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Advances in *Alstroemeria* Biotechnology

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Keywords: genetic transformation, interspecific hybridization, tissue culture

ABSTRACT

The genus *Alstroemeria* belongs to the family Alstroemeriaceae and comprises many ornamental species. This genus, including more than 60 species, is indigenous to South America. Thus far, numerous cultivars, which are used as cut flowers and potted plants worldwide, have been produced by interspecific hybridization and mutation breeding. Recently, biotechnological approaches are being applied in order to improve *Alstroemeria* strains. Interspecific hybrid plants have been produced by ovule cultures. By improving certain culture techniques, sexual incompatibility was overcome in some cross combinations using ovule cultures. Plant regeneration systems that involved the use of explants, immature ovules, leaves, etc., through callus cultures have been reported. Isolation of protoplasts and cultures resulting in plant regeneration were achieved by using the embryogenic callus. Particle bombardment and *Agrobacterium*-mediated procedures were applied for genetic transformation, and some transformed plants with marker genes were produced. The procedure of *in vitro* fertilization using single isolated gametes has been developed to study fertilization and early zygotic embryogenesis in higher plants. This technique will also be utilized as a novel strategy in plant breeding for inducing the fusion of gametes obtained from distantly-related incompatible species and for achieving direct gene transfer into isolated zygotes. In the case of *Alstroemeria*, isolation of egg cells and zygotes from ovules has been attempted in order to develop an *in vitro* fertilization technique.

1. INTRODUCTION

The Alstroemeriaceae family has originated from South America and includes 4 genera – *Alstroemeria*, *Bomarea*, *Leontochir* and *Schickendantzia*. *Alstroemeria*, particularly, is an important ornamental crop used for cut flowers and pot flowers throughout the world. According to Hofreiter and Rodríguez (2006) and Hofreiter and Tillich (2002), the first species of *Alstroemeria* and *Bomarea* were discovered by Feuillée (1714) in Chile and were classified as *Hemerocallis*. Linné (1762) then named them *Alstroemeria ligtu* (**Fig. 1A**), *A. pelegrina* (**Fig. 1B**) and *A. salsilla* after his colleague Claes Alstroemer. Among these species, *A. salsilla* was revised as *Bomarea salsilla* (**Fig. 1C**) by Mirbel (1804).

Alstroemeria was initially classified under the family Amaryllidaceae based on the presence of the inferior ovary. Dumortier (1829) proposed the family Alstroemeriaceae. Hutchinson (1964) also described the family Alstroemeriaceae in the taxonomic book 'The genera of flowering plants'. Karyotype analysis by means of chromosome banding techniques supported the separation of Alstroemeriaceae from Amaryllidaceae (Lakshmi 1976). Based on these studies, Alstroemeriaceae is recognized as an independent family comprising *Alstroemeria*, *Bomarea*, *Leontochir* and *Schickendantzia*.

The distribution of the genus *Alstroemeria* was studied by Bayer (1987) and Aker and Healy (1990). Hofreiter and Rodríguez (2006) summarized the countries in which these species occurred and the number of wild species of *Alstroemeria* based on previous reports by Gereau (1994), Sanso (1996), Assis (2002), Harling and Neuendorf (2003) and Muñoz Schick and Moreira Muñoz (2003). At present, there are 39 species in Brazil; 33 species in Chile; 10 species in Argentina; 2 species in Peru; and 1 species in Bolivia, the Guianas, Paraguay, Uruguay and Venezuela (Hofreiter and Rodríguez 2006). Among the wild species of *Alstroemeria*, *A. ligtu* (**Fig. 1A**), *A. pelegrina* (**Fig. 1B**), *A. aurea* (**Fig. 1D**), *A. magenta* (**Fig. 1E**), *A. psittacina* (**Fig. 1F**), *A. inodora*, *A. pulchra*, A. *violacea*, *A. versicolor* and *A. caryophyllaea* are presumed to be the parents of extant cultivars or candidates for further breeding.

