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Somatic Embryogenesis and Genetic Improvement of Selected Ornamentals (*Chrysanthemum*, *Euphorbia*, *Caladium* and *Cyclamen*) - A Review

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ABSTRACT

Ornamentals are an important group of plants that include herbs, shrubs and trees of annual and perennial types. Beside propagation of a wide range of ornamentals by plant tissue culture, recently, biotechnological methods have been successfully exploited for their genetic improvement. Various *in vitro* technologies often integrated with conventional breeding methods are somatic embryogenesis (SEG), somatic hybridization by protoplast fusion and genetic transformation which exploits the concept of recombinant DNA technology. In this review, the importance of SEG and the application and events of genetic transformation have been discussed in select ornamentals.

Keywords: genetic transformation, proteomics, somatic embryo
Abbreviations: SE, somatic embryo; **SEG**, somatic embryogenesis

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INTRODUCTION

Ornamentals are an important group of plants cultivated worldwide. They are comprised of herbs, shrubs and trees. These plants are in great demand in both domestic and international markets, generating revenue around US$ 27 billion (Tanaka *et al.* 2005; Xia *et al.* 2006). Nearly about 160 ornamental genera are being commercially propagated in different laboratories by plant tissue culture. Beside propagation, the development of new, novel cultivars is an important target for academic and commercial establishments as peoples' taste and aesthetic values vary with time. Conventional breeding methods such as mutation breeding and selection of sports are by far the main sources of genetic variability in ornamentals. In recent times, however, a range of non-conventional techniques such as protoplast, haploid culture, genetic transformation and other associated technologies have been successfully used to raise new cultivars. Currently, the technique of somatic embryogenesis (SEG) has been utilized in genetic transformation-based crop improvement programmes as it offers several definite advantages.

In this review, SEG, the utilization of SEG culture as a target tissue in developing transgenic has been highlighted taking four important ornamentals as model plants.

SEG AND ITS SIGNIFICANCE

Somatic embryos (SEs) are bipolar structures that develop from a cell other than a gamete or a zygote, which is produced by gametic fusion. They have no vascular connection with the maternal tissue of the explant (Haccius 1978). An SE may develop either directly on somatic cells (direct embryo) or intervening callus which is induced prior to the formation of embryogenic structures (indirect embryogenesis). Although the process has become most familiar in micropropagation with potential applications, it has long been known in nature as adventitious embryony, a type of

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SEG offers advantages in areas like mass clonal propagation, genetic engineering, protoplast culture and production of useful somaclonal variants. Many of these applications started to display their influence on breeding that improved traits of agronomic, fruit and forest tree species, so it likely to serve a major role in genetic improvement of other economic groups of plants, including ornamentals (see extensive reviews in Teixeira da Silva 2006a). Since the initial reports (Reinert 1958; Steward *et al*. 1958) various facts about SEG have unfolded in the literature (Meinke 1995). Information on the origin and development of SEs, structural anomalies of developing embryos, maturation and conversion processes, internal and external signals controlling entire embryogeny have been described in a wide range of monocotyledonous and dicotyledonous plants (Thorpe *et al.* 1995; Mujib and Samaj 2006). However, these *in vitro* steps are not always routine for the majority of ornamental species. Rout *et al*. (2006) reviewed and discussed organogenesis and/or embryogenesis in a number of ornamental pot plants. Somatic cell technologies that utilize organogenesis and embryogenesis in developing plants were described in ornamentals in a few current reports (Li and Pei 2006; Teixeira da Silva 2006a, 2006c; Davey *et al*. 2007).

IMPORTANCE OF SEG

There are several advantages and demerits of SEG in large-scale plant multiplication (Jain 2002). In general, SEG as a tool for propagation of ornamentals shows a number of advantages over conventional vegetative propagation and other micropropagation techniques (Jain *et al*. 2006). As mentioned earlier *in vitro* embryogenesis is either direct or indirect. In direct SEG the SE develops on the explant itself while in indirect embryogenesis, callus first occurs from a cultured explant on which SEs form. Indirect embryogenesis has a higher potential for scale-up propagation as embryogenic cultures may easily be developed into fast-growing cell suspensions (Caligari and Shohet 1993), which also shows simultaneous efficient gene transfer ability. But in direct embryogenic cultures, solitary to clumps of embryos only arise directly on a cultivated explant, which is more difficult to manipulate. However, there are numerous cases of direct embryogenic cultures that proliferated as proembryogenic masses, PEMs (Halperin 1966), and are different from embryogenic callus (Halperin and Jansen 1967). The cultures are amenable to scale-up with the same impossibility of gene transfer as was noted in indirect embryogenic cultures (Merkle *et al*. 1990; Wilde *et al*. 1992). Scale-up of micropropagation using embryogenic or non-embryogenic culture in bioreactors was also attempted in ornamentals with fair success (Ziv and Kipnis 1997). While reviewing the significance of embryogenic cultures it should be kept in mind that the cultures of each species have their own inherent set of genetic make up that may make them receptive to an external cultural environment. Indeed, even different cultivars or genotypes of a particular species can vary widely in their response to a given cultural treatment (Kielly and Bowley 1992; Bailey *et al.* 1993).

