropagation as a Source of Planting Material*

(i) *Papaya (Carica papaya L.)*

Papaya, or papaw as it is known in Australia, is grown in coastal districts of south-east and central Queensland and Innisfail in northern Queensland. From a total area of approximately 500 ha, 7000 t of fruit are produced annually for an estimated value of $7 million. Commercial plantations in Queensland consist of dioecious plants that are overplanted and then thinned at flowering to the required ratio of 1 male : 10 female trees which ensures maximum pollination and fruit set. Plants are grown from seed of open-pollinated flowers resulting in a mixture of genotypes with considerable variation in disease susceptibility and in fruit quality and yield. Variability within papaw lines remains one of the major problems facing the industry and hinders consumer acceptance of papaws as a fresh fruit. Micropropagation offers a technique for the production of uniform, superior female lines.

Micropropagation techniques are available for papaw (Litz 1984; Drew and Smith 1986) but three problems have limited the application of these techniques to commercial production. First, tissue culture of elite genotypes entails culture of buds from proven trees in the field. *In vitro* culture of mature buds is more difficult than culture of juvenile buds (Drew and Smith 1986; Rajeevan and Pandey 1986). The second limitation is the reported inability to sustain multiplication. Maintenance of proliferating cultures was lost after 8–13 subcultures (Litz and Conover 1981), and dominance in shoots could not be established after 12–13 subcultures on shoot proliferation medium (Rajeevan and Pandey 1986). Third, there is a high level (>80%) of bacterial contamination in bud explants from field-grown trees (Litz and Conover 1978), thus most cultures have to be discarded.

These problems have largely been overcome (Drew 1987, 1988; Drew and Miller 1989). Contamination problems have been minimised by pre-treatment of cuttings established from selected trees before axillary buds are removed for culture initiation. The stem apex is removed from the glasshouse-grown cuttings when the plants are approximately 30 cm high and a mixture containing 225 mg benzylaminopurine per litre of lanolin is applied to the cut surface. This treatment promotes rapid growth of 6–8 axillary branches, and buds removed from these new flushes of growth are less likely to be contaminated with endogenous bacteria. Considerable attention has been given to sustaining shoot growth and multiplication rates of cultures established from mature trees. The micropropagation procedure currently involves initiation and multiplication on DS (Drew and Smith 1986) medium, a modified DeFossard *et al.* (1974) medium, containing 1 μM benzylaminopurine and 0·25 μM naphthaleneacetic acid, 2% sucrose and 0·8% Dicfo Bacto-agar. Axillary shoot growth deteriorates only if the plantlets are not subcultured every 2–3 weeks or if they are continually subcultured on medium containing benzylaminopurine. For this reason plantlets are alternatively subcultured to hormone-free basal medium which also promotes the formation of apically dominant shoots. Apically dominant shoots produce roots on half-strength DS medium with 10 μM indole-3-butyric acid when incubated at 27°C with 12 h days.

During the last few seasons we have evaluated micropropagated papaw selections in the field. Field plants have been characterised by excessive vegetation at the lower nodes and a reduced juvenile stage (Fig. 4). Young trees have grown vigorously and set flowers and fruit at 30–40 cm above ground level. Seedling dioecious papaws are currently planted in autumn in south-eastern Queensland and have a juvenile section of 1·0–1·5 m before flowering. This early flowering on micropropagated trees, when planted in spring, may extend the harvest period to three seasons, compared with the current two seasons. An unexpected advantage with micropropagated plants was the development of strong root

systems, which have been superior to those of seedling controls, and give the plants more vigour and resistance to strong winds. This may be a consequence of the strong root system produced *in vitro* and influenced by the presence of indole-3-butryic acid in the culture medium. An obvious advantage has been the production of superior female lines, which eliminates the requirement for thinning, and uniformity of plants and fruit have been good.

Because micropropagation has the potential to produce high yielding clones with superior fruit quality, it could be used as a continuing source of planting material with trees being replanted every 2–3 years.

Fig. 4. Micropropagated papaya tree at Redlands Research Station from a clone established from mature tissue. Tree planted in December, set flowers in January and had a markedly reduced juvenile phase. Bar = 0·4 m.

(ii) *Banana (Musa spp.)*

Bananas are one of the major fruit crops grown in Queensland. The banana industry occupies an area of 6000 ha and has an annual production valued in excess of $115 million. Major production areas are located in northern Queensland and on frost-free hillside slopes in south-eastern Queensland. Cavendish (AAA) varieties ('Williams' and 'Mons Mari') account for 90% of the production, while 'Lady Finger', an AAB type, is the only non-Cavendish cultivar with significant commercial production.