An increased production of *Alstroemeria* cultivars worldwide has recently been noted, particularly in the Netherlands, US and Japan since these plants have favourable characteristics such as high ornamental value, long vase life, variation in flower colour and low energy input for cultivation. In order to cultivate *Alstroemeria*, biotechnology-based procedures have been developed in addition to conventional breeding techniques. In this chapter, I overview the advances in biotechnology with regard to *Alstroemeria* cultivation and discuss the prospects for *Alstroemeria* breeding using novel techniques.

Abbreviations: BAP, benzylaminopurine; **DAPI**, 4',6-diamidino-2-phenylindole; **GUS**, β-glucuronidase; **HPT**, hygromycin phosphotransferase; **MS**, Murashige and Skoog (1962) medium; **NPTII**, neomycin phosphotransferase II; **PI**, propidium iodide; **PPT**, phosphinothricin

Fig. 1 Representative flowers of the Alstroemeriaceae family. (**A**) *Alstroemeria ligtu*; (**B**) *A. pelegrina* var. *rosea*; (**C**) *Bomarea salsilla*; (**D**) *A. aurea*; (**E**) *A. magenta*; (**F**) *A. psittacina*.

1.1. Development of ovule cultures for interspecific hybridization

Generally, interspecific hybridizations fail due to post-fertilization barriers or inhibition of pollen tube elongation. By using fluorescence microscopy, de Jeu and Jacobsen (1995) observed the entry of pollen tubes into ovules and the subsequent fertilization and embryo development in interspecific diallel crossing among *A. aurea*, *A. hookeri*, *A. pulchra*, *A. ligtu*, *A. pelegrina*, *A inodora* and *A. brasiliensis*. They described the post-fertilization barrier-induced incompatibility in many interspecific hybridizations of *Alstroemeria*. This result indicated that embryo rescue before abortion is effective in obtaining successful interspecific hybridizations. To ensure success of embryo rescue, many culture conditions were examined, such as using various sucrose concentrations, culture temperatures and basal media. **Table 1** lists the manuscripts that have described successful interspecific hybridizations in *Alstroemeria*.

Buitendijk *et al*. (1995) reported that embryo rescue by half-ovule culture was effective in producing interspecific hybrid plants from diallel crosses of *A. aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora* and *A. psittacina*. They reported that germination of hybrid embryos was induced by harvesting ovules before the onset of endosperm degeneration and preparation of the ovules by cutting them into halves. The alternative culture conditions were as follows: harvesting the ovules 14 days after pollination, removing the chalazal region and using a liquid medium containing 1/4× strength Murashige-Skoog (MS) (Murashige and Skoog 1962) macronutrients, full strength MS micronutrients, vitamins, 400 mg l^{-1} casein hydrolysate and 60 g l^{-1} sucrose; incubation was carried out on a rotary shaker at 21°C in the dark. By using this procedure, a total of 260 hybrid plants were successfully produced across all cross combinations.

de Jeu and Jacobsen (1995) reported that early post-fertilization ovule culture could overcome barriers to interspecific hybridization. They

Table 1 List of manuscripts describing interspecific hybridizations in *Alstroemeria*.

Cross combination	Days after pollination	Culture condition	Reference
Diallel cross *A. aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora*, *A. psittacina*	14	1/4× MS macronutrients MS micronutrients and vitamins 400 mg l^{-1} casein hydrolyzate 60 g l^{-1} sucrose 21°C dark liquid culture on rotary shaker	Buitendijk *et al*. 1995
Diallel cross *A. aurea, A. hookeri, A. pulchra, A. ligtu, A. pelegrina, A. inodora, A. brasiliensis*	2	MS medium 9% sucrose 21°C, 12 h photoperiod	de Jeu and Jacobsen 1995
A. violacea × *A. pelegrina* var. *alba* *A. violacea* × *A. pelegrina* var. *rosea* *A. violacea* × *A. ligtu* ssp. *incarnata* *A. pelegrina* var. *alba* × *A. violacea* *A. violacea* × *A.* 'Rosy Wing' *A.* 'UC12' × *A. violacea* *A. gayana* × *A. violacea* *A.* 'ER292' × *A. pelegrina* var. *alba* *A.* 'UC12' × *A. pelegrina* var. *alba*	7	MS medium 146 mg l^{-1} glutamine 30 g l^{-1} sucrose 6.5 g l^{-1} agar 18°C ± 1°C dark	Lu and Bridgen 1996
A. ligtu hybrid × *A. pelegrina* var. *rosea*	7, 14, 21, 28, 35	MS medium 3% sucrose, 2 g l^{-1} gellan gum 20°C, 16 h photoperiod	Ishikawa *et al*. 1997
A. pelegrina var. *rosea* × *A. magenta*	7–14	MS medium 3% sucrose, 2 g l^{-1} gellan gum 20°C, 16 h photoperiod	Ishikawa *et al*. 2001
Diallel cross *A. angustifolia*, *A. aurea*, *A. diluta*, *A. garaventae*, *A. hookeri*, *A ligtu*, *A. magnifica*, *A. magenta*, *A. pelegrina*, *A. presliana*, *A. pulchra*, *A. versicolor*, *A. zoellneri*, *A. inodora*, *A. psittacina*	14	1/2 MS medium 3% sucrose, 2 g l^{-1} gellan gum 20°C, 16 h photoperiod	Shinoda and Murata 2003

