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Crop improvement through tissue culture

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Plant tissue culture comprises a set of *in vitro* techniques, methods and strategies that are part of the group of technologies called plant biotechnology. Tissue culture has been exploited to create genetic variability from which crop plants can be improved, to improve the state of health of the planted material and to increase the number of desirable germplasms available to the plant breeder. Tissue-culture protocols are available for most crop species, although continued optimization is still required for many crops, especially cereals and woody plants. Tissue-culture techniques, in combination with molecular techniques, have been successfully used to incorporate specific traits through gene transfer. *In vitro* techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in the breeding lines, often via haploid production. Cell culture has also produced somaclonal and gametoclonal variants with crop-improvement potential. The culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars. Large-scale micropropagation laboratories are providing millions of plants for the commercial ornamental market and the agricultural, clonally-propagated crop market. With selected laboratory material typically taking one or two decades to reach the commercial market through plant breeding, this technology can be expected to have an ever increasing impact on crop improvement as we approach the new millenium.

Key words: Breeding, embryo culture, haploids, micropropagation, protoplasts, synthetic seed, transformation, wide hybridization.

Tissue-culture techniques are part of a large group of strategies and technologies, ranging through molecular genetics, recombinant DNA studies, genome characterization, gene-transfer techniques, aseptic growth of cells, tissues, organs, and *in vitro* regeneration of plants, that are considered to be plant biotechnologies. The use of the term biotechnology has become widespread recently but, in its most restricted sense, it refers to the molecular techniques used to modify the genetic composition of a host plant, i.e. genetic engineering. In its broadest sense, biotechnology can be described as the use of living organisms or biological processes to produce substances or processes useful to mankind and, in this sense, it is far from new. The products of plant breeding and the fermentation industries (e.g. cheese, wine and beer), for example, have been exploited for many centuries (Zhong *et al.* 1995). What is new and what has changed in the last two decades is the available technology (Davis & Reznikoff 1992). We no longer have to rely on pollination and cross-fertilization as the only ways to genetically modify plants. That the newer molecular and cellular technologies have yet to make a broad-based significant impact on crop production is not surprising since a plant-breeding process of 10 to 20 years duration is still required to refine a selected plant to the stage of cultivar release (Plucknett & Smith 1986; Kuckuck *et al.* 1991).

The applications of various tissue-culture approaches to crop improvement, through breeding, wide hybridization, haploidy, somaclonal variation and micropropagation, are the subjects of this review.

Plant Breeding and Biotechnology

Plant breeding can be conveniently separated into two activities (Kleese & Duvick 1980): manipulating genetic variability and plant evaluation. Historically, selection of plants was made by simply harvesting the seeds from those

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plants that performed best in the field. Controlled pollination of plants led to the realization that specific crosses could result in a new generation that performed better in the field than either of the parents or the progeny of subsequent generations, i.e. the expression of heterosis through hybrid vigour was observed. Because one of the two major activities in plant breeding is manipulating genetic variability, a key prerequisite to successful plant breeding is the availability of genetic diversity (Kuckuck *et al.* 1991; Villalobos & Engelmann 1995). It is in this area, creating genetic diversity and manipulating genetic variability, that biotechnology (including tissue-culture techniques) is having its most significant impact. In spite of the general lack of integration of most plant-biotechnology and plant-breeding programmes, field trials of transgenic plants have recently become much more common. There are therefore reasons to believe that we are on the verge of the revolution, in terms of the types and genetic make up of our crops, that has been predicted for more than a decade (Bodde 1982).

More than 50 different plant species have already been genetically modified, either by vector-dependent (e.g. *Agrobacterium*) or vector-independent (e.g. biolistic, micro-injection and liposome) methods (Sasson 1993; Anon. 1994). In almost all cases, some type of tissue-culture technology has been used to recover the modified cells or tissues. In fact, tissue-culture techniques have played a major role in the development of plant genetic engineering. For example, four of the seven papers listed by Davis & Reznikov (1992) as classic milestones in plant biotechnology used a range of protoplast, microspore, tissue and organ culture protocols. Tissue culture will continue to play a key role in the genetic-engineering process for the foreseeable future, especially in efficient gene transfer and transgenic plant recovery (Hinchee *et al.* 1994).

