Tissue Culture's Potential for Introducing New Plants

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INTRODUCTION

Methods to modify and improve plants have been practiced for at least 10,000 years. Early farmers produced better crops simply by selecting the seeds of desirable plants. During the past century, plant breeding has become a refined art due to technological advancements. Today, the plant breeder may use genetic engineering to add diversity to plant characteristics and to develop superior plants.

Successful plant development and improvement are dependent on genetic diversity followed by genotype selection and evaluation. Plant tissue culture and plant biotechnology offer new and efficient ways to expedite genetic selection. Plant tissue culture is the art and science of aseptically growing plant cells, tissues, organs, protoplasts, and whole plants on a nutrient medium under controlled environmental conditions. Micropropagation is a major part of plant tissue culture.

Although genetic engineering is more complex than traditional plant breeding both procedures introduced new DNA into the plant's genome. In 1992, the U.S. government determined that genetically engineered products do not pose any unreasonable risk; as a result, the government decided that these products should not be subject to additional federal regulations.

This paper will briefly review the in vitro techniques of micropropagation, somaclonal variation, fertility restoration, gene transfer, embryo culture, anther culture, and in vitro fertilization. All of these procedures can be utilized to develop and introduce new plants.

MICROPROPAGATION

Micropropagation is the mass propagation of plants in vitro under controlled environmental conditions. The goal of micropropagation is **not** to develop new plants but to rapidly multiply clones while maintaining their genetic stability. Micropropagation is indirectly involved with new plant development, since it assists with the production of the new cultivars.

In order for plant micropropagation to be a viable method propagules must retain their developmental integrity and chromosome stability as they grow. In order to accomplish stability, explants that have organized growing points are used as propagules. Two types of organized explants used are: (1) meristems from shoot tips, axillary buds, or rhizome tips; and (2) zygotic embryos from seeds. These kinds of explants are used for regeneration of true-to-type plants and for germplasm preservation because they retain their developmental integrity and chromosome stability as they grow (Murashige, 1974). However, a significant degree of genetic variation can be produced if a high rate of multiplication is used, if inappropriate techniques are used, or if a variant is produced and subsequently propagated in large numbers.

SOMACLONAL VARIATION

When plant tissue culture was in its infancy, it was thought that since plant cells

are totipotent (able to regenerate into whole plants from single cells), they would regenerate plants that are identical to the mother plant. However in the 1980s, this was proven not to be true.

Somaclonal variation is variation that occurs as the result of using tissue culture; the variation may be preexisting or induced (Skirvin et al., 1994) Somaclonal variation can be heritable or epigenetic. Usually the term refers to heritable genetic variation, unfortunately, it is not always possible or feasible to verify the heritable nature. Heritable variation is stable through the seed progeny or repeated asexual propagation. Epigenetic variation is not genetically stable and may not manifest itself when plants are asexually propagated. Although somaclonal variation is a problem for micropropagators, the process can be an asset for plant breeders because it is easy, safe, rapid, economical, and a rich source of genetic variability.

Organized growing points, such as shoot tips and axillary buds, are the most genetically stable explants in tissue culture. Adventitious shoot production systems such as organogenesis or somatic embryogenesis from single cells of unorganized explants such as callus, cell suspensions, and protoplasts are the least genetically stable systems. These adventitious systems are useful for producing genetic variants in vitro through somaclonal variation.

The three ways somaclonal variation may arise in vitro are: (1) pre-existing variation, (2) spontaneous mutations, and (3) induced mutations.

Variation in cells may arise from pre-existing variation from chimeral plants. Chimeras consist of cell sectors or tissues that differ in genetic constitution. Adventitious shoot production from single cells is especially good for chimeral dissociation. Chimeral plants grown in tissue culture can yield variants in high percentages (Skirvin, et al., 1994).

Somaclonal variation can occur as a spontaneous mutation (Brand and Bridgen, 1989). Adventitious plants or somatic embryos arising directly from explants or indirectly from a callus intermediate are especially susceptible to spontaneous mutation. Mutations can also occur either by using high-chromosome or polyploid species or by inducing cultures to grow at high proliferation rates. High concentrations of "strong" growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) will also increase the frequency of variability. Cultures that have been maintained for long periods will also demonstrate higher degrees of variation.

Somaclonal variation can also be induced as a form of mutation breeding with the use of mutagenic agents and selection pressures. Either physical mutagens or chemical mutagens may be used to induce mutations. Examples of physical mutagens are gamma rays and x-rays. Chemical mutagens include colchicine, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), sodium azide (NaN_3), ethidium bromide, ethyl nitroso urea, methyl nitroso urethane, and diethyl sulphate (DES).