During the 1986–87 season 15% of all new banana plantings in northern Queensland, approximately 80 ha, were planted with micropropagated material produced from three commercial tissue culture laboratories. Micropropagated bananas were promoted as having four important advantages: (1) plants could be rapidly multiplied from a mother plant with superior characteristics, (2) micropropagated plants could be provided to the grower free of important banana diseases and pests, (3) the material produced would be true-to-type and conform with the characteristics of the mother plant and (4) 100% establishment could be achieved easily.

While some 150 000 plants were produced for growers in northern Queensland, few published reports of the growth and performance of micropropagated bananas were

available. Indeed the demand for micropropagated planting material at that time outpaced research into the evaluation of micropropagated bananas that would have identified some of the potential problems that eventually arose.

Banana cultures are typically initiated from sword suckers approximately 0·4–1·0 m in height. Sections of material (20 mm × 30 mm) containing the shoot tip are removed and treated for 15 min in a 3·5% solution of sodium hypochlorite containing a few drops of Tween 80. Bleached material is removed aseptically and the explant is further sterilised for 5 min in a 1% solution of sodium hypochlorite containing Tween 80. Bleached material is again removed aseptically to produce an explant 5 mm^3 containing the shoot tip, ensheathing leaf bases and 2–3 mm of basal corm or stem tissue. Media for the growth and multiplication of banana cultures can vary from one research or commercial laboratory to another but we use a Murashige and Skoog (1962) basal medium supplemented with 10 μM benzylaminopurine, 2% sucrose and 0·8% Difco Bacto-agar. This medium supports rapid shoot multiplication and plants are subcultured every 6–8 weeks. Plants are subcultured to a hormone-free medium for root development before being hardened-off in the glasshouse.

Agronomically, micropropagated bananas are capable of performing as well as, or better than, plants derived from conventional planting material (Drew and Smith 1990). However, the most significant problem that arose and continues to prevent the widespread acceptance of micropropagated banana planting material is the presence of somaclonal variants, which are referred to as off-types in the industry. Plantings from commercial laboratories containing as high as 90% off-types have been reported (Walduck *et al.* 1988), with dwarfism being the most common off-type encountered. Dwarfs are prone to 'choke-throat', a physiological condition when the bunch fails to emerge fully from the plant, have a tendency to produce small, undersized fruit and the hands are more closely packed causing difficulty in dehanding.

Smith (1988) reviewed the factors contributing to the formation of off-types in micropropagated bananas and divided these into intrinsic factors and culture-induced factors. Genetic changes induced during micropropagation can be influenced by choice of explant, the composition of culture media (particularly the nature and concentration of phytohormones), number of subcultures or length of time in culture, and the level of tissue organisation during culture (i.e. axillary buds more stable than adventitious buds). Intrinsic factors include the genetic stability of the cultivar or genotype being micropropagated. Therefore while little can be done about intrinsic factors, laboratories can exercise greater control over culture-induced factors.

Tissue culture practices designed to minimise somaclonal variation are being adopted by commercial laboratories and we are currently involved in developing reliable screening and selection techniques for early detection of off-types. Confidence in the redevelopment of a commercial banana micropropagation scheme will take time; however, the advantages outlined earlier, especially the disease-free and pest-free aspect of micropropagated material, are the driving forces behind industry acceptance.

(5) *The Use of Somaclonal Variation and Mutation Breeding for the Genetic Improvement of Banana*

Genetic improvement of bananas poses a paradoxical problem to the plant breeder. Most of the commercially important bananas are parthenocarpic triploids and, by their nature, sterile. Seedlessness of fruit is one characteristic traditionally associated with bananas and a trait that must be retained by the breeder. The difficulty associated with conventional banana breeding is best summed up by the fact, that in over 60 years of continuous breeding endeavours, no new banana cultivar that is commercially acceptable has been produced (Rowe 1984). In fact, bananas are one of very few crops in which only clones derived from natural somatic mutations are cultivated.

While somaclonal variation is detrimental from the viewpoint of rapid clonal propagation, several off-types have been found that may have potential agronomic value. One, from a

micropropagated Cavendish cultivar, 'Mons Mari', has extra-long finger length for all hands. Fruit is 2–3 cm longer than usual and this selection has the potential to boost profitability through sales of extra-large fruit. Another selection is a dwarf type with no obvious choke-throat problems with larger, more open hands and potentially higher wind resistance than 'Williams'. These plants are currently being multiplied for field evaluation in a number of sites in Queensland.

