Plant Cell Tiss Organ Cult (2006) 86:233–238
DOI 10.1007/s11240-006-9110-6

ORIGINAL PAPER

Efficient somatic embryogenesis in *Alstroemeria*

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Received: 17 March 2005 / Accepted: 13 April 2006 / Published online: 7 July 2006

Abstract In *Alstroemeria* high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) were obtained from nodes with axil tissue cultured first on a Murashige and Skoog (MS) medium supplemented with 10 μM thidiazuron and 0.5 μM indole-3-butyric acid and after that on a Schenk and Hildebrandt (SH) medium supplemented with 9.1 μM 2,4-dichlorophenoxy acetic acid and 2.2 μM benzylaminopurine (BA). Both types of callus were maintained on modified MS medium supplemented with 20.8 μM picloram. CEC and FEC formed somatic embryos and subsequently plants when transferred to MS medium supplemented with 2.2 μM BA. Plants were produced after 12 weeks (CEC) or after 16 weeks (FEC) of culture. Regenerated plants were established in the greenhouse and flowered normally.

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Keywords *Alstroemeria* · Compact embryogenic callus · Friable embryogenic callus · Regeneration · Somatic embryos

Abbreviations

2,4-d	2,4-dichlorophenoxy acetic acid
BA	Benzylaminopurine
CEC	Compact embryogenic callus
CIM	Callus induction medium
CPM	Callus proliferation medium
FEC	Friable embryogenic callus
IBA	Indole-3-butyric acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	Murashige and Skoog (1962) medium
NAA	α-naphthaleneacetic acid
NEC	Nonembryogenic callus
PM	Propagation medium
SE	Somatic embryo
SH	Schenk and Hildebrandt (1972) medium
SIM	Shoot induction medium
TDZ	*N*-phenyl-*N*′-1,2,3-thidiazo-5-yl urea or thidiazuron

Introduction

Alstroemeria is an important ornamental worldwide (Van Zaayen 1995), used as cut flower and,

recently, also as pot plant. *Alstroemeria* has a long vase-life and a variety of flower colors. In addition, *Alstroemeria* requires low input of energy during cultivation (Blom and Piott 1990; Chekpkairor and Waithaka 1988; Van Schaik et al. 1996).

Conventional breeding techniques have been used to create new attractive *Alstroemeria* cultivars with other colors, long vase-life, high yield of flowers and resistance to diseases. However, for many agriculturally useful traits, the genes are not present in the *Alstroemeria* gene pool. In those cases, genetic modification can be used. Genetic modification requires regeneration protocols with high efficiency and reproducibility. Plant regeneration in *Alstroemeria* has been mainly accomplished via somatic embryogenesis (Akutsu and Sato 2002; Gonzalez-Benito and Alderson 1992; Hutchinson et al. 1994, 1997; Lin et al. 2000a; Van Schaik et al. 1996) rather than via organogenesis (Lin et al. 1997).

Two types of embryogenic callus have been observed in *Alstroemeria*: compact embryogenic callus (CEC) and friable embryogenic callus (FEC). CEC is induced on zygotic embryos cultured on auxin supplemented medium (Van Schaik et al. 1996), and is a hard callus consisting of embryogenic aggregates larger than 0.5 mm. FEC is initiated from seedling explants (Lin et al. 2000a) or ovules (Akutsu and Sato 2002) cultured on auxin supplemented medium and is a soft callus consisting of embryogenic units, smaller than 0.1 mm in diameter. CEC does not result in the production of transgenic plants (Van Schaik et al. 2000). On the other hand, FEC has been used successfully for the production of genetically modified *Alstroemeria* plants (Lin et al. 2000b). Also in crops as cassava (Munyikwa et al. 1998; Raemakers et al. 2001), *Gladiolus* (Kamo et al. 1995), oat (Somers et al. 1992; Torbert et al. 1995), rose (Robinson and Firoozababy 1993), and wheat (Brisibe et al. 2000), FEC has been used successfully to obtain genetically modified plants. Lin et al. (2000b) used seedlings as starting material for transformation. However, because *Alstroemeria* is a vegetatively propagated, highly heterozygous crop, FEC derived from seedling tissue or ovules cannot be used for improvement of existing variety. Therefore, the development of FEC from tissue taken from adult plants should be established.

Materials and methods

Induction of somatic embryogenesis from nodes with axil tissue

VV024-6 was selected from selfed progenies of VV024 on base of its high multiplication rate (Lin et al. 1998). Plants were maintained by a four weeks subculture regime on propagation medium (PM) consisting of MS (Murashige and Skoog 1962) basal salts plus vitamins, 2.2 μM BA, 40 g l^{-1} sucrose and 2.2 g l^{-1} Gelrite. Plants were cultured at 18°C under a 16 h (light)/8 h (dark) photoperiod provided by Philips white fluorescent lights at 40 μmol $m^{-2}s^{-1}$. Callus induction and maintenance experiments were done in darkness. Regeneration of somatic embryos and plants was done in light. The media were adjusted to pH 6.0 using 1N KOH.

