

Scope and importance in crop improvement

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. The applications of various tissue-culture approaches to crop improvement, through breeding, wide hybridization, haploidy, somaclonal variation and micro propagation are discussed in this chapter.

Plant breeding and biotechnology

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

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. 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. 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. *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. This range includes agricultural crops, such as tobacco, clover, corn, rice, cole, canola, poppy and cotton. The use of delayed pollination, distant hybridization, pollination with abortive or irradiated pollen, and physical and chemical treatment of the host ovary have been used to induce haploidy.

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. The breeding cycle of *Iris* was shortened from 2 to 3 years to a few months by employing embryo rescue technology. A similar approach has worked with orchids and roses and is being applied to banana and *Colocasia*. Interspecific and intergeneric hybrids of a number of agriculturally important crops have been successfully produced, including cotton, barley, tomato, rice, jute, *Hordeum X Secale*, *Triticum x Secale*, *Tripsacum x lea* and some Brassicas. 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. 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 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. Haploid wheat has also been produced by this technique.

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. 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 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.

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*. 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 complementation for chlorophyll synthesis 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 and AC Chang, have been released from the progeny of these protoplast fusion products and are presently grown on approximately 42% of the fluecured tobacco acreage in Ontario, Canada. This represents a value of approx. US\$199,000,000. 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. 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. 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. 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. 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. 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.

Haploids

Haploid plants 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

- (1) Culture of excised ovaries and ovules;
- (2) The *bulbosum* technique of embryo culture; and
- (3) Culture of excised anthers and pollen.

A present, 171 plant species have been used to produce haploid plants by pollen, microspore and anther culture. 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. 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, anther-derived haploids have been used to produce an all-male F₁ 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. 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. 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. The same types of variation obtained from somatic cells and protoplasts can also be obtained from gametic tissue. 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. 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.

Micropropagation

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 and at present micropropagation is the widest use of plant tissue-culture technology. 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.

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. 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. The use of synthetic seeds as an improvement on more traditional micropropagation protocols in vegetatively propagated crops may, in the long term, have tissue *culture* and *crop improvement* 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. 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 are susceptible to over 60 viruses and mycoplasmas and this often necessitates the yearly replacement of mother plants. 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. 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%. As only about 10% of viruses are transmitted through seeds, 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. The excision and culture of apical meristems, coupled with thermo- or chemo-therapy, have been successfully employed to produce virus-free and generally pathogen-free material for micropropagation.

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. The technologies are all directed towards reducing or stopping growth and metabolic activity. Techniques have been developed for a wide range of plants. 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).

Plant tissue-culture technology is playing an increasingly important role in basic and applied studies, including crop improvement. 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.

Questions

1. Pre-zygotic barriers to hybridization are

- a) Failure of pollen to germinate
- b) Poor pollen-tube growth
- c) Both a & b**
- d) None of the above

2. Post-zygotic barriers to hybridization are

- a) Failure of pollen to germinate
- b) Poor pollen-tube growth
- c) Lack of endosperm development**
- d) None of the above

3. Pre-zygotic barriers to hybridization can be overcome by

- a) *In vitro* fertilization**
- b) Embryo culture
- c) Ovule culture
- d) Pod culture

4. Post-zygotic barriers to hybridization can be overcome by

- a) Pod culture
- b) Embryo culture
- c) Ovule culture
- d) All the above**

5. Embryo culture has been successful in overcoming the problems.....

- a) Low seed set
- b) Seed dormancy
- c) Slow seed germination
- d) All the above**

6. Delgold and AC Chang are the commercial varieties of produced by protoplast fusion

- a) Tobacco**
- b) Potato
- c) Tomato
- d) None of the above

7. The production of novel hybrids through protoplast fusion should focus on

- a) Agriculturally important traits
- b) Somatic hybrids integrated into a conventional breeding programme

c) Extension of protoplast regeneration to a wider range of crop species **d) All the above**

8. *In vitro* methods used to generate haploids

- a) Culture of excised ovaries and ovules b) *Bulbosum* technique of embryo culture
c) Culture of excised anthers and pollen **d) All the above**

9. The methods used for *in vitro* propagation

- a) Enhancing axillary-bud breaking b) Production of adventitious buds
c) Somatic embryogenesis **d) All the above**