Potential also exists for the development of lines with improved levels of disease resistance. Plants are currently being evaluated from a mutation breeding program for resistance to Race 4 *Fusarium* Wilt (*Fusarium oxysporum* f. sp. *cubense*) (Smith *et al.* 1990).

The procedure involved the transfer of subcultured plantlets to Petri dishes containing a multiplication medium and incubation at 28°C and 16 h photoperiod for 7 days before exposure to gamma radiation from a ^{60}Co source. Dosage was calculated using a Fricke dosimeter (O'Donnell and Sangster 1970). The LD 50 for micropropagated 'Williams' was approximately 40 Gy; however, shoot multiplication and general vigour of plantlets was poor. The optimal dose range was 20 Gy. At this dosage visual changes were apparent and plant survival (73%) was sufficiently high to make the technique practical on a larger scale. Plants were subcultured for two to three cycles before disease screening. Screening trials involved: (1) the use of *Fusarium* culture filtrates *in vitro*, (2) root dip inoculations and (3) direct planting in a *Fusarium* infested field site in south-eastern Queensland. Any survivors from the first two screening trials were also tested in the field.

One of the greatest difficulties with the Race 4 program is the lack of a reliable screening technique that can operate *in vitro* or in the glasshouse for screening the large number of plants produced from mutation breeding. In our own glasshouse or growth cabinet screening programs, breakdown in resistance to races 1 and 2 in Cavendish clones is sometimes encountered and can be triggered by changes in temperature, light intensity, nature and concentration of inoculum and water stress. In addition, plants exhibiting an apparent resistant reaction *in vitro* or in the glasshouse often succumb to the disease in the field. This casts doubt on the relative effectiveness, and hence potential, in pursuing these selection techniques. Nevertheless, the benefits would be extremely great if a breakthrough was made and research is continuing. Screening of plants directly in the field is expensive and only small populations can be handled with the resources and manpower currently available. Ideally field screening of putative resistant mutants should be only of those plants which survive a screening sequence that may commence at the *in vitro* or glasshouse level.

Opportunities for Plant Propagation and Improvement in Queensland's Horticultural Industries Using Tissue Culture Techniques

Significant opportunities exist for the application of tissue culture in plant propagation and improvement. Tables 2 and 3 give an indication of the problems facing some of the horticultural industries in Queensland and the opportunities for resolving these problems using tissue culture techniques.

Micropropagation has an important role to play in clonal propagation of selected breeding lines or for multiplication of rootstocks. It is expected the biggest single gains in productivity of sub-tropical tree crops can be made via genetic uniformity of planting material. Embryo culture will continue to find application in breeding programs by bringing important characteristics from wild species to cultivated varieties. Anther culture is an area of increasing interest for many vegetable crops as attention focuses on the importance of F_1 hybrids and the ability of anther culture to rapidly produce homozygous breeding lines.

The relative ease by which techniques can be applied depends on the species or cultivars being assessed, as responsiveness in culture varies considerably. Herbaceous plants are less difficult than woody perennials. Juvenile tissues are less difficult than more mature tissues. Culture of organised structures such as buds and shoot meristems is less difficult than regeneration from cells or protoplasts. Despite these difficulties, progress is being made

Table 2. Opportunities for plant propagation and improvement in Queensland's vegetable industries using tissue culture techniques

Crop	Problem	Plant tissue culture resolution
1 Asparagus	Crop is dioecious and females are rogued because of inferior quality. A good male line with superior spear characteristics and yield is required.	a. Micropropagation of XY and/or YY (supermale) line for field planting. b. Micropropagation of selected XX and YY lines for production of F_1 hybrid seed (seed would be uniform XY for field planting). c. Anther culture to produce homozygous XX and YY lines to facilitate breeding and production of good F_1 hybrids.
2 Bamboo (edible)	Crop that has potential for the Asian market and one which lends itself to processing (i.e. canned product). Varietal selection is first needed to identify suitable clones for specific markets and then they must be rapidly multiplied.	a. Meristem culture may be required to free selected lines of viral diseases. b. Micropropagation of selected lines. Bamboo lends itself readily to this technique as it is typically propagated by vegetative means.
3 Brassicas	Maintenance of selected breeding lines by vegetative propagation is sometimes a problem. Brassicas suffer from inbreeding depression. Bud pollination is also necessary to overcome their natural self incompatibility (SI) system. Bud pollination is very laborious and the SI system is likely to breakdown with time or under adverse conditions.	a. Micropropagation of selected breeding lines. b. Anther culture to produce homozygous (inbred) lines for F_1 hybrid seed production. c. Fluorescence microscopy would be advantageous for studying the SI system in brassicas and enable strong SI parental lines to be determined.
4 Capsicum	Determination of trisomics and aneuploids to better define linkage groups. This would support research into identification of chromosomes carrying disease resistance and genes necessary for genetic improvement.	a. Cytological investigations required.