Advantages of SEG

There are several advantages of SEG (Merkle 1997; Jain 2002) that facilitate *in vitro* research; some of them are listed below:

1. An unlimited number of SEs may be produced from single culture or explant, a prerequisite for clonal propagation.
2. Thousands of SEs may be produced in agitated liquid medium; more scale up may be possible if bioreactor technologies are applied to embryogenic systems.
3. SE lowers labour inputs; other *in vitro* propagation systems such as shoot elongation or rooting step are not required.
4. The technology has the potential for direct delivery to the greenhouse or field as encapsulated synthetic seeds.
5. SEs may be used as elite genetic material for long-term storage using cryopreservation techniques.
6. SE cultures or SEs may be used in genetic transformation studies directly, thus may efficiently complement other existing efforts of traditional and molecular breeding programme in generating new cultivars at a fast pace.

Disadvantages of SEG

Although reports of SE are numerous but the ability to generate a large number of normal plantlets (conversion frequency) is still not often very high. The common limitations (Merkle 1997; Jain 2002) which restrict *en masse* plantlet production may include:

1. Low frequency of SE production or poor germination rate.
2. Production of malformed SEs.
3. Difficulty in SE maturation.
4. Unpredicted dormancy.
5. Precocious germination with miniature emblings.
6. Indirect embryogenesis may be associated with unwanted new variation in regenerated somaclones.
7. Over-dependence on genotypes.

ORNAMENTALS AND GENETIC TRANSFORMATION APPROACHES

Agrobacterium- and particle bombardment-mediated genetic transformation methods have been used for gene delivery to ornamentals to introduce desirable traits like novel colour, shelf-life, and increased resistance to diseases (Mol *et al*. 1995; Deroles *et al*. 2002; Hammond 2006; Hammond *et al*. 2006; Teixeira da Silva and Tanaka 2008; Teixeira da Silva 2009). This technology has been successfully utilized in the production of new important ornamentals like blue roses (Yoshikazu 2004), novel carnation, transgenic gladiolus (Kamo *et al*. 1997) chrysanthemums, etc. (Teixeira da Silva 2004a). To date, transgenic ornamentals of over 40 genera have so far been produced by different transformation approaches (Hammond 2006; Hammond *et al*. 2006; Li and Pei 2006a). The exploitation of genetic engineering technologies to manipulate plant's genome was recently reviewed by different research groups (Vainstein 2002; Chen *et al*. 2006). It is sometimes rather difficult to accommodate all of these accomplishments in one brief overview, some of the early and current developments are however, important with regards to history and applications. In this present review, the progress has been summarized (**Table 1**) in selected ornamentals.

CHRYSANTHEMUM

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is used as a pot plant; it is also used as cut flower. The plants are propagated vegetatively (cuttings and suckers), which is a slow process while the conventional breeding methods to improve plants show several limitations such as non-availability of enough gene pool and sexual incompatibility (as the ornamentals are being composed of a mixture of polyploids). Non-conventional breeding programmes have been attempted in order to improve traits like flower pigmentation, fragrance, vase-life, etc. *Chrysanthemum* has proven relatively flexible in its response to different biotechnology techniques. In *Chrysanthemum*, the applications and integration of biotechnology into traditional breeding approach were reviewed at length where the uses of *in vitro* techniques to augment mass-scale propagation and the possibilities for improvement have been highlighted (Rout and Das 1997; Teixeira da Silva 2003, 2005). Creation of new flower shape with novel colour is a major breeding target of ornamentals. Aida *et al.* (2008) claimed modified flower shape in *Chrysanthemum* by suppressing the chrysanthemum AGAMOUS (*CAG*) gene, which may be a *C* gene that

Table 1 Genes and vectors used in genetic transformation of selected ornamental plants.

