Tissue culture in ornamental plant breeding: A review

Luciana do Valle Rego and Ricardo Tadeu de Faria*
Departamento de Agronomia, Universidade Estadual de Londrina (UEL), Caixa Postal 6001, CEP 86051-990, Londrina- Paraná, Brazil. (* Corresponding Author. E-mail: faria @ uel.br)

ABSTRACT

Ornamental plant breeding, as other crop breeding, is considered the art and science of genetic alteration/ transmutation of plants for human consumption. This paper presents a review of the literature on tissue culture in ornamental plant breeding and its applications. The following culture methods are discussed: A) polyploidy induction; B) *in vitro* mutagenesis and somaclonal variation; C) genetic transformation; D) protoplast fusion; E) embryo recovery; F) *in vitro* selection and G) micropropagation.

KEY WORDS: Polyploidy, *in vitro* mutagenesis, genetic transformation, protoplast fusion and ornamental plants.

INTRODUCTION

Floriculture is a dynamic and demanding activity since the quality required from the product by consumers is very high. These demands are further accentuated when products are exported. Flower producers have used the most advanced production, genetic breeding and commercialization techniques to meet this high quality standard. Nowadays, *in vitro* propagation of plants has offered producers enough high quality seedlings to meet this demand in a short period of time (Tombolato and Costa, 1998).

Tissue culture is a process by which small fragments of live tissue, called explants, are cultivated under aseptic conditions in a culture medium. Suitable recipients are kept in environments under controlled luminosity and temperature. This technique is available to breeders and can be used in practically all stages of a breeding program, from preservation and interchange of genetic resources and increase of the genetic variability to the selection and multiplication of superior genotypes (Mantell et al., 1994; Cirino and Riede, 1999).

The systematization of genetic breeding programs started in the beginning of the century with the rediscovery of the basic principles of mendelian segregation which set out the basic laws of genetic heredity. Since then, the application of genetic principles to the development of plants with superior agricultural performance, through the application of most diverse methods, has been systematic. At the same time, there has been considerable progress in *in vitro* plant cell and tissue culture techniques (Binsfeld, 1999).

Experiments with tissue culture began in the nineteenth century when two German biologists, M. J. Shleiden and T. Shwann, reported that the whole plant can be reconstituted whenever cells from some plants were removed (Bonga and Aderkas, 1992). This experiment led to the concept of totipotency, suggesting that each cell is a unit capable of originating a new organism, and that each cell from a multicellular organism retains the information present in the fertilized ovule. Totipotency stimulates the regeneration of plants with small tissue mass and isolated cells and, consequently, undetermined plant cells may show totipotency, plus a high degree of plasticity to physical and environmental stimulus (Mantell et al., 1994; Cirino and Riede, 1999).

The year of 1934 was the turning point for plant tissue culture principles , especially for the potential unlimited and undifferentiated growth principle. White (1934) cultivated tomato roots in a defined nutritive medium

and Gautheret (1934) planted three species of callus (dedifferentiated) from the cambium regions (Mantell et al., 1994).

Plant breeding aims at developing new cultivars adapted to the stable and high yield cultivation conditions required by high quality production. From the methodological point of view, plant breeding is applied genetics and has been considered the art and science of altering plants genetically for human consumption (Binsfeld, 1999; Barros, 1999).

Despite the extraordinary contribution of the conventional methods to plant breeding, there has been a consensus that significant gains cannot be expected from selection by these processes only (Barros, 1999).

In this context, the application of biotechnology techniques allied to conventional plant breeding methods can contribute to the sustainability of agriculture by producing cultivars which are more compatible with the environment. This contribution is especially important to developing countries where the technological resources required to deal with problems related to tropical crops are scarce (Barros, 1999).

Classical methods developed new cultivars until the mid 1980s. Since then, plant breeding has developed several techniques based on plant tissue culture and molecular biology. These techniques became important tools to help breeders look for the allelic diversity needed in a breeding program, enabling them to transcend the primary genetic pool used in classic plant breeding and to incorporate the new alleles into the genome to determine the required characteristics of the culture (Binsfeld, 1999).

Currently, sexual crossing is necessary among wild and cultivated species which are normally incompatible. In these cases, crosses are not possible, and the genetic flow is impeded. However, certain *in vitro* techniques can solve these problems, quickly regenerating many types of useful plants which would otherwise be obtained after many years of intensive breeding cycles (Mantell et al., 1994).

The first group of transgenic cultivars commercialized in several parts of the world were resistant to herbicides, insects and pathogens, and were developed by tissue culture combined with molecular biology methods. Thus the researcher had to define the best strategy to solve the problems at hand, always looking for the simplest and the most practical and economically viable alternatives (Ferreira et al., 1998).

This literature review presents some examples of tissue culture applied to ornamental plant genetic breeding. The genetic breeding techniques discussed in this paper may appear either as complementary to classic genetic breeding programs or as techniques used for the development of new cultivars.

A. Polyploidy induction

Each species has a characteristic number of chromosomes, i.e., most organisms are diploid (2x), with two groups of homologous chromosomes, one from the male parent and the other from the female parent. The gametes of these organisms are haploid (x), i.e., they have only one group of chromosomes. However, variation in the number of chromosomes can be found in nature (Faria and Destro, 1999).