Wide Hybridization

A critical requirement for crop improvement is the introduction of new genetic material into the cultivated lines of interest, whether via single genes, through genetic engineering, or multiple genes, through conventional hybridization or tissue-culture techniques. During fertilization in angiosperms, pollen grains must reach the stigma of the host plant, germinate and produce a pollen tube. The pollen tube must penetrate the stigma and style and reach the ovule. The discharge of sperm within the female gametophyte triggers syngamy and the two sperm nuclei must then fuse with their respective partners. The egg nucleus and fusion nucleus then form a developing embryo and the nutritional endosperm, respectively (Tilton & Russel 1984; Zenkteler 1990). This process can be blocked at any number of stages, resulting in a functional barrier to hybridization and the blockage of gene transfer between the two plants.

Pre-zygotic barriers to hybridization (those occurring prior to fertilization), such as the failure of pollen to germinate or poor pollen-tube growth, may be overcome using *in vitro* fertilization (IVF; Yeung *et al.* 1981). Post-zygotic barriers (occurring after fertilization), such as lack of endosperm development, may be overcome by embryo, ovule or pod culture. Where fertilization cannot be induced by *in vitro* treatments, protoplast fusion has been successful in producing the desired hybrids (see below).

In vitro *Fertilization*

IVF has been used to facilitate both interspecific and intergeneric crosses, to overcome physiological-based self incompatibility and to produce hybrids. A wide range of plant species has been recovered through IVF via pollination of pistils and self- and cross-pollination of ovules (Yeung *et al.* 1981; Zenkteler 1990; Raghavan 1994). This range includes agricultural crops, such as tobacco, clover, corn, rice, cole, canola, poppy and cotton. The use of delayed pollination, distant hydridization, pollination with abortive or irradiated pollen, and physical and chemical treatment of the host ovary have been used to induce haploidy (Maheshwari & Rangaswamy 1965; Zenkteler 1984).

Embryo Culture

The most common reason for post-zygotic failure of wide hybridization is embryo abortion due to poor endosperm development. Embryo culture has been successful in overcoming this major barrier as well as solving the problems of low seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of a symbiotic partner, and the production of monoploids of barley (Raghavan 1980, 1994; Yeung *et al.* 1981; Collins & Grosser 1984; Zenkteler 1990). The breeding cycle of *Iris* was shortened from 2 to 3 years to a few months by employing embryo-rescue technology (Randolph 1945). A similar approach has worked with orchids and roses and is being applied to banana and *Colocasia* (Yeung *et al.* 1981). Interspecific and intergeneric hybrids of a number of agriculturally important crops have been successfully produced, including cotton, barley, tomato, rice, jute, *Hordeum* × *Secale*, *Triticum* × *Secale*, *Tripsacum* × *Zea* and some Brassicas (Collins & Grosser 1984; Palmer & Keller 1994; Zapata-Arias *et al.* 1995). At least seven Canadian barley cultivars (Mingo, Rodeo, Craig, Winthrop, Lester and TB891–6) have been produced out of material selected from doubled haploids originating through the widely-used *bulbosum* method of cross-pollination and embryo rescue (Kasha & Kao 1970; Choo *et al.* 1992). Mingo, in particular, was a breakthrough, as it was the first barley cultivar produced by this technique to be licensed, in 1980. Briefly, *Hordeum vulgare* ($2n = 14$) is pollinated with pollen from *H. bulbosum* ($2n = 14$). Normally, the seeds develop for about 10 days and then abort but, if the immature embryos are rescued and cultured

Table 1. Canadian tobacco crops and the estimated value of Delgold, a protoplast-fusion-derived tobacco cultivar, since Delgold's introduction in 1990.*

Year	Total tobacco crop		Delgold	
	Production (tonnes)	Value (US$)	Proportion of total crop (%)	Value (US$)
1990	56,000	175,000,000	1	1,750,000
1991	70,000	212,000,000	23	48,760,000
1992	59,000	190,000,000	33	62,700,000
1993	71,000	224,000,000	35	78,400,000
1994†	59,000	197,000,000	41	80,770,000

* Values are courtesy of R. Pandeya, the Ontario Flue-cured Tobacco Growers Marketing Board, and the Crop Insurance Commission of Ontario.