Gene	Vector	Recipient plant	Reference
PnMv-derived hp RNA gene constructs	*Agrobacterium* LBA4404	*Euphorbia pulcherrima*	Clarke *et al.* 2008
Lc	*Agrobacterium* LBA4404	*Caladium bicolor* cv. 'Jackie Suthers'	Li *et al.* 2005
Hgh (Human Growth Hormone)		*C. bicolor*	Li *et al.* 2004
nptII, *GUS*	*Agrobacterium* LBA4404, AGL0	*Dendranthema grandiflora* cvs. 'Lineker', 'Shuhou-no-Chikara'	Teixeira da Silva and Fukai 2002a, 2002b
nptII, *pac*1	*Agrobacterium* LBA4404, AGL0	*D. grandiflora* cv. 'Regan'	Ishida *et al*. 2002
nptII, *GUS*	*Agrobacterium* EHA101	*D. grandiflora* cv. 'Kanseisetsu'	Shirasawa *et al.* 2000
nptII, *RCC2*	*Agrobacterium* C58, MP90	*D. grandiflora* cv.'Yamabiko'	Takatsu *et al.* 1999
nptII, *TSWV N*	*Agrobacterium* EHA 105	*D. grandiflora* cv. 'Polaris'	Sherman *et al.* 1998
gus, NPT II	*Agrobacterium* EHA105	*D. grandiflora* cvs. 'Polaris', 'Hekla', 'Iridon'	Sherman *et al.* 1998
nptII, *Lc*	*Agrobacterium* LBA4404	*D. grandiflora* cv. 'Peach Margaret'	Boase *et al*. 1998
gus, *ocs*	*Agrobacterium* B6S3	*D. grandiflora* cv. 'White Snowdon'	Benetka and Pavingerová 1995
nptII, *CHS*	*Agrobacterium* LBA4404	*D. grandiflora* cv. 'Moneymaker'	Courtney-Gutterson *et al.* 1993
npt II, *GUS*, opines	*Agrobacterium* LBA4404, LBA9402	*D. grandiflora* cv. 'Parliament'	Van Wordragen *et al*. 1992a
gus	*Agrobacterium* AGL0, LBA4404	*Cyclamen persicum* cv. 'Anneke'	Hvoslef-Eide *et al.* 2000
gus	*Agrobacterium* EHA105	*C. persicum* cv. 'Halios'	Sironi *et al*. 2000
gus	*Agrobacterium* LBA4404, AGL0	*C. persicum* cv. 'Anneke'	Aida *et al.* 1999
gus	*Agrobacterium* LBA4404	*C. persicum* cv. 'Sierra Rose'	Boase and Borst 1997
gus	*Agrobacterium* LBA4414	*C. persicum* cv. 'Sierra Rose'	Boase and Borst 1995

disrupted floral determinacy by converting stamen and pistil into corolla-like tissues. Kumar *et al*. (2008) selected resistant callus lines and obtained *Chrysanthemum* plantlets resistance to pathogenic fungus *Septoria obesa*. The effect of NH_4/NO_3, air volume, air temperature, photo flux, inoculum density on the growth and quality of plantlets was recently investigated in bioreactor and identified the key environmental factors which can improve large-scale propagation in *Chrysanthemum* (Sivakumar *et al*. 2005). Teixeira da Silva (2003) reviewed tissue culture applications, which summarized organogenesis and SEG-based plant regeneration in *Chrysanthemum*. Induction of SEs from ray floret and plant regeneration there from in *Chrysanthemum* by utilizing plant growth regulator (PGR) combinations of IAA and KIN was achieved and finally established in greenhouse (Tanaka *et al*. 2000). SEs were earlier induced from leaf midrib (May and Trigiano 1991) in which the concentration of sucrose influenced the morphogenetic response; with low levels (3-6%) produced shoots and root, whereas higher levels (9-18 %) induced only SEs in culture. It was also observed that the SEs did develop from the epidermal or hypodermal layers of the cultured leaf. Although SEs were produced in 12 cultivars (out of 23), plantlet recovery after successful germination of SEs was only achieved in 5 cultivars. The SE-regenerated plants were phenotypically similar to parent plants as leaf morphology, growth and flower colour remained the same. Plant regeneration from transformed calli of *C. grandiflora* via SE was also earlier reported (Pavingerová *et al.* 1994). In *Chrysanthemum*, the genotype is still the key issue to be used as experimental material for embryogenic research. Teixeira da Silva (2004a) reviewed the impact of biotechnology on the improvement of *Chrysanthemum*. Earlier, the technique of promoting axillary shoots or adventitious shoots was reported from various explant tissues; nodal sections were cultured and buds were produced for micropropagation of *Chrysanthemum* (Lawrence 1981). Adventitious shoots were regenerated either directly from the explant or from callus which was induced from ray florets (Bush *et al*. 1976; Khalid *et al*. 1989), leaves (Khalid *et al*. 1989; Bhattacharya *et al*. 1990) and shoot tips (Ben-Jaacov and Langhans 1972; Earle and Langhans 1974a, 1974b), all calli were cultured *in vitro* to produce adventitious shoots. Direct adventitious shoot formation was reported from several cultured explants like petals (Khalid *et al*. 1989), stems (Miyazaki *et al*. 1976; Lu *et al*. 1990) and flower pedicels (Roest and Bokelmann 1975). Histological examination revealed that the adventitious shoots were developed from different tissues such cortical cells of stem in *Chrysanthemum morifolium* 'Ramat.' (Miyazaki *et al*. 1976; Kaul *et al.* 1990), shoots also emerged from individual epidermal cells of flower pedicel in the same cultivar (Broertjes *et al.* 1976). Leaf and stem for a number of different cultivars were evaluated for their regeneration efficiency, stems proved to be more responsive compared to leaf (Kaul *et al.* 1990).