FERTILITY RESTORATION

Colchicine is a chemical that can be used without difficulty to double chromosome numbers. Historically, it was applied to the growing points of whole plants or seedlings. It is now being applied in vitro to double chromosome numbers for somaclonal variation or fertility restoration of sterile diploids.

Our laboratory has successfully used colchicine in vitro to double chromosome numbers of *Alstroemeria*. Young, vigorously growing cultures of either rhizomes or shoots and buds with 2-3 shoots/buds each are treated with 0.2% to 0.6% colchicine in 2% DMSO (a penetrant) solution for 6 to 24 h. The cultures are shaken periodically during treatment.

After the treatment, cultures are rinsed five times with sterile deionized/distilled water to remove surface colchicine; each rinse lasts 30 min. The explants are then cultured on a normal proliferation medium.

Proof of chromosome doubling can be shown by: (1) observing the ploidy level of the regenerated plant with root squashes followed by cytological examinations, (2) examining the morphological characteristics, (3) determining the leaf stomatal index [stomatal density divided by (stomatal density + epidermal cell density)] ×100, (4) measuring the guard cell size, or (5) estimating the fertility of the plants by examining pollen viability.

GENE TRANSFER

Plant biologists created the first transgenic plants about 10 years ago and since then, genetic engineering has been applied to more than 50 plant species. This technique has helped researchers gain critical insights into the fundamental processes that govern the development of plants. Now, the first commercial introduction of such genetically modified plants has been accomplished.

The first practical system for genetic engineering of plants relied on the bacterium *Agrobacterium tumefaciens*. This bacterium is able to transfer a portion of its DNA into plant cells by introducing a set of genes into one or more DNA fragments. The fragments, called transfer DNA (T-DNA), integrate into chromosomes of infected plant cells and induce the cells to produce elevated levels of plant hormones. These hormones cause the plant to form novel structures, such as tumors or prolific root masses, which provide a suitable environment and nutrient source for the *Agrobacterium* strain. The bacterial infections are called crown gall disease and hairy root disease, respectively (Gasser and Fraley, 1992).

For the bacterium to be an effective vehicle for DNA transfer, the disease-causing genes are removed or "disarmed". This was first accomplished in 1983 by using traditional DNA recombination to delete the genes that cause tumors. Disarming eliminates the bacterium's ability to cause disease, but the mechanism of DNA transfer remains intact.

Although the method previously described is simple and precise, many plant species, especially monocots, are not natural hosts for *Agrobacterium* and are not readily transformed. As a result, extensive efforts have been made to develop alternative systems. One of these systems was to introduce free DNA into plant protoplasts. Protoplasts are plant cells that have had their cell walls removed by enzymes. Cell walls must be removed because their pores are too small to allow the easy passage of DNA. Polyethylene glycol can penetrate the only remaining barrier, the plasma membrane, to transport DNA. It is the most commonly used chemical delivery agent. Electroporation can also transport DNA across the plasma membrane. Short, high-voltage pulses briefly produce pores in the protoplast membrane so that DNA molecules can enter through these spaces. Unfortunately, the regeneration of plants from isolated protoplasts has proven to be very problematic.

To increase the efficiency of gene delivery Dr. John C. Sanford (1990) has developed a method to bombard many plant cells with genetic material. This process is known as the Biolistic™ bioparticle delivery system, the microprojectile bombardment method, the gene gun method, and the particle acceleration method. Sanford surmised that small metal particles about one or two microns in diameter could be coated with DNA and then sufficiently accelerated to penetrate the walls of intact cells to deliver the DNA. Because small holes in cell walls and membranes rapidly heal by themselves, the punctures are temporary and do not irreversibly compromise the integrity of the cells. The nucleic acids and other substances that are introduced into the cells remain in the cytoplasm but are too small to interfere with any cellular functions. The introduced DNA is expressed within days after transfer and becomes, to a low extent, stably integrated into the plant genome. Diverse applications for the biolistic process are rapidly being found for research with genetic engineering.

The Biolistic™ bioparticle delivery system (a term coined from biological and ballistics) offers wider applicability than existing gene transfer technologies including direct DNA uptake, cell fusion, microinjection, and *Agrobacterium*-mediated transformation (Table 1) (Kikkert, J.R. 1993).

EMBRYO CULTURE

Intervarietal and interspecific crosses followed by selection have accounted for the improvement in quality and yield potential of practically all major crops. One biotechnological technique which has been very beneficial in this area is embryo culture.