† A second, protoplast-derived cultivar, A.C. Chang, introduced in 1994, occupied about 1% of the crop acreage, with an estimated value of US$1,970,000.

on basal growth medium, plants can be recovered. The plants resulting from this cross-pollination/embryo rescue are haploids rather than hybrids and are the result of the systematic elimination of the *H. bulbosum* chromosomes (Kasha & Kao 1970; Ho & Kasha 1975). Haploid wheat has also been produced by this technique (Bajaj 1990).

Protoplast Fusion

Protoplast fusion has often been suggested as a means of developing unique hybrid plants which cannot be produced by conventional sexual hybridization. Protoplasts can be produced from many plants, including most crop species (Gamborg *et al.* 1981; Evans & Bravo 1983; Lal & Lal 1990; Feher & Dudits 1994). However, while any two plant protoplasts can be fused by chemical or physical means, production of unique somatic hybrid plants is limited by the ability to regenerate the fused product and sterility in the interspecific hybrids (Evans *et al.* 1984; Gleddie *et al.* 1986; Pandeya *et al.* 1986; Schieder & Kohn 1986; Evans & Bravo 1988) rather than the production of protoplasts. Perhaps the best example of the use of protoplasts to improve crop production is that of *Nicotiana*, where the somatic hybrid products of a chemical fusion of protoplasts have been used to modify the alkaloid and disease-resistant traits of commercial tobacco cultivars (Pandeya *et al.* 1986). Somatic hybrids were produced by fusing protoplasts, using a calcium-polyethylene glycol treatment, from a cell suspension of chlorophyll-deficient *N. rustica* with an albino mutant of *N. tabacum* (Douglas *et al.* 1981a, b). The wild *N. rustica* parent possessed the desirable traits of high alkaloid levels and resistance to black root rot. Fusion products were selected as bright green cell colonies, the colour being due to the genetic complemention for chlorophyll synthesis in the hybrid cells. Plants recovered by shoot organogenesis showed a wide range of leaf alkaloid content but had a high level of sterility. However, after three backcross generations to the cultivated *N. tabacum* parent, plant fertility was restored in the hybrid lines, although their alkaloid content and resistance to blue mould and black root rot were highly variable. Interestingly, neither parent was known to possess significant resistance to blue mould. Two commercial varieties, Delgold (Pandeya & White 1994) and AC Chang, have been released from the progeny of these protoplast fusion products (R. Pandeya, unpublished work) and are presently grown on approximately 42% of the flue-cured tobacco acreage in Ontario, Canada. This represents a value of approx. US$199,000,000 (Table 1).