Genetic transformation in *Chrysanthemum*

Several genera including *Chrysanthemum,* which respond and proliferate well in culture, are still proving very difficult to transform. It is a prime criterion that the plant cells need to be transformed show efficient plant regeneration ability, thus are uniquely targeted as tissue for the gene delivery system. *Chrysanthemum* is a good host for *A. tumefaciens* (Smith and Townsend 1907) which is systemic (Jones and Raju 1988). A total of 237 cultivars of *Chrysanthemum* were co-cultured with isolate of *A. tumefaciens* or with strain B6, 12% were noted to be resistant to *Chrysanthemum* isolate, 41% were resistant to B6 while 9% were resistant to both (Miller *et al.* 1975). Screening of cultivars against a range of *Agrobacterium* strains is therefore essential to start with major transformation programme. The first successful report of *C. grandiflora* transgenic plants was regenerated from peduncle callus (Lemieux *et al.* 1990). The idea was conceived with cv. 'Indiapolis White Giant', transformed with *A. tumefaciens* strain LBA4404 (Hoekema *et al*. 1983), contained the binary vector pJJ3499 incorporating *nptII gus* A gene. In another approach transgenic plants of cv. 'Moneymaker' were produced with vectors that had cDNA of the *Chrysanthemum chs* gene, constructed either sense or antisense fashion. The transformed plants produced violet and white flowers, as against pink produced by parent, flower colour changes were attributed to the insertion and the expression of the introduced genes as was indicated by over accumulation of marker acids like caffiec and coumaric acid resulting from a block at chalcone synthase (CHS).

There are several other reports of *Chrysanthemum* genetic transformation using *Agrobacterium*-based gene vectors (Ledger *et al.* 1991; Van wordragen *et al*. 1992a, 1992b; Lowe *et al*. 1993; Kudo *et al.* 2002). Three cultivars of *C. grandiflora* were similarly transformed with *Agrobacterium* EHA 105 (pB1121) (Urban *et al*. 1992), an efficient, high frequency transformation protocol for cvs. 'Iridon', 'Hekla' and 'Polaris' were later established. The transformed shoots were rooted on medium with 50 mg/ml kanamycin. *Agrobacterium*-mediated transformation and regeneration from leaf explants in *C. grandiflora* was achieved (Seo *et al*. 2003) and four different methods of gene transfer for stable transgene expression in *Chrysanthemum* was efficiently

attempted with a 2- to 10-fold increase in transformation efficiency (Teixeira da Silva and Fukai 2002a, 2002b). The genotype, low regeneration rate, variable shoot regeneration capacity on antibiotics added selection media are some of the limitations require to be resolved before large-scale applications. The use of different antiobiotics (Teixeira da Silva and Fukai 2004) or carbon sources (Teixeira da Silva 2004b), however, plays a negative role in the efficiency of the regeneration of chrysanthemum.

EUPHORBIA (POINSETTIA)

Euphorbia pulcherrima Willd. is one of the most popular household plants and is a native of Central America; this fast growing plant is grown in other parts of the world as well. It has brilliant coloured bracts ranging from scarlet, crimson, yellow, red and white. The ability of these spectacular bracts to remain fresh and intact for longer period of time may add its value more as an ornamental. The latex of *E. pulcherrima* was reported to be poisonous to livestock (Anonymous 1978). However, in veterinary medicine it is used to kill maggots in the wounds of livestock.

This shrub is conventionally propagated by seeds and cuttings, however, seeds lose their viability on storage while propagation is highly seasonal, and cuttings take about 6-8 weeks to root (Sochacki and Chimid 1994). These methods of clonal propagation are not sufficient to meet the growing demand. An *in vitro* study on *E. pulcherrima* was reported as an alternative (Langhe *et al.* 1974). An efficient method of plant regeneration was recently established in *Euphorbia* (Xu *et al.* 2008) where the effect of various cultural conditions on the induction of callus and shoot regeneration was evaluated. Castellanos *et al.* (2008) reviewed the biotechnological applications on *Euphorbia* in which the role of genetic engineering and protoplast culture was highlighted. In another effort, SEs were obtained from stem nodal explant on α-naphthaleneacetic acid (NAA) and isopentenyl adenine (2-iP)-amended MS medium (Castellanos *et al.* 2006). Earlier, embryogenic and non-embryogenic cultures were analysed and compared (Brandau *et al*. 1997): embryogenic cultures were rich in extracellular proteins, which also showed higher peroxidase activity compared to non-embryogenic cultures. The activity and behaviour of polyphenol oxidase (PPO, E.C. 1.10.3.1) of various tissues, including embryogenic and non-embryogenic cell suspension cultures of *E. pulcherrima*, was investigated (Carotin *et al.* 1995). Embryogenic cells showed high PPO activity and demonstrated a strong membrane bound activate potential enzyme affinity. Non-embryogenic materials revealed low PPO activity with poor activation potential.