provided data on 7 *Alstroemeria* species, 5 from Chile (*A. aurea*, *A. hookeri*, *A. pulchra*, *A. ligtu* and *A. pelegrina*) and 2 from Brazil (*A. inodora* and *A. brasiliensis*). Based on histological observations of developing embryos and endosperm in interspecific crosses, they employed the early post-fertilization ovule culture technique, in which young ovules were cultured *in vitro* 2 days after pollination. This technique produced seeds that were a cross between *A. inodora* and *A. pelegrina*, as well as in reciprocal crosses between *A. inodora* and *A. brasiliensis*.

Lu and Bridgen (1996) provided detailed data on the effect of genotype, developmental stage of an embryo and the culture medium on interspecific hybrids of *Alstroemeria*. They reported that harvesting ovules 7 days after pollination and culturing them on MS medium (Murashige and Skoog 1962) supplemented with 146 mg l^{-1} glutamine and 30 g l^{-1} sucrose resulted in a higher rate of embryo germinations (53.3%) in responsive genotypes. Furthermore, Lu and Bridgen (1997) succeeded in producing amphidiploid plants that were crosses between *A. aurea* and *A. caryophyllaea* through interspecific hybridization and chromosome doubling with colchicine treatment. As a result, the fertility of the amphidiploid plants was not restored, although an increased pollen fertility of up to 12% was observed when examined using aniline-blue-lactophenol.

Ovule cultures have been applied for other cross combinations, e.g., *A. ligtu* hybrid × *A. pelegrina* var. *rosea* (Ishikawa *et al*. 1997) and *A. pelegrina* var. *rosea* × *A. magenta* (Ishikawa *et al*. 2001). They harvested the ovules 7-14 days after cross pollination and cultured them under a 16-h photoperiod at 20°C on 2 g l^{-1} gellan gum-solidified MS medium (1962) supplemented with 30 g l^{-1} sucrose. In order to restore fertility, amphidiploid plants were produced by colchicine treatment (Ishikawa *et al*. 1999) from interspecific crosses of *A. ligtu* hybrid and *A. pelegrina* var. *rosea*. The results showed that the amphidiploid plants could produce seeds by self-pollination and the progenies revealed amphidiploid characteristics. Moreover, the amphidiploid plants that were crossed with *A. ligtu* hybrid, *A. pelegrina* var. *rosea*, *A. aurea*, *A. paupercula* and *A. psittacina* produced triploid progeny plants.

Recently, Shinoda and Murata (2003) carried out great number of cross hybridizations through ovule cultures using *A. angustifolia*, *A. aurea*, *A. diluta*, *A. garaventae*, *A. hookeri*, *A. ligtu*, *A. magnifica*, *A. magenta*, *A. pelegrina*, *A. presliana*, *A. pulchra*, *A. versicolor*, *A. zoellneri*, *A. inodora* and *A. psittacina*. The ovule cultures after diallel crossing showed interspecific incompatibility in many combinations; hybrid embryos germinated in 196 out of 210 crossing combinations. In particular, *A. angustifolia*, *A. ligtu*, *A. magenta* and *A. pelegrina* showed high cross-compatibility as ovule parents. Therefore, *Alstroemeria* breeding through interspecific hybridization has been successful. The present detailed study will contribute in producing interspecific hybridization in *Alstroemeria*, resulting in progress in breeding novel cultivars.