Embryo culture involves the sterile isolation and growth of an immature or mature zygotic embryo on an aseptic nutrient medium with the goal of obtaining a viable plant. The basic premise for this technique is that the integrity of the hybrid genome is retained in either a developmentally arrested or an abortive embryo and the embryo's potential to undergo normal growth may resume if it is supplied with the proper growth substances. The technique is dependent on the isolation of the embryo without injury, formulation of a suitable nutrient medium, and the induction of continued embryogenic growth and seedling formation.

The culture of immature embryos is used to rescue embryos that would normally abort or not undergo the progressive sequence of ontogeny. This process is difficult due to the tedious dissection necessary and the complex nutrient medium requirements. The chance of success of this type of culture depends strongly on the developmental stage of the embryo when isolated.

The culture of mature embryos from ripened seeds is used to eliminate inhibitors of seed germination; to shorten the breeding cycle if, for example, dormancy is a problem; and to prevent embryo abortion of inter- or intraspecific hybrids. The culture of mature embryos is easy and only requires a relatively simple nutrient medium with agar, sugar, and minerals.

ANTHER CULTURE

With sexual reproduction, the number of chromosomes in a cell are reduced to half as a result of meiosis and then doubled again by fertilization when the male and female gametes fuse. Culturing the flower's anthers which contain haploid pollen can produce androgenic embryos in vitro. The number of chromosomes of the

Table 1. Methods of gene transfer into plant cells and their advantages and disadvantages.

Technique	Advantage	Disadvantage
Biological transfer (*Agrobacterium* mediated)	High probability T-DNA will integrate into plant genome.	Only a small number of cells integrate & express introduced DNA.
	"Markers" are available.	Bacterium needs disarmed.
	Relatively simple.	Many plant species are not readily transformed (success primarily with dicots).
	May not require callus phase.	
	Relatively "clean" insertions into the plant genome.	DNA sequences are essential for T-DNA replication and transfer.
		False positives from growth of *Agrobacterium*.
Direct gene transfer: (naked DNA transfer)		
A. Microinjection of protoplasts	PEG mediates transfer into protoplasts.	Difficult to regenerate plants.
B. Electroporation	Does not need to use protoplasts.	Special equipment is needed.
C. Microprojectile bombardment (Biolistics)	Adaptable to most species.	Low probability of integration into genome.
	DNA introduced into intact cell and tissues.	Multiple rearranged copies of the DNA may be inserted.
	Improved recovery of transformed plants.	Special equipment needed.
	Very useful for transient expression studies. Plasmic construction is simplified. No false positives.	
Protoplast cell fusion	Simple	Difficult to regenerate plants.

sterile haploid plant can doubled spontaneously or be induced with colchicine to produce a fertile homozygous diploid individual with two identical sets of chromosomes. Homozygous inbred lines are valuable for breeders because they give rise to identical gametes by meiosis. When two genetically different homozygotes are crossed, the progeny will be identical but hetorozygous.

In anther culture, flower buds are removed from the mother plant when the anthers are at the uninucleate stage. Buds are surface sterilized with bleach, rinsed in sterile water, and the anthers aseptically removed. Anthers are checked under the microscope to confirm the stage of development and then cultured. In 4 to 8 weeks, haploid plants form from the microspores.

The success of anther culture depends on several factors. (1) The stage of development of the anthers at the time of culture is important—pollen within anthers should be at the late uninucleate to early binucleate stage. (2) Genotype is also important since certain plant families, such as the Solanaceae, respond better than others. (3) Anthers should not be damaged before culture. (4) Pretreatments, such as cold or hot thermal traumas, sometimes enhance the androgenic response. (5) A simple nutrient medium with 0.5% to 1% activated charcoal and 2% to 4% sucrose is best.

Although the promise of anther culture for new cultivar development seemed great 20 years ago, its impact on cultivar release is just now being realized (Veilleux, 1994). In addition, problems have been associated with anther-derived haploids, such as reduction in vigor, gametoclonal variation (somaclonal variation of haploids), and elevated DNA contents. However, as the procedures for anther culture become more conventional, more applications to cultivar development will be seen.

IN VITRO FERTILIZATION

Fertilization fails to occur when the pollen does not germinate on the stigma, pollen tube growth stops or stagnates, the fertilized egg aborts, the embryo and endosperm are incompatible, there is poor endosperm development, or ovaries abscise prematurely. In vitro pollination and fertilization appears very promising to overcome pre-fertilization barriers and generate new genotypes.

In vitro fertilization of the egg inside the ovule can occur by test tube fertilization, the development of seeds through in vitro pollination of exposed ovules, or in vitro stigmatic pollination of cultured whole pistils. This process is used when self incompatibility or cross incompatibility are problems.

Two decades ago, there was interest in this technique, but little research. To date, there is still little research for a very promising procedure.

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