In *E. pulcherrima* young nodal explants were used as explant and cultured on MS with various combinations of auxins (NAA and 2,4-dichlorophenoxyacetic acid (2,4-D)) and cytokinins (kinetin (KIN), isopentyladenine); 2iP at 9.8 µM and NAA at 2.69 µM yielded maximum amount of embryogenic callus (Razdan 1993). The induction of callus was observed at the cut surfaces of the explant and clumps of SEs were induced on friable and red-pigmented callus while white, compact callus failed to produce SEs. The former embryogenic callus continuously produced more SEs on the same medium. These SEs germinated and produced well-developed plants on MS amended with 9.8 µM 2iP and 4.03 µM NAA, and were transplanted to the field with 87% of the regenerants showing normal morphological characteristics. A similar report of SEG in *E. pulcherrima* was achieved from hypocotyls on MS when amended with 2.0 mg/l indole-3-acetic acid (IAA); nearly 1400 embryos were induced from calli (Osternack *et al.* 1999) in which only 8% were developed into normal plantlets. In most cases shoots were rooted in PGR-free medium.

A cell suspension culture of *E. pulcherrima* was initiated to produce SEs by culturing the cells in medium containing 2,4-D and 6-benzyladenine (BA) (Kleffel and Preil 1986). An approach with vibro-mixer design equipped with silicone tubes for bubble-free aeration was established that produced 100,000 *E. pulcherrimma* SEs/l of suspension when the suspension was plated on filter paper and kept for 4 weeks (Preil *et al* 1988). Raising the dissolved oxygen level in the bioreactor from 10 to 60% dramatically increased cell proliferation rates, although a direct relationship of dissolved oxygen with SE production was not established. In another effort, Luttman *et al*. (1994) used silicone tubing system for oxygenation of different bioreactor types to promote growth of poinsettia and *Clematis tangutica* embryogenic cultures.

Genetic transformation in *Euphorbia*

In poinsettia there are only a few reports describing genetic transformation: one was the US patent 7119262, filed by Smith *et al*. (1997) using biolistic transformation approach, while the other two were electrophoresis based transformation attempts (Vik *et al*. 2001; Clarke *et al*. 2006). *Agrobacterium*-mediated transformation in *Poinsettia* was recently developed (Clarke *et al*. 2008) where internodal stem explants (5-15 mm long) from cv. 'Millennium' were excised and used to produce SEs prior to transformation. The induced SEs were matured in medium when amended with 0.05 mg/l BAP and 30 g/l sucrose. Later roots were induced on ½ MS supplemented with 2 mg/l IAA, 20 g/l sucrose or on PGR-free ½ MS supplemented with 20 g/l sucrose (Clarke *et al*. 2008). The disarmed *A. tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) was utilized in which 3 virus-derived hairpin (hp) RNA gene constructs named pCP, pR2 and pR3 were inserted into appropriate restriction sites to induce RNA silencing-mediated resistance to *Poinsettia mosaic virus* (PnMV) (Clarke *et al.* 2008). The internode explants were used and co-cultured with *Agrobacterium* for 5 min, about 3.5% transformation efficiency was registered with pR3.

Biolistic transformation requires the use of gene gun device which tends to generate transformants with high transgene copy number, complex transgene loci and unpredictable silencing of the transgene (Herrera-Estrella *et al.* 2004). Electrophoresis of DNA into living meristem was described as a simple method to generate transformants by avoiding tedious tissue culture work and was utilized in producing transgenic orchid (Griesbach 1994). However, no stable transgenic poinsettia was ever produced with electrophoresis regardless of the strong transient expressions that were detected in both studies (Vik *et al*. 2001; Clarke *et al.* 2006).

CALADIUM

Caladium is an important ornamental comprising 12 species. Caladiums are widely distributed but many of the genera are only restricted to New World Tropics (Bown 1988); these are mostly used as landscape and potted plants. Wilfret (1986, 1993) reported over 2000 cultivars in *Caladium* but the identification of such cultivars is difficult as several of them show similar kind of leaf morphology. Characterization of caladiums has not been made at the molecular level except a report of amplified fragment length polymorphism (AFLP)-based identification of *Caladium biocolor* cultivars (Loh *et al.* 1999) where two different species of *Caladium* were studied that distinguished the species (including six cultivars) by their unique and different banding patterns.

These *Caladium* cultivars are propagated asexually but can also be propagated by seeds. Hybridization produces new cultivars in sexually propagated plants but in ornamentals the application of sexual breeding methods is rather limited (Repellin *et al.* 2001). The need to improve the genetic base of *Caladium* has been felt lately as the cultivars are very susceptible to a number of viruses (Zettler and Hartman 1987; Lesemann and Winter 2002; Rivas *et al*. 2005). Several methods including molecular and biotechnological tools have been used to raise plants with new extra traits, these include foliage colouration, growth habit, tuber