Polyploidy generally refers to all natural or induced variations in the number of chromosomes, also known as the numerical chromosome mutations. Although inexpressive in animals, polyploidy has been of major importance to plant evolution and culture, and has gained importance in agriculture. It is estimated that a third of the angiosperms (flower bearing plants) shows more than two groups of chromosomes, i.e., polyploidy (Faria and Destro, 1999).

Polyploidy in orchids produces desirable characteristics such giantism (an increase in floral piece), an intensification in flower coloring , durability and resistance to diseases (Griesbach, 1985; Watrous and Wimber, 1988).

Numerical chromosome mutations can be of two types: aneuploidy and euploidy.
- **Aneuploidy:** is a variation in the number of chromosomes that affects part of the genome.

The chromosomes carry genes and each species has a characteristic number. The non-disjunction of chromosomes at meiosis explains most of the aneuploid variations.

- **Euploidy:** is a variation in the basic number (x) of chromosomes involving the whole genome. The n represents the number of chromosomes present in the gamete while x represents the number of chromosomes in the genome (Vieira, 1964 quoted by Faria and Destro, 1999). In hexaploid wheat (6x = 42 chromosomes), the "n" is equal to 21 chromosomes and the x is equal to 7. This means that the cell or individual possesses a multiple number of the genome of the species it belongs to. Euploids are classified in monoploidy (x) and polyploidy (3x, 4x, 5x, 6x, 7x ...).

Treatments with different temperatures and with colchicine or orizaline can induce polyploidy.

1. Temperature

One of the techniques used to induce polyploidy consists in submitting the tissue of recently germinated seeds to cold water, at a temperature between 1 and 3°C. The treatment with cold water is more effective than with hot water since the heat can damage the treated tissue (Dermen, 1940).

2. Colchicine

Colchicine ($C_{22}H_{25}N$), an alkaloid extracted from the seeds and bulbs of a lilac tree (*Colchicum autumnale*), acts at the end of the mitotic prophase, either by inhibiting the development of the mitotic spindle or by leading it to an abortive spindle by precipitation of the proteins that make up its fiber (Jackson, 1976).

Menninger (1963) induced tetraploids in *Cymbidium* genus orchids by treating a pseudobulb shoot with colchicine solution.

Colchicine has limitations as a polyploidy inducing agent in *in vitro* plant culture since it becomes toxic to the plant tissues at high concentration or under very prolonged treatments. Thus, the appropriate evaluated for each species and type of material to be treated (Hammill et al.,1992).

In an *in vitro* experiment with *Dendrobium*, Vajrabhaya (1983) obtained a high number of plants (72%) with duplicated chromosomes when a 0.05 and 0.20% colchicine concentration was added to culture medium, while, Watrous and Wimber (1988) obtained 50% of tetraploids from a *Paphiopedilum,* cultured meristem tissue.

3. Orizaline

Orizaline, a well known herbicide such as the dinitroaniline, is another polyploid plant inducing agent. Several authors have found that the orizaline can be less phytotoxic than the colchicine and also more efficient . It increases the number of tetraploids and increases the number of chimera (Tosca et al., 1995).

Example 1
Somatic embryos of *Camellia japonica* were differentiated from single cells of hypocotyl explants and placed on a MS medium containing GA3. Secondary polyploid embryos developed on the hypocotyl of primary embryos whenever these embryos were treated with 0.1% colchicine for 1 week. Polyploid embryos proliferated in a similar manner on the MS + GA3 medium, during subsequent subcultures. These polyploid embryos developed into plantlets with autopolyploid morphological characteristics (Kato, 1989).

Example 2
Bulb scales of *L. davidii* var. willmottiae were irradiated with gamma rays at 500-2000 R and sections were cultured on a MS medium with 1-4 mg colchicine/litre, BA and NAA. Regenerated plantlets were also treated with gamma rays at 100-500 R and 1-4 mg colchicine/litre. Plantlet regeneration was reduced by treatment of scales with gamma rays or colchicine. During subculture, proliferation of plantlets was also reduced by colchicine, especially at 2 mg/litre, and irradiation at 400 R , while irradiation at 300 R enhanced proliferation. The LD50 for gamma irradiation of the plantlets was 400-500 R. The combined inhibitory effects of gamma irradiation and colchicine on plantlet proliferation were greater than those of the treatments alone. In plantlets treated with colchicine, with or without irradiation, variations in leaf thickness, leaf colour, bulb size and other attributes were found. These variations were maintained in subcultures and in plants transplanted to the field (Wang et al, 1989).

Example 3
Anthers of an ornamental shrub (*Iochroma warscewiczii* Rangel) were cultured on a Nitsch & Nitsch medium containing varying concentrations of IAA and benzyl adenine (BA). Two types of embryogenesis were noted, one involving the vegetative cell, and the other starting with 2 equal cells in the pollen grains. In all media tested, initiation was highest when anthers contained pollen at the first mitosis, or close to it, at inoculation. High sucrose (7%) and Ca (11.3 mM) concentrations were best suited for androgenesis induction. The addition of 0.5 mg BA/litre improved callus and embryoid production. Plantlet development from pollen embryos required lower levels of sucrose (3%) and a combination of 0.1 mg BA and 0.5 mg gibberellic acid/litre in the culture medium. Cytological analysis of 55 regenerated plantlets showed that about 49% were haploids, but diploid (49%) and triploid (2%) plantlets were also obtained (Canhoto et al., 1990).