5 Carrot	Processing carrots are prone to a physiological disorder known as black ring. Some individual carrots do not show this characteristic and are distinguished by a domed appearance at the crown.	a. Somatic embryogenesis from selected lines. This research is currently in progress and second generation selection of seeded progeny from the original regenerates is underway. b. Regeneration from carrot cell cultures is a routine process and thus lends itself to a range of tissue culture applications. Currently *in vitro* selection for glyphosate resistant cell lines is in progress.
6 Cucurbits	Susceptible to watermelon mosaic virus (zucchini yellows and papaya ringspot viruses also cause problems). Wild species show resistance to these viral diseases.	a. Embryo culture of seeds obtained from wide crosses increases the recovery of resistant plants significantly. The program is currently in progress.
7 Garlic	Viral disease in parent stock.	a. Meristem culture for virus elimination and indexing of regenerated plants.
8 Lettuce	Susceptible to downy mildew but wild species have resistance.	a. Embryo culture of seeds obtained from wide crosses to develop more resistant varieties.
9 Melons	Susceptible to a race of *Fusarium* wilt (rock-melon). Wild species show resistance. Seedless types may find some market acceptance.	a. Embryo culture of seeds obtained from wide crosses to develop more resistant varieties. b. Colchicine treatment to produce fertile tetraploids. Diploid and tetraploid crosses then necessary to produce seedless triploid types.
10 Sweet potato	Viral disease in parent stock presents a quarantine risk.	a. Meristem culture for elimination of viruses and indexing of re-generated plants.
11 Tomato	Susceptible to *Fusarium* wilt (Race 3) and potato virus yellows. Wild species show resistance. Production of F_1 hybrids[A].	a. Embryo culture of seeds obtained from wide crosses to develop more resistant varieties. b. Anther culture to produce homozygous (inbred) lines for F_1 hybrid seed production[A].

[A] This applies to any vegetable crop where the production of F_1 hybrid seed is a desired goal.

Table 3. Opportunities for plant propagation and improvement in Queensland's fruit and nut industries using tissue culture techniques

Crop	Problem	Plant tissue culture resolution
1 Avocado	Conventional propagation difficult and costly. Uniformity of rootstocks and scion material would be of immense benefit to the industry.	a. Micropropagation of rootstocks and compatible scions. Clonal propagation of disease resistant rootstocks (e.g. *Phytophthora*-resistant) would be beneficial to the industry.
2 Banana	Conventional breeding in bananas is an expensive process as commercial cultivars are seedless. There is an urgent need, however, for clones which are resistant to race 4 *Fusarium oxysporum* f. sp. *cubense* (Panama disease) and *Mycosphaerella fijiensis* (Black Sigatoka).	a. Micropropagation of overseas cultivars and breeding lines that may show resistance to these diseases. In addition mutagenesis of select clones may produce lines with the desired resistance.
	Transmission of pests and diseases (e.g. nematodes, beetle borer, Panama disease and Bunchy Top disease) through conventional planting material.	b. Micropropagated bananas can be supplied disease free. However, a potential threat exists with virus diseases, particularly Bunchy Top. The virus responsible for Bunchy Top disease can be transmitted through micropropagated material and plants maintained in tissue culture show no symptoms of the disease until they are planted out.
	With the large numbers of tissue cultured plants produced for the industry, high numbers of off-types have occurred. These off-types are commercially unacceptable.	c. A satisfactory system for micropropagation of bananas is needed to reduce the incidence of off-types and suitable screening and selection techniques are needed to rouge off-types before they reach the field.
3 Cacao	Need for superior cultivars specially selected for various Queensland locations.	a. Micropropagation of selected lines.
4 Citrus	Need for selected dwarfing rootstocks and for seedless lines, particularly with mandarins which have good export potential.	a. Micropropagation of rootstocks.
		b. Regeneration of plants from triploid endosperm or by triploid embryo rescue for production of seedless lines.
	Conventional breeding practices are long term—any techniques that can speed-up the process would be beneficial.	c. Zygotic embryo rescue in embryonic lines would provide an increased number of female plants for plant breeding. Anther/ovule culture for production of haploid plants (with subsequent doubling) would also aid the breeder.
	Need for virus-free citrus budlines.	d. Meristem culture and micropropagation of virus-free budlines.
5 Coffee	Mechanical harvesting and clonal propagation of rust-resistant cultivars are the principal requirements for an economically viable industry.	a. Micropropagation of rust-resistant cultivars.
		b. Somaclonal variation/mutation breeding may produce useful genetic variants.