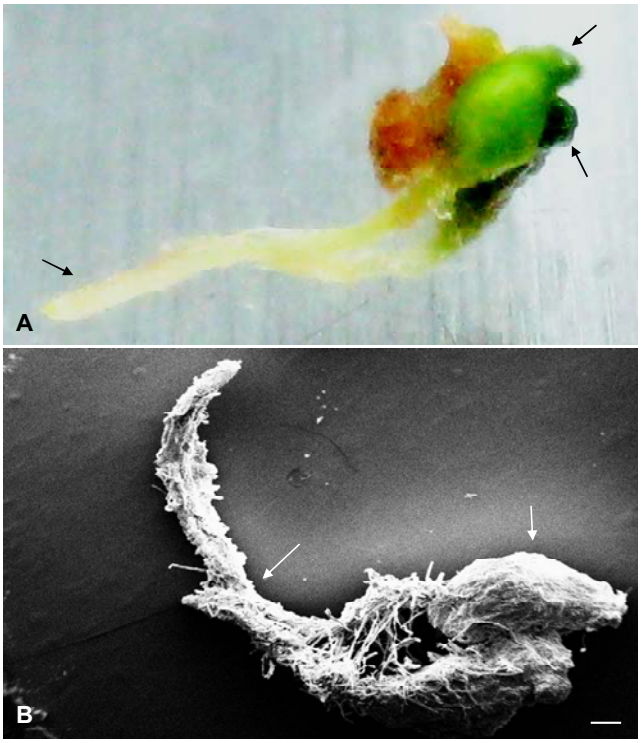

Fig. 1 Isolated somatic embryos in *Caladium bicolor* cv. 'Bleeding Heart' (**A**) Somatic embryo showing green cotyledon and root ends (arrowheads). (**B**) Scanning electron microscopic view of somatic embryo (arrowheads).

production, disease and pest resistance (Wilfret 1993). In order to apply biotechnological method an efficient plant regeneration system is essential that could generate true to type plant and transgenic in number and at fast pace. Tissue culture derived plantlet variation was obtained earlier in *C. biocolor* where the regenerated plants showed altered plant morphology, regenerated plant populations from rhizome calli also demonstrated a wide range of variation in chromosome number (Mujib *et al.* 2000, 2008) that could be used as important raw material to develop a new cultivar. In this plant, 2,4-D induced profuse callus from various explant sources, NAA and BAP combinations were also observed to be very effective in inducing callus and in the same PGRs levels, a steady callus growth was obtained (Mujib *et al* 2000). In our earlier reports, we mentioned that these groups of plants responded better to a variety of NAA and BAP combinations compared to other PGRs applied (Mujib *et al*. 1991, 1996, 1998). The regeneration ability was also very high as BAP alone or in combination with NAA produced a large number of shoots. Similar effect of PGRs in promoting shoots from apical meristem was recently observed in MS, amended with 1.0 mg/l BAP + 0.25 mg/l NAA (Ali *et al.* 2007). Cultured leaves of *Caladium* produced SEs; morphologically the embryos were bipolar, 'mango-like' structures with a cotyledonary and root axis; the cotyledonary end shows a broad, swollen base with a narrowed apex (**Fig. 1A, 1B**). The origin, maturation and germination of somatic embryos were all completed on the same embryo induction medium (**Fig. 2A, 2B**). In agitated liquid medium, the growth of shoot was even better compared to solid (**Fig. 2C, 2D**). Attempt was made to sterilize culture medium with a disinfectant calcium hypochloride, instead of autoclaving, which suppressed microbial activity in culture but, did not affect shoot multiplication in *C. bicolor* (Ahmad *et al.* 2007).

Genetic transformation in *Caladium*

A protocol was established in *C. bicolor* that showed enhanced anthocyanin synthesis when the maize anthocyanin regulatory gene *Lc* was introduced into an albino cultivar of

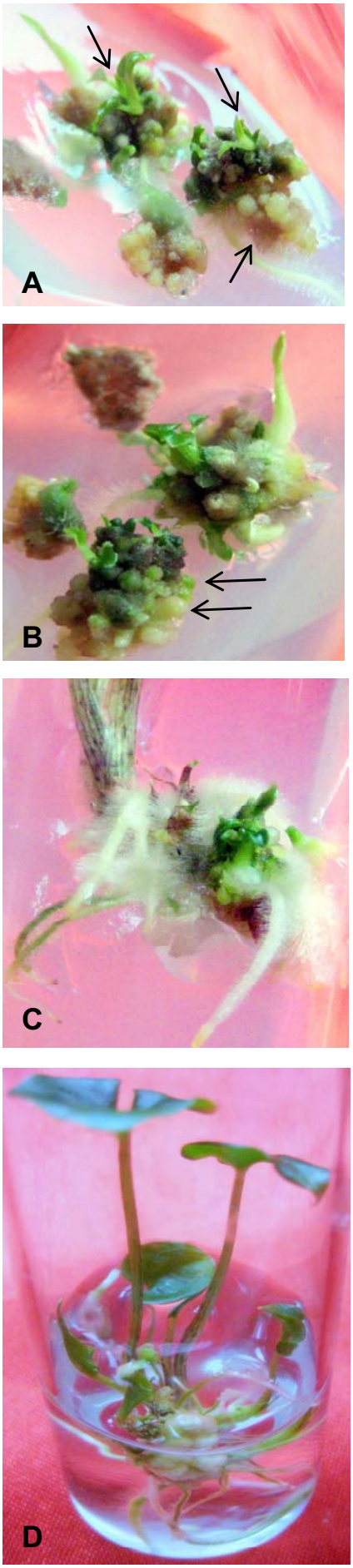

Fig. 2 Plant regeneration in *Caladium bicolor* cv. 'Bleeding Heart' by somatic embryogenesis pathway. (**A, B**) Somatic embryos (arrowheads) developed on leaf callus. (**C, D**) Plantlet regeneration in solid and in liquid medium.