Example 4
The transfer of disease resistance, especially to black spot (*Diplocarpon rosae*), from wild diploid Rosa species to modern rose cultivars has become high priority in rose (*Rosa* spp.) breeding research. Amphidiploids (2n = 4x = 28) were induced with colchicine from five interspecific diploid (2n = 2x = 14) hybrids involving the black spot resistant diploid species *R. wichuraiana, R. roxburghii, R. banksiae, R. rugosa* subsp. rubra, *and R. setigera*. Two application procedures (agitation of excised nodes in colchicine solution or tissue culture of shoots on a medium with colchicine), five colchicine concentrations (0.0, 1.25, 2.50, 3.76, and 5.01 mmol), and five periods (2, 3, 5, 8, and 10 days) were used. After the colchicine treatment, the materials were cultured *in vitro* and the surviving explants were examined for the "gigas" characteristics, typical of doubled diploids. Chromosome counts of morphologically suspect genotypes confirmed 15 amphidiploids among the 1109 plants that survived the colchicine treatment. Although the effect of the colchicine treatment varied somewhat among interspecific hybrids, 2.50 mmol for 48 h of node agitation or 1.25 mmol for at least 5 days of shoot culture were optimal (Ma-Yan et al., 1997).

Example 5
Protocorms of spring orchid (*Cymbidium*) developed by tissue culture of aerial parts/ shoots, seeds and stems, were treated with ultraviolet (UV) radiation and colchicine. After UV irradiation, the cell nucleous became deformed. Cell division frequencies increased with the UV dose, causing phenotypic variation among regenerated plants. Similar phenotypic variation was observed in plants regenerated from material treated with colchicine (Lin et al., 1997).

B. *In vitro* mutagenesis and somaclonal variation

In recent times, developments in *in vitro* mutation induction and the advances in cell and molecular biology techniques in plant breeding have appeared very quickly. Spontaneous mutations, recombinations and selection are the main evolution factors. Plant breeding, in this sense, is considered an evolution factor controlled by man (Tulmann Neto et al., 1998).

Mutation is a permanent heritable change in the primary structure of the genetic material which consists of the total genome of a cell or plant. This concept includes the deletion or addition of DNA and the chromosome rearrangements by DNA inversion of translocation. A change in the primary DNA structure may result in an altered phenotype, a mutant phenotype, which has four characteristics: it remains stable over consecutive cell generations; it occurs at relatively low frequencies (10^{-6} to 10^{-10}) which can be increased by mutagenesis; it should be correlated whenever possible with specific genetic products, and it should be transmitted by sexual crosses (Mantell et al., 1994).

Genetic variability is the first requirement for the improvement of any trait. The variability available to breeders comes from spontaneous or artificially induced mutations. Plant breeding can involve genetic variability amplification procedures, desirable genotype selection, selected genotype assessment, and finally, new cultivar multiplication and release (Montalvan, 1999).

According to Fehr (1987), artificial or induced mutation can be a practical and efficient genetic breeding technique to be used with cultivated plants. Artificial mutation induction is carried out using physical and chemical mutagens which can increase the mutation frequency when compared to its spontaneous occurrence. It is used mainly to obtain mutants for qualitative genes and ,on a smaller scale, for quantitative genes. However, the breeding process for quantitative genes has been successful in detriment of the genetic variability found in the populations used. The high efficiency of mutant production is essential for its extensive use in plant breeding. Thus the use of any mutagenic agent depends not only on its mutagenic effectiveness but also on its efficiency. Mutagenic efficiency is the production of desirable changes free of association with unwanted genetic alterations (Montalvan, 1999).

1. *In vitro* mutagenesis

Since the beginning of the century, when Stadler (1928 a,b) reported that X rays caused mutation in plants, breeders have used other tools to broaden genetic variability. Nowadays there are several ways of using mutation induction in plant breeding. Periodic reviews of the literature, Konzak, 1984 and IAEA, 1993 quoted by Tulmann Neto et al., 1998 have shown a constant increase in the number of cultivars obtained directly or indirectly by the use of induced *in vivo* mutation. However, advances in cell and tissue culture techniques have opened up a new field for the use of the *in vitro* mutagenic.

Mutation induction with cell and tissue culture techniques have increased progressively since a large population of haploid and diploid cells can be handled in a small space, developing new individuals in a short period of time. *In vitro* treatments with chemical mutagenic agents occur more uniformly than *in vivo* treatments in which a controlled environment and culture medium are used (Constantin, 1984).
One of the limitations of using *in vivo* mutagenic agents in plants with plant propagation is that they have difficulty to penetrate the tissues to be treated. However, this can be minimized by *in vitro* mutagenesis. One advantage of using *in vitro* techniques is the possibility of working with haploid cells, protoplasts and cells in suspension (Tulmann Neto et al., 1998). According to Chaleff, 1983, *in vitro* selection involves the use of a large cell population which makes the search for dominant mutations, which occur at low frequencies when compared to the recessive mutations, more efficient. Thus, *in vitro* mutagenesis studies are restricted to species that can grow and regenerate under these conditions (Constantin, 1984).

When mutation induction is used in plant breeding (Brock, 1977), the probability of success is greater, since conventional techniques take a lot of time and effort to develop the desired genotype.