Where mutant cell lines of donor plants are not available for use in a genetic complementation selection system, it has been demonstrated that mesophyll protoplasts from donor parents carrying transgenic antibiotic resistance can be used to produce fertile somatic hybrids selected by dual-antibiotic resistance (Sproule *et al.* 1991). The fusion of protoplasts from 6–azauracil-resistant cell lines of *Solanum melongena* (aubergine) with protoplasts from the wild species *S. sisymbriifolium* yielded hybrid, purple-pigmented cell colonies that underwent regeneration via organogenesis (Gleddie *et al.* 1986). As protoplasts from the parental cell suspension cultures could not be regenerated, hybrids could be screened by their 6–azauracil resistance, capacity to synthesize anthocyanins (purple pigment) and ability to undergo shoot organogenesis. The restoration of regeneration ability through complementation has also been observed in *Nicotiana* cell-fusion products (Douglas *et al.* 1981a; Gleddie *et al.* 1983). The hybrids resulting from this study were found to be resistant to root knot nematodes and spider mites, important agricultural traits. However, they were also completely sterile and could not be incorporated into an aubergine-breeding programme. Two possible ways of solving this sterility problem, 'back' fusions of somatic hybrids with the cultivated parents and initiation of suspension cultures of the hybrid cells so that more of the wild-species chromosomes can be eliminated, have so far been unsuccessful with these hybrids (S. Gleddie, unpublished work). Selection of hybrids and use of protoplast fusion for hybridization in crop plants has been reported in Brassicas, citrus, rice, carrot, canola, tomato, and the forage legumes alfalfa and clover (Akagi *et al.* 1989; Bajaj 1989; Tanno-Suenaga *et al.* 1988; Vardi *et al.* 1989; Kao *et al.* 1991).

Evans & Bravo (1988) have recommended that production of novel hybrids through protoplast fusion should focus on four areas: (1) agriculturally important traits; (2) achieving combinations that can only be accomplished by protoplast fusion; (3) somatic hybrids integrated into a conventional breeding programme; and (4) the extension of protoplast regeneration to a wider range of crop species. In the case of the above-mentioned example of *Nicotiana*, all of these criteria were met although this took 12 years from

the isolation of the fusion product in 1978 to the release of the first variety in 1990; this underlines the often overlooked fact that it takes 10 to 20 years to take initial research results to the stage of a recognized cultivar (Plucknett & Smith 1986; Kuckuck *et al.* 1991).

Haploids

Haploid plants have the gametophytic (one-half of the normal) number of chromosomes (Atanassov *et al.* 1995; Zapata-Arias *et al.* 1995). They are of interest to plant breeders because they allow the expression of simple recessive genetic traits or mutated recessive genes and because doubled haploids can be used immediately as homozygous breeding lines. The efficiency in producing homozygous breeding lines via doubled *in vitro*-produced haploids represents significant savings in both time and cost compared with other methods. Three *in vitro* methods have been used to generate haploids (Bajaj 1990): (1) culture of excised ovaries and ovules; (2) the *bulbosum* technique of embryo culture; and (3) culture of excised anthers and pollen. At least 171 plant species have been used to produce haploid plants by pollen, microspore and anther culture (Evans *et al.* 1984; Hu & Zeng 1984; Bajaj 1990). These include cereals (barley, maize, rice, rye, triticale and wheat), forage crops (alfalfa and clover), fruits (grape and strawberry), medicinal plants (*Digitalis* and *Hyoscyamus*), ornamentals (*Gerbera* and sunflower), oil seeds (canola and rape), trees (apple, litchi, poplar and rubber), plantation crops (cotton, sugar cane and tobacco), and vegetable crops (asparagus, brussels sprouts, cabbage, carrot, pepper, potato, sugar beet, sweet potato, tomato and wing bean). Haploid wheat cultivars, derived from anther culture, have been released in France and China (Bajaj 1990). Five to 7 years were saved producing inbred lines in a Chinese maize-breeding programme by using anther culture-derived haploids. A similar saving has been reported for triticale and the horticultural crop *Freesia*. In asparagus (Dore 1990), anther-derived haploids have been used to produce an all-male F_1 hybrid variety in France.

Somaclonal Variation

In addition to the variants/mutants (cell lines and plants) obtained as a result of the application of a selective agent in the presence or absence of a mutagen, many variants have been obtained through the tissue-culture cycle itself. These somaclonal variants, which are dependent on the natural variation in a population of cells, may be genetic or epigenetic, and are usually observed in the regenerated plantlets (Larkin & Scowcroft 1981). Somaclonal variation itself does not appear to be a simple phenomenon, and may reflect pre-existing cellular genetic differences or tissue-culture-induced variability. The variation may be generated through several types of nuclear chromosomal re-arrangements and losses, gene amplification or de-amplification, non-reciprocal mitotic recombination events, transposable element activation, apparent point mutations, or re-activation of silent genes in multigene families, as well as alterations in maternally inherited characteristics (Larkin *et al.* 1985; Scowcroft *et al.* 1987; Karp 1994).