Caladium by using *Agrobacterium* (Li *et al.* 2005). Similarly, a genetic transformation approach was previously attempted with a human growth hormone gene which expressed in regenerated *Caladium* transgenics (Li *et al*. 2004). The efforts to induce *Caladium* transgenics is however, less and more and more attention is needed as to improve this clonal group of plants.

CYCLAMEN

Cyclamen is a member of the family Primulaceae, and is used as a common pot plant in Europe. It is also found in Mediterranean region and extends to north and east of it. *Cyclamen* is propagated sexually as vegetative propagation (e.g. via cuttings) is either slow or has several limitations. *In vitro* clonal propagation has been considered as a good alternate method for propagation of true-to-type plant. SEG of *C. persicum* is a well developed system for mass propagation of plants; the same system is also used to study *in vitro* SEG, particularly in dicotyledonous plants. The first incidence of SEG in *Cyclamen* was reported in 1984 (Wicart *et al*. 1984), since then it has been induced in various cultivated tissues, i.e. callus derived from anthers, ovaries and zygotic embryos (Kiviharju *et al*. 1992). Hohe *et al*. (2001) and Schwenkel (2001) also reported *in vitro* propagation of *C. persicum* using embryogenic cell suspension culture. Mass-scale propagation of *Cyclamen* via SE was highlighted lately (Püschel *et al*. 2003). More recently, experiment on desiccation of *Cyclamen* somatic embryo for developing artificial seed was conducted as to preserve germplasm (Winkelmann *et al*. 2004; Seyring and Hohe 2005).

The *in vitro* embryogenic response in *Cyclamen* was earlier noted to be depend upon specific genotype (Takamura and Tanaka 1996; Schwenkel and Winkelmann 1998), suggesting thereby a genetic control over regeneration. In 4 out of 13 *Cyclamen* cultivars, the differentiation of SEs were observed (Takamura and Miyajima 1997); Schwenkel and Winkelmann (1998) reported 29 genotypes (out of 30) with SE-inducing ability when various genotypes were tested for their *in vitro* responses. In one of the studied cultivars, ovules from closed flower buds were used as explants on which embryogenic calluses were induced. In another genotype, induced callus was observed to be very soft, could be propagated for long term by using suspension (Winkelmann *et al.* 1998) or involving bioreactor (Hohe *et al*. 1999a, 1999b; Hvoslef-Eide 2005). A second genotype RS8, was included in several experiments; although callus was formed on cultured ovules, it never differentiated into SEs (Püeschel *et al.* 2003). Variable DNA content was observed in the regenerated plants of *Cyclamen* which were regenerated via SEG (Borchert *et al.* 2007).

A plant regeneration protocol from protoplast was attempted from embryogenic suspension in *Cyclamen* by enzymatic digestion with cellulase and macerozyme (Winkleman *et al.* 2006). Plantlets were successfully regenerated from protoplasts via SEG and transferred to soil. A similar approach was earlier made to establish protoplast culture from *in vitro*-raised adventitious shoots that showed fast cell division and produced callus (Morgan 1999).

Analysis by Expressed Sequence Tag (EST) transcript of embryogenic *Cyclamen* tissue was conducted by Rensing *et al*. (2005), who screened a total of 2083 ESTs (499 average base length), transcripts were annotated, compared the homology with the genes associated with SEG. They observed a high proportion of homology to genes involved in carrot SEG was also found in *Cyclamen* transcript collections.

Shoot formation was first achieved from tuber segments on MS when supplemented with 1.1 µM NAA (Mayer 1956). Subsequently similar response was also observed by other working groups (Okumoto and Takabayashi 1969; Pierik 1975) from the same tuber explant. Geier (1977) noted that the shoots and roots were formed on medium containing 14.3-28.6 µM IAA and 0.9-2.3 µM KIN, other explants were observed to be less responsive and showed poor morphogenetic potential compared to tuber tissue. Since then *in vitro* cloning of *C. persicum* has been continuously accumulated in literature (Geier 1978; Geier *et al*. 1983; Dillen *et al.* 1996; Schwenkel 2001).