The main attributes of mutant ornamental plant cultivars obtained through direct propagation of induced mutants are listed below (Kawai, 1986) (the number in brackets indicates the number of cultivars with such attributes):
- **Annual ornamental plants**: flower color (123), more flowers (15), flower shape (15), leaf shape (120), number of flower petals (10), large leaf (50), large plant (5), small plant (5), large flower (3), plant type (2), growth rate (2), number of branches (1), ornamental novelty (1), regeneration skill (1) and flower longevity (1).
- **Ornamental plants with roots and tubers**: flower color (520), flower shape (18), plant type (13), long stem (7), leaf color (7), neutrality to photoperiod (5), early blooming(3) large flower (2) and stem color (2).
- **Perennial ornamental plants**: flower color (28), short stem (4), small flower petals (1), striped leaf (3), vigorous growth (4), early blooming (1), more branches (1), greater branch density (1) and more flowers (1).

2. Somaclonal variation

There are many *in vitro* culture techniques, including somaclonal variation induced by mutagenic agents (Tulmann Neto et al., 1998). Somaclonal variation is a term introduced by Larkin and Scowcroft (1981) to designate all types of variation which occur in plants regenerated from plant tissue culture.

There are many cases of somaclonal variation in tissue cultures carried out with cultivated plants

as well as with ornamental plants species, but according Larkin and Scowcroft (1981), the causes for this variation, however, are not very clear, and may differ for each plant.

Several mechanisms may be responsible for the somaclonal variation induction. Such mechanisms include gross karyotypic changes that accompany *in vitro* culture via callus formation, cryptic chromosome rearrangements, somatic permutation with changes of parts among sister chromatides, transposition of elements, genetic amplification or decrease , and several combinations of these processes (Mantell et al., 1994). In short, somaclonal variation is the sum of the genetic variations (chromosome and genetic mutations) that are incorporated in the regenerated plants of a species. Part of such variation may exist prior to the *in vivo* culture and is produced in the *in vitro* culture (D'amato, 1986).

There are many studies on ornamental plants involving mutation by somaclonal variation and *in vitro* mutagenesis involving different types of plants for different commercial interests. The following examples demonstrate some of the practical results obtained by this technique as well as its great potential for producing new cultivars.

Example 1
In 1984, Horn carried out an *in vitro* experiment with mutagenic agents in *Chrysanthemum* and *Kalachoe blossfeldina*. *Chrysanthemum* callus and cell suspension from five different clones were irradiated with gama rays (800 to 1800 R). A clone with SEM (0.5 to 1.5%) was submerged in a recipient with the callus and cell suspensions in the mutagenic solution, for two hours. Pieces of *Kalanchoe* leaf, callus and cell suspensions were treated with X rays (1,500 to 3,000 R). All treatments, in both cultures, were observed until flowering. Although there was great spontaneous variation in the controls, a significant increase in mutations was found in the treatments with mutagens. The percentage of chimerical mutants in *Chrysanthemum* was small in plants derived from cell mutations and in *Kalanchoe*; leaf explants resulted in lower chimera. Thus, regenerated plants were in general derived from a single cell, creating an ideal situation for mutation induction.

Example 2
Kohleria ornamental plant internodes, obtained *in vitro*, were treated with NMH chemical mutagen. Then, adventitious sprouts were obtained on a suitable culture medium. A mutant was identified with a shorter internode and smaller leaves (Geier, 1989).

Example 3
In vivo and *in vitro* studies were carried out on *Chrysanthemum morifolium* to obtain flower color mutants. Pieces of undeveloped flower buds were inoculated in an appropriate culture medium and irradiated with a 8Gy dose of gamma rays. Regeneration was observed by adventitious sprouts, and the plants were selected as coloring mutants. Mutants were not observed in the control, but a 6.5% mutant frequency was observed in the treatment with irradiation for bronze, champagne, dark pink and paler pink colorings (Rodrigo, 1994).

Example 4
Morphological and genetic variations in 1360 *Phalaenopsis* True Lady 'B79-19' flowering semiclones, derived from tissue culture were evaluated. No apparent difference was found in the shape of the leaves, whereas the flowers in some somaclones were deformed. Thirty-eight selected random primers were used to generate amplified segments of genomic DNA and to differentiate polymorphisms of somaclonal variations in *Phalaenopsis*. The random amplified polymorphic DNA (RAPD) data indicated that normal and variant somaclones are not genetically identical. Banding patterns of aspartate aminotransferase (AAT) and phosphoglucomutase (PGM) were analyzed in young leaves of variant and normal somaclones of *Phalaenopsis*. As for AAT, three distinct banding patterns were found in normal somaclones and only two-banded phenotypes were detected in variant somaclones. Three to four bands were detected in normal somaclones and two to three bands in variant somaclones when the banding patterns of PGM isoenzymes were compared (Chen et al., 1998).

C. Genetic transformation

Genetic transformation is the transference (introduction) of a DNA sequence or, more specifically, of a gene to an organism without

fertilization or crossing. The genetically transformed organisms are called transgenic. Therefore, genetically transformed plants are called transgenic plants (Bespalhok, 1999). Genetic introduction is defined as being the controlled introduction of nucleic acids in a receiver genome without fertilization (Tacchini and Walbot, 1986). *In vitro* plants must present good regeneration results for the introduction to be performed.