Consequently, the most reliable embryo rescue techniques for the production of interspecific hybridizations in *Alstroemeria* are summarized as follows:

(1) Ovaries are harvested 2-14 days after pollination.

(2) Ovules are cultured on 2 g l^{-1} gellan gum-solidified MS medium or 1/2 strength MS medium supplemented with 30-90 g l^{-1} sucrose.

(3) The ovules are kept under 12-16-h photoperiod at 20°C.

1.2. Tissue culture techniques

Recent advances in plant regeneration systems in *Alstroemeria* are summarized in **Table 2**. In order to induce callus harbouring regeneration capability, different explants and culture media were examined. In most cases, mature or immature embryos in ovules were used for inducing callus growth, and subsequent plant regeneration was achieved from the embryo-derived callus. Culture conditions differed considerably among the different works. In particular, plant growth regulators used in the initial media for callus induction were different (**Table 2**). However, one

Table 2 Summary of recent manuscripts describing plant regeneration from several explants in *Alstroemeria*

Plant material	Explant	Initial culture	Regeneration medium	Reference
cv. 'Butterfly'	Mature embryo	MS medium 2 or 4 mg l^{-1} 2,4-D 2 or 4 mg l^{-1} picloram 0.7% agar	MS medium 4 mg l^{-1} BAP 0.7% agar	Gonzalez-Benito and Alderson 1992
Tetraploid cultivar (*A. pelegrina* × *A. psittacina*)	Mature zygotic embryo	MS salts B5 vitamins 10 or 20 µM NAA 20 µM kinetin 3% sucrose 0.3% Gelrite	MS salts B5 vitamins 3% sucrose 0.3% Gelrite	Hutchinson *et al*. 1994
A. inodora accession P007 Tetraploid cultivar '118'	Immature embryo in half-ovule	MS medium 50 g l^{-1} sucrose 0.4 g l^{-1} casein hydrolysate 1 mg l^{-1} 2,4-D 0.5 mg l^{-1} BAP 2 g l^{-1} Gelrite	MS medium 30 g l^{-1} sucrose 0.4 g l^{-1} casein hydrolysate 2 mg l^{-1} BAP 2 g l^{-1} Gelrite	Van Schaik *et al*. 1996
Tetraploid cultivar (*A. pelegrina* × *A. psittacina*)	Mature zygotic embryo	MS salts B5 vitamins 40 µM NAA 20 µM kinetin 3% sucrose 0.3% Gelrite	1/2 MS medium 250 mg l^{-1} casein hydrolysate 0.15% Gelrite	Hutchinson *et al*. 1997
Tetraploid cultivar genotype VV024	Leaf	MS medium 10 µM TDZ 0.5 µM IBA 3% sucrose 0.74% Daishin agar	MS medium 2.2 µM BAP 3% sucrose 0.7% micro agar	Lin *et al*. 1997
Tetraploid cultivar genotype VV026	Leaf axil	MS medium 10 µM TDZ 0.5 µM IBA 3% sucrose	MS medium 2.2 µM BAP 3% sucrose	Lin *et al*. 1998
Tetraploid cultivar genotype VV024, VV2410, VV2434, VV2435, VV2452, VV2454	Leaf axil	MS medium 10 µM TDZ 0.5 µM IBA 3% sucrose 0.74% Daishin agar	MS medium 2.2 µM BAP 3% sucrose 0.7% micro agar	Lin *et al*. 2000a
Tetraploid cultivar genotype VV024, BT207	Stem segment	MS medium 4 mg l^{-1} 2,4-D 0.5–1.0 mg/l BAP 30 g l^{-1} sucrose 7 g/l micro agar ↓ MS medium 0.5 mg l^{-1} BAP 30 g l^{-1} sucrose 3 g l^{-1} Gelrite ↓ MS medium (PCA medium in original paper) 10 mg l^{-1} picloram 20 g l^{-1} sucrose 3 % Gelrite	MS medium 0.1 mg l^{-1} BAP 30 g l^{-1} sucrose 3 g l^{-1} Gelrite	Lin *et al*. 2000b
cv. 'Sweet Laura' Butterfly-type 'A30'	Rhizome splitting	MS medium 8.9 µM BA 3% sucrose 0.25% Phytagel	MS medium 1.1 µM NAA 3% sucrose 0.25% Phytagel (for rooting)	Chiari and Bridgen 2000
'Yellow King'	Floral apices	1/2 MS medium 2.5 mg l^{-1} kinetin 1.5 mg l^{-1} BA 1.0 mg 1^{-1} NAA 30 g l^{-1} sucrose filter paper to support	MS medium 1.0 mg 1^{-1} BA 30 g l^{-1} sucrose (for shoot multiplication)	Padraza-Santos *et al*. 2006
VV024-6	Nodes with axil tissue	MS medium 10 µM TDZ 0.5 µM IBA 30 g l^{-1} sucrose 7.5 g l^{-1} Microagar ↓ Schenk and Hildebrandt medium (SH medium) 9.1 µM 2,4-D 2.2 µM BA 30 g l^{-1} sucrose 8 g l^{-1} Microagar ↓ Modified MS medium 20.8 µM picloram	MS medium 2.2 µM BA 40 g l^{-1} sucrose 2.2 g l^{-1} Gelrite	Kim *et al*. 2006