6	Custard apple	Conventional propagation difficult and costly. Uniformity of rootstocks and scion material would be of immense benefit to the industry.	a. Micropropagation of rootstocks and compatible scions.
7	Date	Need for superior cultivars specially selected for various Queensland locations.	a. Micropropagation of selected lines.
8	Ginger	Requirement for green ginger lines for the export market.	a. Micropropagation of selected lines for more rapid field and processing evaluation.
9	Grape	Requirement for virus elimination and rapid clonal multiplication for release of new and improved varieties, particularly subtropical varieties, for central Queensland.	a. Meristem culture. b. Micropropagation of selected lines.
10	Macadamia	Conventional propagation difficult and costly. Uniformity of rootstocks and scion material would be of immense benefit to the industry.	a. Micropropagation of rootstocks and compatible scions.
11	Mango	Conventional breeding practices are long-term—any techniques that speed-up the process would be beneficial.	a. Micropropagation of monoembryonic lines which do not reproduce true-to-type from seed. b. Zygotic embryo rescue in polyembryonic cultivars would be useful in understanding embryogenesis and as an aid in plant breeding.
12	Papaya	Variability from seedling lines is hindering the industry. Uniformity of planting material would be of immense benefit.	a. Micropropagation of selected female lines.
13	Passionfruit	The passionfruit industry has been devastated by Passionfruit Woodiness Virus and there is an urgent need for virus-free planting material. Conventional propagation is difficult and costly.	a. Meristem culture for virus elimination. b. Micropropagation of disease-resistant and cold-tolerant rootstocks.
14	Pineapple	Requirement to assess new and improved lines, particularly *Phytophthora* resistant lines.	a. Micropropagation of selected lines for more rapid field evaluation and release of new varieties. b. Assess the use of somaclonal variation and mutation breeding of important local accessions for *Phytophthora* resistance.
15	Pome fruit	Virus elimination from imported cultivars.	a. Meristem culture and micropropagation of selected lines.
16	Strawberry	Requirement for early release of new varieties that are certified disease (especially virus) free.	a. Meristem culture and micropropagation of selected lines.

and the list of plants amenable to tissue culture increases every year. Ultimately a more conceptual understanding of the genetics and developmental biology of regenerability is required.

Plant tissue culture offers exciting prospects for future improvements in crop productivity. Combined with the powerful techniques of plant molecular biology and integrated with well established plant breeding practices, these closer collaborative ties should strengthen our efforts in achieving a common goal.

Acknowledgments

Various funding authorities have allowed us to pursue the application of tissue culture for plant propagation and improvement. We gratefully acknowledge the support of Australian Centre for International Agricultural Research (ACIAR), Australian Special Rural Research Fund (ASRRF), Committee of Direction of Fruit Marketing (COD), International Board for Plant Genetic Resources (IBPGR), Queensland Banana Industry Protection Board, and Reserve Bank, Rural Credits Grant.

References

Adams, A. N. (1972). An improved medium for strawberry meristem culture. *Journal of Horticultural Science* **47**, 263–4.

Bhojwani, S. S., and Razdan, M. K. (1983). 'Plant Tissue Culture: Theory and Practice.' (Elsevier: Amsterdam.)

Bhojwani, S. S., Evans, P. K., and Cocking, E. C. (1977). Protoplast technology in relation to crop plants: progress and problems. *Euphytica* **26**, 343–60.

Biggs, B. J., Smith, M. K., and Scott, K. J. (1986). The use of embryo culture for the recovery of plants from cassava (*Manihot esculenta* Crantz) seeds. *Plant Cell, Tissue and Organ Culture* **6**, 229–34.

Binding, H. (1986). Regeneration from protoplasts. In 'Cell Culture and Somatic Cell Genetics'. (Ed. I. K. Vasil.) Vol. 3, pp. 259–74. (Academic Press: Orlando.)

Burnham, C. R. (1962). 'Discussions in Cytogenetics.' (Burgess Publishing Co.: Minneapolis.)

Caplan, A., Herrara-Estrella, L., Inze, D., Van Haute, E., Van Montagu, M., Schell, J., and Zambryski, P. (1983). Introduction of genetic material into plant cells. *Science* **222**, 815–21.

Carlson, P. S. (1973). The use of protoplasts for genetic research. *Proceedings of the National Academy of Sciences of the U.S.A.* **70**, 598–602.