Different genetic transformation techniques have been established with the development of tissue culture techniques and genetic engineering (Brasileiro and Carneiro, 1998). Recently, several gene transference methods for plant cells have been published, including the use of *Agrobacterium tumefaciens*, particle acceleration (biolistics), polyethulenoglicol, electroporation, sinication, silica carbonate microparticles, microlaser, micro and macro injection and direct DNA application (the first two being the most used). All these plant genetic transformation techniques can be grouped into two categories, indirect and direct gene transference. Indirect transference takes place when a vector such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* is used to mediate the transformation. Transformation via *Agrobacterium tumefaciens* has been the method most commonly used to obtain transgenic plants. However, many species, especially the monocotyledons, are not susceptible to infection by this bacteria which has influenced the research and development of other transformation methods, also known as direct methods. Direct DNA transference is based on physical or chemical methods, generally adapted from others already established animal cell transformation methods. The transformation with polyethylene glycol (PEG) electroporation and the particle acceleration methods have been largely used in gene promotion and expression studies (Brasileiro and Dusi, 1998).

One of the limitations of these methods is the lack of control over the DNA integration in the genome, which occurs randomly. The other limitation is the silencing process of genes and the interaction among different transgenes, which result in unexpected expression patterns in the introduced genes. Several independent transformations with a specific construction are necessary to obtain a transgenic plant with a desirable expression pattern (Siemens and Schieder, 1996).

In 1994, the FLAVR SAVR tomato derived from plant genetic engineering, was commercialized in the USA. Since then, genetic engineering has expanded, and, today, several transgenic materials are found in the North America, Chinese and the United Kingdom markets (Torres et al., 1998). In Australia, transgenic carnation and violet plants have been developed with a half post harvest period (Redenbaugh, 1997). There has been a considerable increase in the number of laboratories doing research on transgenic plants in Latin America in the last 5 years (REDBIO, 1998). Studies have been developed on the most cultivated plants such as rice and maize as well as on some ornamental plants such as sunflower (used for oil extraction) and the *Anthurium* (Brasileiro and Dusi, 1998).

The following are the necessary steps in genetic transformation: 1) isolation of a useful gene; 2) introduction of this gene into the plant cell; 3) integration of this gene in the plant genome; 4) fertile plant regeneration; 5) expression of the introduced gene in the regenerated plants; 6) transmission of the induced gene from generation to generation. This method offers breeders some advantages such as: 1) it can change one characteristic without modifying the others; 2) it requires fewer generations and it is faster than backcrossing, and 3) it is more flexible, i.e., it allows the introduction of new characteristics from other plant species and even animal species and microorganisms.

1. The use of *Agrobacterium tumefaciens* as vector

DNA transference by *Agrobacterium* is the most frequently used method in dicotyledon plant transformation. *Agrobacterium* is a gram-negative bacteria with a Ti plasmid (extra chromosomal DNA) that can transfer one part of its DNA to the plant cell that is being infected, becoming a T-DNA. When the T-DNA is transferred to the plant cell, it produces substances that serve as food for the pathogen,

multiplying the cells and developing tumors or callus. By the genetic manipulation of the Ti plasmid, it was possible to introduce genes of interest in the plasmid transference (T-DNA). Thus, when the *Agrobacterium* infects a cell, it transfers the gene of interest to the transformed cell (Bespalhok, 1999).

As a method to obtain transgenic plants, the *Agrobacterium* and plant interaction system derives from the basic studies associated with plant diseases caused by soil bacteria. Researchers are trying to use the knowledge derived from the plant genetic breeding interaction in the development of pharmaceutical products and in developing plants that protect and improve the environment, as in the case of detoxifying plants (Stalker et al., 1988; Cárceres, 1991).

2-. Protoplast transformation technique

Protoplasts are plant cells without a cell wall that can be transformed by co-cultivation with *Agrobacterium* or by direct DNA introduction. However, there must be pores in the protoplast cell membrane for direct DNA introduction to occur. This can be done by electroporation (amplification of a high intensity electric field) or by the use of certain chemical substances such as the polyethylene glycol. One of the limitations of this technique is the need to regenerate plants from protoplasts which is still impossible in many species (Bespalhok, 1999).

3. Bombardment technique

The particle bombarding technique was reported for the first time in 1987 by Sanford et al. (1987) and has become the genetic engineering method of choice for plant species, which, until recently, could only be treated by genetic manipulation. Many plants have been transformed by this method including vegetables, cereals, forest trees, and ornamental plants. The advantages of the bombarding technique over the alternative gene transference methods and organized tissue transference is the possibility of getting independent transformation of varieties by working with few tissue cultures and cycles with quick responses (Christou, 1996).

The method consists of accelerating microparticles which cross the cell wall of the plasmatic membrane, in a non lethal manner, carrying substances adsorbed from the cell (Klein et al., 1987; Sanford, 1988).

Among the transformation methods such as the protoplast electroporation and the infection by *Agrobacterium*, biolistics, a technique for gene transference to different types of organisms and for efficiently obtaining transgenic plants of different species, has shown greater efficiency. As it is a relatively recent technique, several parameters can be modified and the control of expression and the exogen gene stability should be considered in the transgenic plants and in their progeny (Lacorte et al., 1998).

Example 1
Transgenic carnation (*Dianthus caryophyllus*) plants were obtained after infection of petal explants with the supervirulent *Agrobacterium tumefaciens* strain AGLO. Southern blot techniques confirmed the transgenic nature of four transformed plants. The expression of the gus [uidA] gene was verified in these plants by histochemical assays on selected shoots. It was very difficult to transfer the transgenic plants to the greenhouse due to vitrification and premature flowering (Altvorst et al., 1996).