condition was maintained across most works, i.e., the use of benzylaminopurine (BAP), which was effective in regenerating plantlets. Plant growth regulators can influence callus induction by affecting the genotype-dependence and the possible cultivars or species that can be used are limited. New culture systems need to be developed for further progress in *Alstroemeria* biotechnology.

Lin *et al*. developed a plant regeneration system from leaf and stem explants (Lin *et al*. 1997, 1998, 2000a, 2000b). Kim *et al*. (2006) also reported on somatic embryogenesis from nodes with axil tissue. Organogenesis from leaf and stem has not been considered possible in monocotyledons. The development of culture system from leaf and stem in *Alstroemeria* is valuable, although successful organogenesis from leaf and stem is strongly genotype-dependent at the present. These results could expand the possibilities of use of culture systems for *Alstroemeria*.

By utilizing the embryogenic callus culture in *Alstroemeria*, isolation of protoplasts, subsequent protoplast culture and regeneration of plants from protoplasts were achieved (Kim *et al*. 2005). Such techniques will promote research on somatic hybridization between cross-incompatible species as well as on direct gene transfer with electroporation.

1.3. Genetic transformation

Particle bombardment and *Agrobacterium*-mediated procedures were applied for genetic transformation, resulting in the generation of transformed plants containing the marker genes. The first attempt at genetic transformation in *Alstroemeria* was reported by van Schaik *et al*. (2000). They confirmed transient gene expression in embryogenic callus after particle bombardment. Foreign gene expression examined with a β-glucuronidase (GUS) assay indicated the reducible transformation conditions in which the *GUS* gene was driven by the maize ubiquitin promoter; suitable parameters for the assay equipment have been reported. Although stable transformed calluses were obtained after visual selection based on luciferase activity, no plant regeneration was observed during the 2 years of maintenance.

Subsequently, Lin *et al*. (2000c) succeeded in producing transgenic plants by particle bombardment. They used a *luciferase* gene from a firefly as the reporter gene under the control of the maize ubiquitin promoter (*Ubi1*) and attempted transferring this gene into friable embryogenic calluses and proembryos. Following visual selection, transgenic plantlets were regenerated through embryogenesis. Moreover, another selection by phosphinothricin (PPT) helped in obtaining *bar* and *GUS* gene-incorporated plants after particle bombardment. The successful transformation obtained by Lin *et al*. (2000c) appears to be due to the selection of elite embryogenic callus lines with a high regeneration potential and maintaining these cells till abiolistic procedure was performed.