Chaleff, R. S. (1983). Isolation of agronomically useful mutants from plant cell cultures. *Science* **219**, 676–82.

Collins, G. B., and Genovesi, A. D. (1982). Anther culture and its application to crop improvement. In 'Application of Plant Cell and Tissue Culture to Agriculture & Industry'. (Eds D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha and R. L. Peterson.) pp. 1–24. (The University of Guelph: Guelph.)

Collins, G. B., and Grosser, J. W. (1984). Culture of embryos. In 'Cell Culture and Somatic Cell Genetics of Plants'. (Ed. I. K. Vasil.) Vol. 1, pp. 241–57. (Academic Press: Orlando.)

Damiano, D., Faedi, W., and Cobianchi, D. (1983). Nursery runner plant production, fruiting and behaviour of micropropagated strawberry plants. *Acta Horticulturae* **131**, 193–9.

DeFossard, R. A., Myint, A., and Lee, E. C. M. (1974). A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum* L.) pith tissue callus. *Physiologia Plantarum* **31**, 125–30.

Drew, R. A. (1980*a*). Tissue culture in horticultural crops. *Queensland Agricultural Journal* **106**, 6–12.

Drew, R. A. (1980*b*). Pineapple tissue culture—unequalled for rapid multiplication. *Queensland Agricultural Journal* **106**, 447–51.

Drew, R. A. (1987). The effects of medium composition and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya* L.). *Journal of Horticultural Science* **62**, 551–6.

Drew, R. A. (1988). Rapid clonal propagation of papaya *in vitro* from mature field-grown trees. *HortScience* **23**, 609–11.

Drew, R. A., and Miller, R. M. (1989). Nutritional and cultural factors affecting rooting of papaya (*Carica papaya* L.) *in vitro*. *Journal of Horticultural Science* **64**, 767–73.

Drew, R. A., and Smith, N. G. (1986). Growth of apical and lateral buds of papaw (*Carica papaya* L.) as affected by nutritional and hormonal factors. *Journal of Horticultural Science* **61**, 535–43.

Drew, R. A., and Smith, M. K. (1990). Field evaluation of tissue cultured bananas in south-eastern Queensland. *Australian Journal of Experimental Agriculture* **30** (in press).

Drew, R. A., Herrington, M. E., Greber, R. S., and Duncalfe, F. (1986). Tissue culture and subsequent field evaluation of strawberry. *Queensland Journal of Agricultural and Animal Sciences* **43**, 91–6.

Evans, D. A. (1989). Somaclonal variation—genetic basis and breeding applications. *Trends in Genetics* **5**, 46–50.

Evans, D. A., and Bravo, J. E. (1986). Phenotypic and genotypic stability of tissue cultured plants. In 'Tissue Culture as a Plant Production System for Horticultural Crops'. (Eds R. H. Zimmerman, R. J. Griesbach, F. A. Hammerschlag and R. H. Lawson.) pp. 73–94. (Martinus Nijhoff: The Netherlands.)

Gebhart, F. (1985). Plant Genetics gets synthetic seed patent. *Genetic Engineering News* **5**, 18.

George, E. F., and Sherrington, P. D. (1984). 'Plant Propagation by Tissue Culture.' (Exegetics Ltd: Basingstoke, U.K.)

Heinz, D. L., Kirshnamurthi, M., Nickell, L. G., and Maretzki, A. (1977). Cell, tissue and organ culture in sugarcane improvement. In 'Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture'. (Eds J. Reinert and Y. P. S. Bajaj.) pp. 3–17. (Springer-Verlag: Berlin.)

Herrington, M. E., Greber, R. S., and Persley, D. M. (1988*a*). Response of progeny from the cross *Cucurbita moschata* × *C. ecuadorensis* to infection with papaya ringspot virus type W and watermelon mosaic virus type 2. *Queensland Journal of Agricultural and Animal Sciences* **45**, 151–6.

Herrington, M. E., Teakle, D. S., Greber, R. S., and Brown, P. J. (1988*b*). Inhibition of mechanical transmission of virus by sap, and infection of *Cucurbita ecuadorensis* with papaya ringspot virus type W. *Australian Journal of Experimental Agriculture* **28**, 651–4.

Herrington, M. E., Byth, D. E., Teakle, D. S., and Brown, P. J. (1989). Inheritance of resistance to papaya ringspot virus type W in hybrids between *Cucurbita ecuadorensis* and *C. maxima*. *Australian Journal of Experimental Agriculture* **29**, 253–9.