Many of the changes observed in plants regenerated *in-vitro* have potential agricultural and horticultural significance. These include alterations in plant pigmentation, seed yield, plant vigour and size, leaf and flower morphology, essential oils, fruit solids, and disease tolerance or resistance. Such variations have been observed in many crops, including wheat, triticale, rice, oats, maize, sugar cane, alfalfa, tobacco, tomato, potato, oilseed rape and celery (Thorpe 1990; Karp 1994). The same types of variation obtained from somatic cells and protoplasts can also be obtained from gametic tissue (Evans *et al.* 1984; Morrison & Evans 1988).

One of the major potential benefits of somaclonal variation is the creation of additional genetic variability in co-adapted, agronomically useful cultivars, without the need to resort to hybridization (Scowcroft *et al.* 1987). This method could be valuable if selection is possible *in vitro*, or if rapid plant-screening methods are available. It is believed that somaclonal variants can be enhanced for some characters during culture *in vitro*, including resistance to disease pathotoxins and herbicides and tolerance to environmental or chemical stress. However, at present few cultivars of any agronomically important crop have been produced through the exploitation of somaclonal variation (Karp 1994).

Micropropagation

Propagation of Plants

During the last 30 years it has become possible to regenerate plantlets from explants and/or callus from all types of plants. As a result, laboratory-scale micropropagation protocols are available for a wide range of species (Debergh & Zimmerman 1991) and at present micropropagation is the widest use of plant tissue-culture technology. Murashige (1990) reported that there were over 300 commercial operators World-wide in 1990. In Europe, there were 172 micropropagation firms and about 1800 different tissue lines (species and varieties) in culture amongst the 501 plant tissue-culture laboratories identified in 1993 (O'Riordain 1994). For example, of the 88 European laboratories using potato in tissue culture, 58 were listed as using *in-vitro* multiplication, 49 were involved in the elimination of pathogens, 45 were using tissue-culture simply to store germplasm, 44 were involved in genetic modification and 26 had plant-breeding programmes. The role of micropropagation in crop improvement has been recently reviewed in considerable detail (Bajaj 1991a, 1992a, b, c). Along with

the impressive successes there are several limiting factors to its use (Wang & Charles 1991). The cost of the labour needed to transfer tissue repeatedly between vessels and the need for asepsis can account for up to 70% of the production costs of micropropagation. Problems of vitrification, acclimatization and contamination can cause great losses in a tissue-culture laboratory. Genetic variations in cultured lines, such as polyploidy, aneuploidy and mutations, have been reported in several systems and resulted in the loss of desirable economic traits in the tissue-cultured products.

There are three methods used for micropropagation: (1) enhancing axillary-bud breaking; (2) production of adventitious buds; and (3) somatic embryogenesis. In the latter two methods, organized structures arise directly on the explant or indirectly from callus. Axillary-bud breaking produces the least number of plantlets, as the number of shoots produced is controlled by the number of axillary buds cultured, but remains the most widely used method in commercial micropropagation and produces the most true-to-type plantlets. Adventitious budding has a greater potential for producing plantlets, as bud primordia may be formed on any part of the inoculum. Unfortunately, somatic embryogenesis, which has the potential of producing the largest number of plantlets, can only presently be induced in a few species (Thorpe 1990). Nevertheless, the production of somatic embryos from cell cultures presents opportunities not available to plantlets regenerated by the organogenic routes, such as mechanization (Ammirato 1983). One approach envisages the use of bioreactors for large-scale production of somatic embryos and their delivery in the form of seed tapes or artificial seeds (see below). No commercial operation based on somatic embryogenesis exists (Thorpe 1990) but such embryogenesis is playing an important role in improving herbaceous dicots (Brown *et al.* 1995), herbaceous monocots (KrishnaRaj & Vasil 1995) and woody plants (Dunstan *et al.* 1995).