Example 2
A method is described for producing genetically transformed plants from explants of three scented *Pelargonium* spp. (*P. fragrans, P. odoratissimus* and *P. quercifolia*). Transgenic hairy root lines were developed from *Pelargonium* spp. leaf explants and microcuttings after inoculation with *Agrobacterium rhizogenes* strains derived from the agropine A4 strain. Hairy root lines grew prolifically on a growth regulator-free medium. Transgenic shoots were regenerated from hairy roots and the plantlets were successfully transferred to soil. The phenotype of the regenerated plants was characterized as having abundant root development, more leaves and internodes than the controls, short internodes, and highly branched roots and aerial parts. Southern blot analyses confirmed the transgenic nature of these plants(Pellegrineschi and Davolio-Mariani, 1996).

Example 3
Root explants from *in vitro* grown plants of *Anthurium andreanum* cv. Alii and *Anthurium* interspecific hybrid UH1060 produced multiple shoots under weak light on a modified Murashige & Skoog medium containing 2.2 µM benzyladenine (BA). Regenerated UH1060 plants grew normally and flowered within 16 months after transfer to the greenhouse. Co-cultivation of root cuttings with *A. tumefaciens* plasmid LBA4404 carrying the binary vector pCa2Att resulted in kanamycin-resistant shoots of cv. Aneuneu transgenic for neo and att and recovered more than one year after culture on selection media. Transformation efficiency (number of explants with transgenic shoots per total explants) was 1.3%. Other cultivars (UH1003, UH1060, Rudolph and Mauna Kea) failed to produce shoots under the transformation conditions employed (Chen et al., 1997).

Example 4
Using the biolistics PDS 1000/He system, transgenic lily (*Lilium longiflorum*) plants were developed via microprojectile bombardment with plasmids containing the uidA reporter gene and the phosphinothricin acetyltransferase (PAT) gene of morphogenic calluses derived from bulblet scales, followed by a bialaphos selection. Parameters which gave the highest transient uidA expression were: a bombardment pressure of 1100 psi, a target distance of 6 cm and a 48-h preculture on a medium with 3% sucrose. A total of 1800 morphogenic calluses were co-bombarded with the plasmids. After bombardment, the calluses were exposed to 2 mg bialaphos/litre. Only 72 of the shoot-forming calluses (4%) survived. The 72 shoot clusters produced 342 shoots on a elongation medium containing 0.5 mg bialaphos/litre. Only 55 plantlets survived exposure to 2.0 mg bialaphos/ litre. PCR analysis indicated that 19 of these plantlets contained the PAT transgene. Southern blot analysis of 3 of the plants indicated the presence of the PAT gene (Watad et al., 1997).

Example 5
An *in vitro* plant regeneration system from thin transversal epicotyl sections of orange (*Citrus sinenis* L Osbeck cvs Pera, Valencia and Folha Murcha) was developed. Thin section explants (1-2 mm) from seedlings produced adventitious shoot buds on a MS medium, supplemented with BA (0.5-5 uM). Optimal shoot induction was achieved on 2-5 uM BA. Explants with buds were transferred to elongation media to improve the recovery of normal shoots. Higher number of elongated shoots was obtained on a medium with 0.5 uM BA. Elongated shoots were rooted on a half strength MS medium without growth regulators and transferred to soil for acclimatization. The advantages of this system for transformation are debatable (Bespalhok, 2001)

D. Protoplast fusion

Protoplasts are plant cells without a cell wall, denominated a cell transitory state, obtained in the laboratory. Plant cells may be manipulated similarly to animal and microorganism cells, still preserving the potentials of complete plant cells (Carneiro et al., 1998).

Protoplasts have been widely used in basic plant physiology, molecular biology and cell studies. Research applied to biotechnology includes plant development, gene expression and regulation, biochemical studies, studies on cell wall synthesis and the pathogen host interaction mechanisms in the cells (Carneiro et al., 1998).

Cocking (1960) pioneered protoplast isolation. The author isolated protoplasts from tomato root tips using cells extracted from the *Myrothecium verrucaria* fungus that digests cell walls. Since then protoplasts have been isolated in a range of plant tissues and organs, including leaves (Power and Cocking, 1970), fruits (Raj and Herr, 1973), petioles (Bidney and Shepard, 1980), cotyledons (Kirby and Cheng, 1979), stems (Potrykus et al., 1977), floral pedicels (Flick and Evans, 1983), somatic embryos (Nomura et al., 1982) and cell suspensions (Davey and Power, 1988**)**. Cell suspensions, however, are the most used since they are easy to manipulate and have high isolation efficiency. Embryonic cells are always recommended, particularly in monocotyledon plants (Horn et al., 1988; Shillito et al., 1989; Megia, 1993), when the objective is to regenerate plants from protoplasts.

Several parameters such as the species, the physiological state of the plant, the explant type and age and the isolation conditions of the protoplasts (Carneiro et al., 1998) should be taken into consideration when developing protoplasts. Protoplasts should be manipulated in aseptic conditions. When plants not kept *in vitro* are used, the tissue should be previously disinfected.

Plant cell walls consist basically of cellulose, hemicellulose and pectin where the cellulose fiber and hemicellulose give rigidity to the wall while the pectin maintains the adjacent cells together (Carneiro et al., 1998).

The most used method consists of degrading the components of the wall with pecto-cellulotic enzymes. The physiological state of the plant, the osmotic pressure and the composition of the digestion medium can interfere greatly in this process. The most used enzymes are Onozuka R10 cellulase, extracted from *Trichoderma viride*; Macerozyme R10 pectinase, from *Rhizopus* sp.; Driselase cellulase from the *Irpex lacteus* fungus; and Cellulysin cellulase, Rhozyme hemicellulase and Pectolyase Y23 pectinase. The optimum period for cell wall digestion in enzyme solution should be determined for each genotype, explant type and enzymatic mixture (Carneiro et al., 1998).