Hu, H., and Zeng, J. Z. (1984). Development of new varieties via anther culture. In 'Handbook of Plant Cell Culture, Volume 3, Crop Species'. (Eds P. V. Ammirato, D. A. Evans, W. R. Sharp and Y. Yamada.) pp. 65–90. (Macmillan Publishing Co.: New York.)

Hussey, G. (1978). The application of tissue culture to the vegatative propagation of plants. *Science Progress, Oxford* **65**, 185–208.

Hussey, G. (1983). *In vitro* propagation of horticultural and agricultural crops. In 'Plant Biotechnology'. (Eds S. H. Mantell and H. Smith.) pp. 111–38. (Cambridge University Press: Cambridge.)

Kanta, K., Rangaswamy, N. S., and Maheshwari, P. (1962). Test-tube fertilization in a flowering plant. *Nature* **194**, 1214–17.

Karnosky, D. F. (1981). Potential for forest tree improvement via tissue culture. *BioScience* **31**, 114–20.

Kartha, K. K. (1985). 'Cryopreservation of Plant Cells and Organs.' (CRC Press Inc.: Boca Raton.)

Kimber, G., and Riley, R. (1963). Haploid angiosperms. *Botanical Review* **29**, 480–531.

Larkin, P. J., Taylor, B. H., Gersmann, M., and Brettell, R. I. S. (1990). Direct gene transfer to protoplasts. *Australian Journal of Plant Physiology* **17**, 291–302.

Larkin, P. J., and Scowcroft, W. R. (1981). Somaclonal variation—a novel source of variability from cell culture for plant improvement. *Theoretical and Applied Genetics* **60**, 197–214.

Litz, R. E. (1984). Papaya. In 'Handbook of Plant Cell Culture. Vol. 2, Crop Species'. (Eds W. R. Sharp, D. A. Evans, P. V. Ammirato and Y. Yamada.) pp. 349–68. (Macmillan: New York.)

Litz, R. E., and Conover, R. A. (1978). *In vitro* propagation of papaya. *HortScience* **13**, 241–2.

Litz, R. E., and Conover, R. A. (1981). Effect of sex type, season, and other factors on *in vitro* establishment and culture of *Carica papaya* L. explants. *Journal of the American Society of Horticultural Science* **106**, 792–4.

Maheshwari, S. C., Tyagi, A. K., Malhotra, K., and Sopory, S. K. (1980). Induction of haploidy from pollen grains in angiosperms—the current status. *Theoretical and Applied Genetics* **58**, 193–206.

Mantell, S. H., Matthews, J. A., and McKee, P. A. (1985). 'Principles of Plant Biotechnology. An introduction to genetic engineering in plants.' (Blackwell Scientific Publications: Oxford.)

Maretzki, A. (1987). Tissue culture: Its prospects and problems. In 'Sugarcane Improvement Through Breeding'. (Ed. D. J. Heinz.) pp. 343–84. (Elsevier: Amsterdam.)

Mazur, B. J., and Falco, S. C. (1989). The development of herbicide resistant crops. *Annual Review of Plant Physiology* **40**, 441–70.

Micke, A., Donini, B., and Maluszynski, M. (1987). Induced mutations for crop improvement—a review. *Tropical Agriculture (Trinidad)* **64**, 259–78.