Synthetic Seed

A synthetic or artificial seed has been defined as a somatic embryo encapsulated inside a coating and is considered to be analogous to a zygotic seed (Redenbaugh 1993). There are several different types of synthetic seed: somatic embryos encapsulated in a water gel; dried and coated somatic embryos; dried and uncoated somatic embryos; somatic embryos suspended in a fluid carrier; and shoot buds encapsulated in a water gel. No large-scale system for producing such seeds has yet been developed, although pilot studies of moderate size, using somatic embryos encapsulated in a water gel, have been conducted in Japan with F_1 hybrids of celery and lettuce (Sanada *et al.* 1993). Several applications for synthetic seeds have been reviewed (Deunff 1993). The use of synthetic seeds as an improvement on more traditional micropropagation protocols in vegetatively propagated crops may, in the long term, have a cost saving, as the labour intensive step of transferring plants from *in vitro* to soil/field conditions may be overcome. Other applications include the maintenance of male sterile lines, the maintenance of parental lines for hybrid-crop production, and the preservation and multiplication of elite genotypes of woody plants that have long juvenile developmental phases (Villalobos & Engelmann 1995). However, before the widespread application of this technology, somaclonal variation will have to be minimized, large-scale production of high quality embryos must be perfected in the species of interest, and the protocols will have to be made cost-effective compared with existing seed or micropropagation technologies.

Pathogen Eradication

Crop plants, especially vegetatively propagated varieties, are generally infected with pathogens. Strawberry plants, for example, are susceptible to over 60 viruses and mycoplasms and this often necessitates the yearly replacement of mother plants (Boxus 1976). In many cases, although the presence of viruses or other pathogens may not be obvious, yield or quality may be substantially reduced as a result of the infection (Bhojwani & Razdan 1983). In China, for example, virus-free potatoes, produced by culture *in vitro*, gave higher yields than the normal field plants, with increases up to 150% (Singh 1992). As only about 10% of viruses are transmitted through seeds (Kartha 1981), careful propagation from seed can eliminate most viruses from plant material. Fortunately, the distribution of viruses in a plant is not uniform and the apical meristems either have a very low incidence of virus or are virus-free (Wang & Charles 1991). The excision and culture of apical meristems (the meristem with one to three of the subjacent leaf primordia), coupled with thermo- or chemo-therapy, have been successfully employed to produce virus-free and generally pathogen-free material for micropropagation (Kartha 1981; Bhojwani & Razdan 1983; Wang & Charles 1991; Singh 1992).

Germplasm Preservation

One way of conserving germplasm, an alternative to seed banks and especially to field collections of clonally propagated crops, is *in vitro* storage under slow-growth conditions (at low temperature and/or with growth-retarding compounds in the medium) or cryopreservation or as desiccated synthetic seed (Harry & Thorpe 1991; Villalobos & Engelmann 1995). The technologies are all directed towards reducing or stopping growth and metabolic activity. Techniques have been developed for a wide range of plants (Bajaj 1991b). The most serious limitations are a lack of a common method suitable for all species and genotypes, the high costs and the possibility of somaclonal variation and non-intentional cell-type selection in the stored material (e.g. aneuploidy due to cell division at low temperatures or

non-optimal conditions giving one cell type a selective growth advantage).

Concluding Thoughts

Plant tissue-culture technology is playing an increasingly important role in basic and applied studies, including crop improvement (Thompson & Thorpe 1990; Thorpe 1990; Vasil & Thorpe 1994). In modern agriculture, only about 150 plant species are extensively cultivated. Many of these are reaching the limits of their improvement by traditional methods. The application of tissue-culture technology, as a central tool or as an adjunct to other methods, including recombinant DNA techniques, is at the vanguard in plant modification and improvement for agriculture, horticulture and forestry.

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