Protoplasts must be purified to maintain viability and to avoid that any possible undigested cell groups or cell fragments are removed. Isolated protoplast viability should also be determined by using stains such as Evans Blue (Kanay and Edward, 1973) and phenolsafraine (Karanaratne and Scott, 1981) where the intact protoplasts exclude the stains and the broken protoplasts are permeable to them.

After purification, the protoplasts are cultivated in a nutritive medium containing auxin and cytosine to stimulate cell wall regeneration and division.

The fact that a plant can be regenerated from a single protoplast makes the transformation via protoplast method advantageous compared to the other methods . In addition, it also does not present chimeras.

Plant transformation from protoplasts is carried out mainly by direct free DNA transference systems. These methods of plant genome modification are used in breeding programs and gene expression studies. After the exogenous DNA is introduced, the protoplast cell membrane is destabilized either by treating with glycol polyethylene (PEG) or by applying heat shock (eletroporation). Protoplasts and their derived cells constitute an ideal system to obtain a large number of mutant cells .

The alteration of the genetic material of a cell may be induced by radiation or chemical mutagenic agents. This technique consists in exposing a protoplast culture to a mutagenic agent and then transfer it to a selective medium that favors mutated cell growth but does not permit the growth of original cells.

The dosage of the selective agent can be neither low to the point of letting the wild cultures grow, nor too high as to inhibit the mutated cell growth (Negrutiu et al., 1984). In conclusion, protoplasts are excellent resources used to improve a species by introducing a gene by mutant induction or by introducing interspecific hybrids of incompatible crosses.

Example 1

Wide hybridization that cannot be attained through conventional sexual crosses, can now be handled by somatic hybridization. Protoplasts of *Iris ensata* and *Iris germanica* were fused by electrofusion. For the selection of somatic hybrids, protoplasts of *I. ensata*, which did not develop colonies in protoplast culture, and protoplasts of *I. germanica,* which had regeneration ability for albino shoot only, were used in the symmetric fusion. On the other hand, the protoplasts of *I. ensata* and *I. germanica*, which were inactivated by iodoacetamide (IOA) treatment were used in the asymmetric fusion. Five to six months after cell fusion, green plants were obtained in the symmetric and asymmetric fusion. In the random amplified polymorphic DNA (RAPD) analysis, the green plants had bands specific to both parental species. Therefore, these plants were somatic hybrids between *I. ensata* and *I. germanica* (Shimizu et al., 1999).

Example 2
Cyclamen persicum cv. Sierra Rose protoplasts were isolated from adventitious shoots and *in vitro* regenerated from petiole and leaf explants with yields of 1.3 X 10^6 protoplasts/g fresh weight of tissue. Protoplasts were embedded within agarose lenses bathed in a modified KM8p medium, supplemented with 1.0 mg l^{-1} NAA, 0.1 mg l^{-1} 2.4-\small_cap\D and 0.5 mg l^{-1} BA. Cell division was observed after 4-5 days. After 6 weeks, calluses had grown out of the lenses which were transferred to a modified MS medium for further callus growth. The fastest callus growth took place on the medium containing 0.1 mg l^{-1} NAA and 10 mg l^{-1} TDZ (Morgan, 1999).

Example 3
Effects of glucose concentrations, different sugars and combinations of 2,4-D and kinetin on cell division and colony formation were examined in cultures of protoplasts isolated enzymatically from suspension cultures of *Iris hollandica*. N6 medium was used, supplemented with 1.0-mg 2,4-D, 1.0-mg kinetin, 200.0-mg casein hydrolysate, 250.0-mg proline per litre, 0.3-0.5 M glucose and 20.0-g/litre agarose , suitable for cell division and colony formation. When colonies were transferred to a hormone-free MS medium, many shoots were induced. In addition, when induced shoots were transferred to a MS medium with 1.0 mg NAA/litre, root induction was observed (Hida et al., 1999).

Example 4
Application of micropropagation is reviewed for orchids, flowering tropical species, tropical foliage plants, ferns, bromelias, carnivorous plants and cacti and other succulents. Use of other biotechnological methods such as protoplast fusion and genetic transformation are also discussed (Read et al., 1998)

E. Embryo recovery

Interspecific and intergeneric crosses are frequently carried out in genetic breeding to transfer genes of interest from wild to cultivated species. Incompatibilities are often found in such crosses which result in seeds with abortive embryos (Yeh-Hu and Ferreira, 1998).

Embryo recovery is recommended when carrying out interspecific or intergeneric crosses. The objective of such crosses is to transfer alleles which confer disease resistance, environmental stress tolerance, high yield potential or other desirable characteristics of species or genus to traditionally used cultivars (Cirino and Riede, 1999).

One of the objectives of this technique is to recuperate rare hybrids derived from incompatible crosses as well as to overcome seed dormancy by studying the nutritional and physiological aspects of embryo development and by testing seed viability. These rare hybrids are also a source of explants with high totipotency tissues (Yeh Hu and Ferreira, 1998).