Hoshino *et al*. (2000) reported on the genetic transformation of *Alstroemeria* via co-cultivation of embryogenic suspension cells with *Agrobacterium tumefaciens*. The process was illustrated in **Figure 2**. Embryogenic cell suspension cultures were established via ovule culture of an interspecific cross between *A. pelegrina* and *A. magenta*. Cell suspension cultures could be initiated from the callus using liquid MS media containing picloram. An *Agrobacterium*-mediated transformation system was developed by using the embryogenic suspension cells. The suspension cells were co-cultivated with *A. tumefaciens* strain EHA101/pIG121Hm or LBA4404/pTOK233, both of which harboured the binary vector carrying the *neomycin phosphotransferase II* (*NPTII*), *hygromycin phosphotransferase* (*HPT*) and the intron-containing *β-glucuronidase* (*intron-GUS*) genes in the T-DNA region. Transgenic plants were obtained from co-cultivated cells after selection on a hygromycin-containing medium.

Further research is needed before useful genes can be successfully incorporated into *Alstroemeria* and the safety of genetically modified plants can be evaluated. Subsequently, transgenic techniques will contribute in improving the plant species.

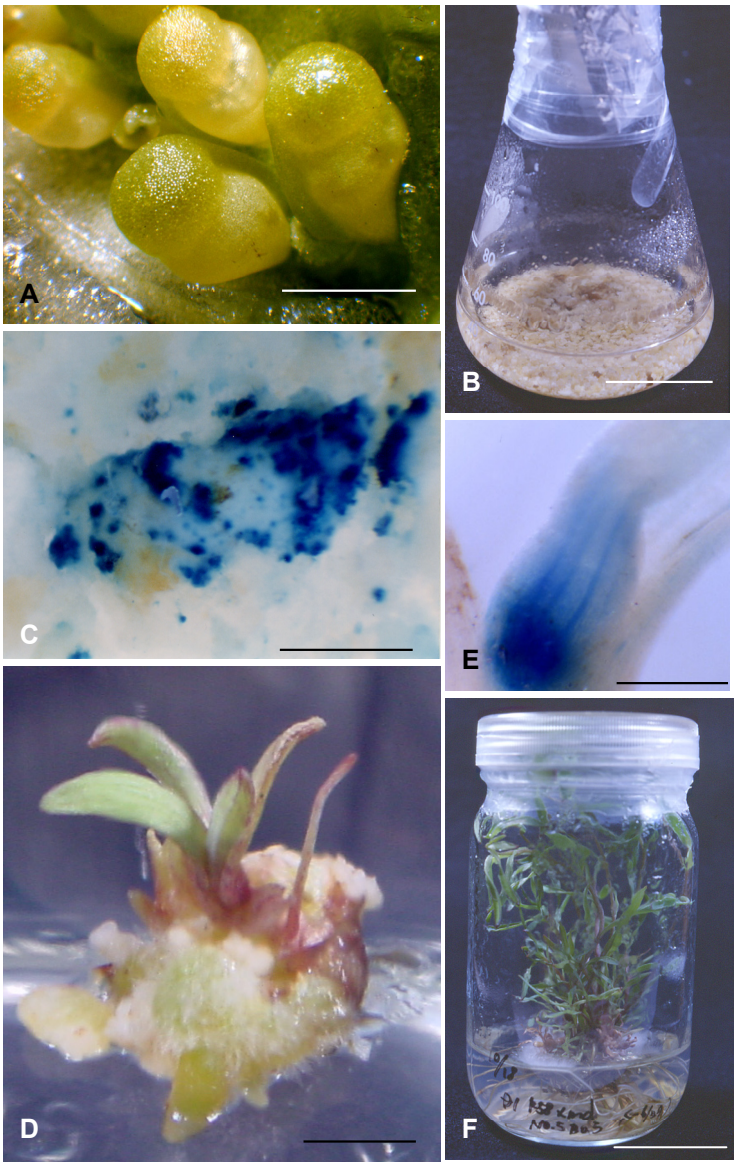

Fig. 2 Regeneration of transgenic *Alstroemeria* plant from cell suspension culture after co-cultivation with *Agrobacterium tumefaciens*. (**A**) Ovules 14 days after pollination (*Alstroemeria pelegrina* × *A. magenta*) were cultured for callus induction. Bar = 2 mm. (**B**) Cell suspension culture induced from ovule materials. Bar = 3 cm. (**C**) GUS expression of *Alstroemeria* suspension cells 7 days after co-cultivation with *A. tumefaciens*. Bar = 1 cm. (**D**) Shoot regeneration showing hygromycin-resistance after infection of *Agrobacterium*. Bar = 1 cm. (**E**) GUS expression of regenerated *Alstroemeria* shoot. Bar = 0.5 cm. (**F**) Regeneration of transgenic *Alstroemeria* plant. Bar = 3 cm.

1.4. Attempts to develop novel procedures for *Alstroemeria* improvement

The ovules of *A. aurea* were used in an attempt to develop a reliable protocol for isolating living egg cells and zygotes involved (Hoshino *et al*. 2006). Fresh ovule sections were prepared with a surgical blade and treated with an enzyme solution. Subsequently, these ovule sections were dissected using a hand-made glass needle with fine tip under an inverted microscope. The egg cells successfully isolated by this procedure were collected using microcapillaries connected to a computer-controlled micropump. For zygote isolation, ovules were excised from the ovaries 24 h after self-pollination. By treating the excised ovules with an enzyme solution and subsequently dissecting them by using a glass needle, the zygotes were successfully isolated from the ovules and collected with a microcapillary. Egg cells and zygotes were viable for up to 2 h following isolation, as determined by fluorescein diacetate staining. These procedures may be utilized for *in vitro* fertilization, enabling artificial fusion between a single isolated egg cell and sperm cell. This concept can be applied for obtaining crosses of sexually incompatible *Alstroemeria* species through *in vitro* fertilization.