Genetic transformation in *Cyclamen*

Genetic engineering technologies provide opportunities to insert novel characters into *Cyclamen* but reproducible reliable gene transfer systems need to be established first for the target cultivars. There are several previous studies on genetic transformation of *C. percisum*. These include transient GUS expression in hypocotyls and cotyledons in cv. 'Sierra Rose' after inoculation by the armed *A. tumefaciens* strains C58 and A281 and the disarmed strains LBA4414, all containing PKIWI110 (Boase and Borst 1995). The production of plants with tumours expressing GUS, 20 days of inoculation in 'Sierra Rose' by strain A28 with pKIWI110 was also reported (Boase and Borst 1995). Subsequently transformation as evidenced by GUS expression in etiolated hypocotyl with strain LBA4404 with binary vector pMOG410 was available. Likewise, transient GUS expressed etiolated hypocotyls of 'Sierra Rose' infected by different strains of *A. tumefaciens* (A281, A772, EHA 105 and LBA4404) harbouring plasmid pMOG410 or pTOK233 were reported (Boase and Borst 1997). The disarmed strain LBA4404 gave the highest gene transfer frequencies in these experiments. Transgenic *C. persicum* has been produced from etiolated petiole segments of 'Anneke' using *Agro* strains AGL0 and LBA4404 with the binary vector pIGI21Hm (Aida *et al.* 1999). Explants were precultured and later co-cultured with *Agrobacterium* in regeneration media containing 100 µm acetosyringone for 6 days. GUS-positive transgenic shoots were selected with 5 mg/l hygromycin or with 100 mg/l kanamycin in regeneration medium added with 1 mg/l TDZ, 1 mg/l 2, 4-D and 300 mg/l ticarcillin. Transformation efficiencies based on GUS-positive plant ranged from 0% with strain LBA4404 in 100 mg/l kanamycin aided selection to 19% with strain AGL0 and 5 mg/l hygromycin. The expression of the *gus* gene under 35S CaMV promoter in suspension cells of 'Purple Flame', two days after bombardment with plasmid p1515 coated with gold particles was earlier reported (Hvoslef-Eide *et al.* 2000). Plantlets of 'Halios' expressing the *gus* gene and showing resistance to kanamycin could be regenerated after transformation with *Agrobacterium* strain EHA105 carrying pKIWI105 or strain GV2260 carrying the *gus* gene (Sironi *et al*. 2000). Etiolated petiole explants were co-cultivated for two days with *Agrobacterium* and then transformants were screened on selected medium aided with 100 mg/l kanamycin and 100 mg/l cefotaxime. Transgenic plantlets were regenerated via SE in PGR-free medium.

Proteomics in *Cyclamen*

In order to understand the proteomics of SEG, total proteins were characterized in embryogenic and non-embryogenic cell lines. MALDI-TOF-MS analysis detected about 1200 cyclamen proteins, of which 943 proteins were present in both lines, while about 27 protein spots were proposed as SE-specific proteins (Lyngved *et al*. 2008). Winkelman *et al*. (2006) studied systemic comparison of the proteomes of zygotic embryos, SEs and endosperms, 74% of the proteins expressed in zygotic embryos were found to be similar in abundance with SEs that were cultivated in medium with 60 g/l sucrose. Following mass spectrometry (nano-LC-MS/MS), four enzymes (UDP glucose pyrophosphorylase, fructose biphosphate aldolase, triosephosphate isomerase, and glyceraldehyde 3-phosphate dehydrogenase) were specifically identified in SEs. Similarly, one protein 11S globulin was present in high levels in all three studied samples of SEs, zygotic embryos and endosperms. Other protein 7S globulins were detected in endosperm and zygotic embryos but not in SEs; xyloglucan, a seed storage compound was

found to be highly accumulated in endosperm tissues only. Proteomic studies, of which there are not too many in ornamentals (Hajduch *et al.* 2008), may help to understand the seed and germination physiology (including somatic embryo) of plants.

CONCLUSION

Biotechnological technologies offer several important avenues for the genetic improvement of plants. In ornamentals, in particular, improvement focuses on specific traits like alteration of morphology and form, flower sizes with their improved vase life, persistent fragrance, modification of floral pigmentations, etc. Although genetic transformation has been practised in a wide array of ornamentals (reviewed in Teixeira da Silva 2006b) the number of transformed plants is not very high compared to other model groups of plants such as cereals, fruits or vegetables. In this modification approach the early embryogenic cells, suspensions or isolated protoplasts may act as potential recipient tissues for up taking foreign DNA to generate genetically manipulated ornamentals. The modification of floral pigment has been however, considered to be a difficult task as it requires information of complete and complex metabolic pathways, the understanding of the enzyme's regulation in developing and distribution of pigments which determine flower colour. Similarly, alteration of ethylene biosynthesis may prolong vase life of cut flowers. Except a few preliminary reports, information on modification of flower pigment and improved vase life are unfortunately very limited (e.g. Satoh *et al*. 2006). Molecular analyses of genes which transform vegetative cells into embryos especially in ornamentals are still found inadequate where proteomics can play an important role in unravelling some of the fundamental basics of ornamental biotechnology.

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