Fertilization occurs and normally results in embryo formation in many interspecific or intergeneric crosses and in crosses between diploids and tetraploids. However, due to bad endosperm formation the embryo degenerates. On the other hand, these embryos can frequently grow and give rise to normal hybrid plants if a supplementary *in vitro* endosperm is provided. The embryo is recovered from the ovule some days after fertilization and cultivated *in vitro*. Some embryos are difficult to dissect. The development of a viable plant from an embryo depends on many factors such as genotype, embryo development stage at the moment of isolation, growth condition of the mother plant, culture medium composition, oxygen concentration, light and temperature (Cirino and Riede, 1999).

The ideal embryo recovery time varies from species to species or from one species cultivar to another. Successful incompatible, interspecific and intergeneric crosses in species were reported by Raghava (1977) and Collins and Grosser (1984).

F. *In vitro* selection

The great advantage of *in vitro* selections is that the unpredictable environment effect is avoided. Another advantage is that a certain genotype can be assessed simultaneously for several characteristics as explants of a specific genotype can be obtained and submitted to different stress factors in several culture media. In addition, a

large number of individuals can be handled easily in a very small space (Cirino and Riede, 1999). However, lack of in depth plant physiology knowledge has made *in vitro* selection more an art than a scientific tool since the results obtained frequently cannot be reproduced (Wenzel and Fouroughi-Wehr, 1993).

According to Cirino and Riede (1999), *in vitro* selection has been widely used in genetically modified plant selection and as well as in the selection of plants derived from mutations and somaclonal variation, whether induced or not.

G. Micropropagation

Flower and ornamental *in vitro* plant tissue culture for propagation was initially developed in England and France in the 1960s. Orchids, chrysanthemums and carnations dominated the initial phase. In fact, the *in vitro* propagation process of the *Cymbidium* genus orchid was optimized by stem apex culture and later regeneration of protocorm that gave rise to many embryos. Each protocorm can regenerate a new plant that, if mutation does not occur, will be exactly the same as the parent (Morel, 1960; 1965). This technique was extended to other genera and found immediate practical application in the beginning of the 1970s. Currently, many commercial laboratories in Europe, North America and Southeast Asia produce millions of orchid plants annually at low cost (Bornman, 1993).

Commercial scale clonal propagation of flowers and ornamental plants was made possible after studies by Holdgate and Aynsley (1977) and Murashige (1974).
In vitro clonal multiplication, also called *in vitro* micropropagation, has caused great impact on commercial seedling production of flowers and ornamental plants.

Micropropagation can be used for the intensive propagation of new varieties and species (Faria et al., 1996), the cleaning of virus and pathogen (bacteria and fungus) which can seriously affect flower and ornamental plant yield and quality, and the maintenance of germplasm and its applications in genetic breeding programs (Tombolato and Costa, 1998).

Furthermore, the certification systems of flower and ornamental plant seedlings are highly dependent on maintaining basic materials where health is a fundamental requirement. Depending on the pathogen affecting the clones, meristem culture techniques can be associated with other complementary techniques such as thermotherapy or chemotherapy (Seregen, 1995).

Nutritive mediums are formed from multiple components and vary greatly according to plant species and explant origin (Faria and Illg, 1995; Illg and Faria, 1995). There is no single formula that can promote and/or maintain optimum growth of different tissues, cells and organs. Formulas by Murashige and Skoog (1962), Quorin and Lepoivre (1977), White (1963), Gamborg (1968) have been used as starting points in several plant species from which significant alterations are made in the components to meet specific cases. Sucrose or any other sugar source is the only component always present in the culture media and its concentration varies from 3 to 12%.
Well nourished mother plants, supplied with sufficient water throughout their growth period until explant removal, give the best results. The physiological stage of the mother plant at the explant removal stage influences the growth and the quality of virus-free shoots. Meristem should generally be removed from new shoots during the active growth phase of the mother plant.

The main explants used in flower and ornamental plant propagation are meristem, bulbs, leaves and seeds. According to Mantell et al.(1994), there is a range of explants that can be as large as seedlings and organs (such as ovule and embryo culture) or as small as isolated cells and protoplasts.

Commercial production laboratories should be managed very carefully and skilled personnel should be carefully selected to perform culture medium preparation, subcultures in light flow chamber and shoot climatization.

The main species of ornamental plants propagated in private laboratories in Brazil are orchids (11 laboratories); *Spathiphyllum* (5 laboratories), *Anthurium* (5 laboratories), violet (5 laboratories) and samambaia (fern) (4 laboratories). Other species that are also micropropagated on a smaller scale are *alstromeria*, amaryllis, banana trees, cyclamen, calla (arum) lilies, carnation, eucalyptus, philodendron, geranium, *gerbera*, gloxinia, Gypsophila, *heliconia*, lily, palm tree, plumbago, statice, syngonium, *zingiberaceas*. Recently, Brazilian research laboratories have been developing and adapting protocols for many ornamental species (Tombolato and Costa, 1998).

RESUMO

Cultura de tecidos no melhoramento de plantas ornamentais: Uma revisão

O melhoramento de plantas ornamentais, assim como o de outras culturas, é considerado a arte e a ciência de alterar geneticamente as plantas em benefício da humanidade. Este trabalho representa uma breve revisão sobre a utilização da cultura de tecidos no melhoramento das plantas ornamentais, tendo como objetivo apresentarr as suas aplicações nesta área. Os tópicos abordados nesta revisão foram: A) indução de poliploidia; B) mutagênese *in vitro* e variação somaclonal; C)transformação genética; D)fusão de protoplastos; E) resgate de embriões; F) seleção *in vitro* e G) micropropagação.

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