2. CONCLUSIONS

Evaluation of the genetic diversity of wild species of Alstroemeriaceae and *Alstroemeria* cultivars has been attempted using chromosomal research and molecular markers. Karyotype analysis has been carried out and applied to taxonomic research. The chromosome numbers of wild *Alstroemeria* species have been determined (Strasburger 1882; Taylor 1926; Hang and Tsuchiya 1988; Stephens *et al*. 1993). According to these studies, the somatic chromosome number is 2n = 2X = 16. Sanso and Hunziker (1998) revealed that the basic chromosome number of *Alstroemeria* (n = 8) and *Bomarea* (n = 9) is critical for discriminating between the genera *Alstroemeria* and *Bomarea* that have similar morphological features.

Buitendijk and Ramanna (1996) showed that Giemsa C-banding and Feulgen staining could be used to identify *A. angustifolia*, *A. aurea*, *A. ligtu*, *A. magnifica*, *A. pelegrina*, *A. philippii* and *A. psittacina*. Furthermore, many individual chromosomes in the interspecific hybrids of these plants were differentiated on the basis of chromosome morphology and C-banding pattern.

de Jeu *et al*. (1997) characterized the repetitive DNA sequences of *A. aurea* and identified the species-specific clones A001-I and A001-II. Analysis of *A. aurea*-specific repeats using fluorescence *in situ* hybridization (FISH) revealed distinct localization patterns in the heterochromatic regions of all *A. aurea* chromosomes. FISH was also applied to the chromosomes of *Alstroemeria* species by Kamstra *et al.* (1997). Species-specific repetitive DNA and highly conserved rDNA sequences were localized on metaphase chromosomes of the Chilean species *A. aurea* and the Brazilian species *A. inodora*. The FISH patterns showed obvious differences between these 2 species. Furthermore, Kamstra *et al*. (1999) revealed that FISH performed using species-specific probes could detect homoeologous chromosome pairing in the distant hybrid *A. aurea* × *A. inodora* and the genome constitution of its BC_1 progeny plants. Additionally, genomic *in situ* hybridization (GISH) enabled the identification of parental genomes in the following interspecific hybrids: *A. magnifica* × *A. inodora*, *A. inodora* × *A. magnifica*, *A. pelegrina* × *A. inodora*, *A. pelegrina* × *A. magnifica*, *A. aurea* × *A. ligtu*, *A. aurea* × *A. inodora*, *A. pelegrina* × *A. aurea* and *A. magnifica* × *A. aurea* (Kuipers *et al*. 1997).