Morel, G. M. (1960). Producing virus-free cymbidiums. *American Orchid Society Bulletin* **29**, 495–7.
Morrish, F., Vasil, V., and Vasil, I. K. (1987). Developmental morphogenesis and genetic manipulation in tissue and cell cultures of Gramineae. *Advances in Genetics* **24**, 431–99.
Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–97.
Narayanaswamy, S. (1977). Regeneration of plants from tissue cultures. In 'Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture'. (Eds J. Reinert and Y. P. S. Bajaj.) pp. 179–248. (Springer-Verlag: Berlin.)
Ng, T. J. (1986). Use of tissue culture for micropropagation of vegetable crops. In 'Tissue Culture as a Plant Production System for Horticultural Crops'. (Eds R. H. Zimmerman, R. J. Griesbach, F. A. Hammerschlag and R. H. Lawson.) pp. 259–70. (Martinus Nijhoff: The Netherlands.)
O'Donnell, J. H., and Sangster, D. F. (1970). 'Principals of Radiation Chemistry.' (Edward Arnold: London.)
Ozias-Akins, P., and Lorz, H. (1984). Progress and limitations in the culture of cereal protoplasts. *Trends in Biotechnology* **2**, 119–23.
Pierik, R. L. M. (1987). '*In Vitro* Culture of Higher Plants.' (Martinus Nijhoff Publishers: Dordrecht.)
Provvidenti, R., Robinson, R. W., and Munger, H. M. (1978). Resistance in feral species to six viruses infecting *Cucurbita*. *Plant Disease Reporter* **62**, 326–9.
Purcifull, D. E., Edwardson, J., Hiebert, E., and Gonsalves, D. (1984). Papaya ringspot virus. Commonwealth Mycological Institute/Association of Applied Biologists. Descriptions of Plant Viruses No. 292 (No. 84 revised) 8 pp. (Commonwealth Mycological Institute: Kew, U.K.)
Quak, F. (1977). Meristem culture and virus-free plants. In 'Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture'. (Eds J. Reinert and Y. P. S. Bajaj.) pp. 598–615. (Springer-Verlag: Berlin.)
Raghavan, V. (1976). 'Experimental Embryogenesis in Vascular Plants.' (Academic Press: New York.)
Rajeevan, M. S., and Pandey, R. M. (1986). Lateral bud culture of papaya (*Carica papaya* L.) for clonal propagation. *Plant Cell, Tissue and Organ Culture* **6**, 181–8.
Randolph, L. F. (1945). Embryo culture of *Iris* seed. *Bulletin of the American Iris Society* **97**, 33–45.
Redenbaugh, K., Viss, P., Slade, D., and Fujii, J. A. (1987). Scale-up: artificial seeds. In 'Plant Tissue and Cell Culture'. (Eds C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer.) pp. 473–93. (Alan R. Liss, Inc.: New York.)
Rose, R. J., Thomas, M. R., and Fitter, J. T. (1990). The transfer of cytoplasmic and nuclear genomes by somatic hybridisation. *Australian Journal of Plant Physiology* **17**, 303–21.
Rowe, P. (1984). Breeding bananas and plantains. *Plant Breeding Review* **2**, 135–55.
Shepard, J. F., Bidney, D., and Shahin, E. (1980). Potato protoplasts in crop improvement. *Science* **208**, 17–24.
Shepard, J. F., Bidney, D., Barsby, T., and Kemble, R. (1983). Genetic transfer in plants through interspecific protoplast fusion. *Science* **219**, 683–8.
Smith, M. K. (1988). A review of factors influencing the genetic stability of micropropagated bananas. *Fruits* **43**, 219–23.
Smith, M. K., Hamill, S. D., Langdon, P. W., and Pegg, K. G. (1990). *In Vitro* mutation breeding for the development of bananas with resistance to race 4, *Fusarium* Wilt (*Fusarium oxysporum* f. sp. *cubense*). In '*In Vitro* Mutation Breeding of Bananas and Plantains'. Vienna, *IAEA-TECDOC* (in press).
Tilton, V. R., and Russell, H. (1984). Applications in *in vitro* pollination/fertilization technology. *BioScience* **34**, 239–42.
Tomes, D. T., and Swanson, E. B. (1982). Application of *in vitro* selection to plant improvement. In 'Application of Plant Cell and Tissue Culture to Agriculture & Industry'. (Eds D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha and R. L. Peterson.) pp. 25–43. (The University of Guelph: Guelph.)
Walduck, G., Daniells, J., and Gall, E. (1988). Results of survey of off-types in tissue cultured Cavendish bananas in north Queensland. *Bananatopics* **8**, 11–12.
Wall, J. R., and York, T. L. (1960). Gametic diversity as an aid to interspecific hybridization in *Phaseolus* and in *Cucurbita*. *American Society for Horticultural Science, Proceedings* **75**, 419–28.
Wilkins, C. P., and Dodds, J. H. (1983). The application of tissue culture to plant genetic conservation. *Science Progress, Oxford* **68**, 259–84.
Williams, E. G., Verry, I. M., and Williams, W. M. (1982). Use of embryo culture in interspecific hybridisation. In 'Plant Improvement and Somatic Cell Genetics'. (Eds I. K. Vasil, W. R. Scowcroft and K. J. Frey.) pp. 119–28. (Academic Press: New York.)

Withers, L. A. (1989). *In vitro* conservation and germplasm utilisation. In 'The Use of Plant Genetic Resources'. (Eds A. H. D. Brown, O. H. Frankel, D. R. Marshall and J. T. Williams.) pp. 309–34. (Cambridge University Press: Cambridge.)

Zhu, Z., and Wu, H. (1979). *In vitro* production of haploid plantlets from the unpollinated ovaries of *Triticum aestivum* and *Nicotiana tabacum*. *Acta Academia Sinica* **6**, 181–3.

Manuscript accepted 14 March 1990