Kuipers *et al*. (1998) detected Ty1-*copia*-like retrotransposons in some *Alstroemeria* chromosomes by *in situ* hybridization and discussed the contributions of these retrotransposons to genome evolution and diversity. Kuipers *et al*. (2002) also reported the molecular cloning of highly repetitive DNA sequences from the Brazilian species *A. psittacina* and *A. inodora*. *In situ* hybridization using the repetitive DNA sequences revealed specific localization patterns in the chromosomes of *A. psittacina* and *A. inodora*.

Random amplified polymorphic DNA (RAPD) analysis was utilized to analyse the genetic relationships among *Alstroemeria* cultivars (Dubouzet *et al*. 1997) and between wild *Alstroemeria* species and cultivars (Dubouzet *et al*. 1998). Aros *et al*. (2006) used RAPD markers in combination with morphological descriptors and demonstrated the genetic diversity between commercial cultivars and wild *Alstroemeria* species. Molecular markers have also been developed for amplified fragment length polymorphism (AFLP) by Han *et al*. (1999). By using AFLP markers, their group constructed a linkage map for *A. aurea* (Han *et al.* 2002) and demonstrated the differences between the Chiliean and Brazilian *Alstroemeria* species (Han *et al*. 2000). The sequences of the *rps16* and *rbcL* introns were analyzed to investigate the phylogeny of the Alstroemeriaceae family, including 23 *Alstroemeria*, 3 *Bomarea* and 1 *Leontochir* species (Aagesen and Sanso 2003). Based on the results, the *Alstroemeria* species were roughly divided into 3 subclades according to their geographical area of occurrence, i.e. northern Chile, central Chile and Brazil.

As mentioned above, taxonomic and phylogentic analyses using molecular markers and karyotype analysis have yielded accurate data regarding the genetic relationships and diversity in Alstroemeriaceae. In addition, the nuclear content in the *Alstroemeria* species and some of their hybrids was estimated by flow cytometry using PI and DAPI (Buitendijk *et al*. 1997). These results are useful for further breeding of *Alstroemeria* through interspecific cross combination.

In this chapter, many attempts to produce interspecific hybrids in *Alstroemeria* have been described. Novel cross combinations using related species are necessary for further cultivation. Recently, intergeneric hybrids have been produced in the Colchicaceae by crossing *Sandersonia aurantiaca* and *Gloriosa rothschildiana* (Nakamura *et al*. 2005). The hybrid plants are expected to have an increased ornamental value. With regard to *Alstroemeria*, the related genus *Bomarea* is one of the candidates for hybridization because of its ornamental value. Phylogenetically, *Bomarea* is related to *Alstroemeria* (Sanso and Xifreda 2001). Future studies should focus on the production of hybrids by cross combinations between distantly related genera such as *Alstroemeria* and *Bomarea*. Several barriers need to be overcome before hybrid varieties of *Alstroemeria* species can be obtained. In order to overcome post-fertilization barriers, further improvements in hybridization techniques are necessary.

Recently, *in vitro* fertilization studies have been carried out in higher plants (Kranz *et al*. 2004). This technique can be utilized as a novel strategy in plant breeding for inducing the fusion of gametes between distantly related incompatible species and for achieving direct gene transfer into isolated zygotes. In *Alstroemeria*, *in vitro* fertilization using isolated egg cells and zygotes from ovules has been attempted (Hoshino *et al*. 2006). In breeding programs for *Alstroemeria*, it is expected that the hybrid plants will possess traits such as virus and disease resistance, attractive flower colours, novel flower shapes, flower longevity, dwarfness and attractive scent. Furthermore, flowering control is important for

commercial production. Further studies combining recent biotechnological techniques are necessary to produce valuable cultivars *Alstroemeria* with the above-mentioned traits.

ACKNOWLEDGEMENTS

I would like to thank Dr. K. Shinoda (National Agricultural Research Center for Hokkaido Region) for helpful suggestions and providing plant materials. Technical supports from Y. Kashihara and members of my laboratory are greatly appreciated.

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