Nodes with axil tissue (Lin et al. 1997) were excised from in vitro plants and cultured on shoot induction medium (SIM) consisting of MS basal salts and vitamins, 10 μM TDZ, 0.5 μM IBA, 30 g l^{-1} sucrose and 7.5 g l^{-1} Microagar (Duchefa Biochemie B.V., Haarlem, The Netherlands) for ten days (Lin et al. 1997). Explants were then transferred to callus induction medium (CIM), containing SH (Schenk and Hildebrandt 1972) basal salts and vitamins, 30 g l^{-1} sucrose, 8 g l^{-1} Microagar (Duchefa Biochemie B.V., Haarlem, The Netherlands) and four different combinations of growth regulators (9.1 μM 2,4-d; 9.1 μM 2,4-d + 2.2 μM BA; 8.3 μM picloram; 8.3 μM picloram + 2.2 μM BA). The explants were subcultured after four weeks. Formation of friable embryogenic callus (FEC) and compact embryogenic callus (CEC) was evaluated after eight weeks of growth.

CEC and FEC were maintained in a three weeks subculture regime on callus proliferation medium (CPM) consisting of MS basal salts and vitamins, supplemented with 0.2 g l^{-1} myo-inositol, 20 g l^{-1} sucrose, 18.2 g l^{-1} mannitol, 0.48 g l^{-1} MES, 0.1 g l^{-1} casein hydrolysate, 0.08 g l^{-1} adenine sulphate, 0.5 mg l^{-1} d-calcium-panthotenate,

0.1 mg l^{-1} choline chloride, 0.5 mg l^{-1} ascorbic acid, 2.5 mg l^{-1} nicotinic acid, 1.5 mg l^{-1} pyridoxine–HCl, 10.1 mg l^{-1} thiamine–HCl, 0.5 mg l^{-1} folic acid, 0.05 mg l^{-1} biotin, 2.5 mg l^{-1} glycine, 0.1 mg l^{-1} L-cystein, 0.25 mg l^{-1} riboflavine and 20.8 μM picloram.

Plant formation

CEC and FEC cultures were transferred to PM for plant formation. After four weeks the explants were transferred to fresh medium. After eight weeks somatic embryos were isolated, transferred to fresh PM and cultured for an additional period of four weeks. Primary and secondary embryos with two or more normal shoots considered to be regenerated into plants. These plants were transferred to a rooting medium composed of MS basal salts and supplemented with 2.7 μM NAA, 45 g l^{-1} sucrose, and 2.2 g l^{-1} Gelrite (pH 6.0). Rooted plants were transferred to the greenhouse.

Statistical analysis

All experiments were repeated at least three times with 40 replicates per treatment. Results were analyzed weekly over a period of eight weeks. The data are presented as the mean ± standard error (SE). Statistical analysis was carried out using analysis of variance (ANOVA) comparing the treatments. Treatment means were separated using least significant differences (LSD) test at the 5% probability level and analyzed using SPSS Windows version 10 (SPSS Inc., Chicago, IL, USA).

Results

Induction of CEC and FEC from nodes with axil tissue

After two weeks of culture on CIM, callus appeared at the wound sites of the explants. Callus was formed in the axil at the position of attachment to the stem (Fig. 1A). Three different types of callus were observed simultaneously: soft and watery callus (nonembryogenic callus: NEC), compact embryogenic callus (CEC), and friable embryogenic callus (FEC). FEC was yellowish, round-shaped, fast growing and could easily be divided into single units. CEC (Fig. 1B) was initially white and later turned yellow. CEC did grow rather slowly and was difficult to divide into single units. NEC turned brown and died soon after initiation. Almost 50% of the explants produced directly somatic embryos. More than 60% of the directly induced somatic embryos died, whereas the remaining 40% developed into weak plants.

All four media induced the formation of both CEC and FEC. 2,4-d was more efficient in producing CEC and FEC than picloram. Addition of BA to both picloram and 2,4-d slightly increased the formation of CEC (Table 1). The addition of BA to 2,4-d had a slight positive effect on the induction of FEC, whereas the addition of BA added to picloram doubled the formation of FEC.

After three months of culture, a total of 150 CEC and 100 FEC lines had been selected from 400 nodes with axil tissue explants. The lines were maintained on CPM. CEC and FEC did not produce somatic embryos on either media. About 15% of the CEC also produced FEC. In all cases, FEC initiated new FEC without further organization into CEC.

Somatic embryo germination and plant establishment in the greenhouse

CEC and FEC lines either induced directly from nodes with axil tissue or indirectly from CEC were maintained for four months on CPM before culturing for plant regeneration. In general, FEC did not initiate somatic embryos directly on PM. About 80% of the FEC units developed into CEC units after three weeks of culture. These CEC units were either relatively large and white in color or were small and yellow. Only yellow CEC units did regenerate somatic embryos. Again, three weeks later, (pre-) globular embryos developed from CEC units. Up to six globular somatic embryos were initiated per CEC unit. These embryos were isolated from the CEC and cultured on fresh regeneration medium for further development. After two weeks, the shoot apex broke through the cotyledon, developing into a shoot.

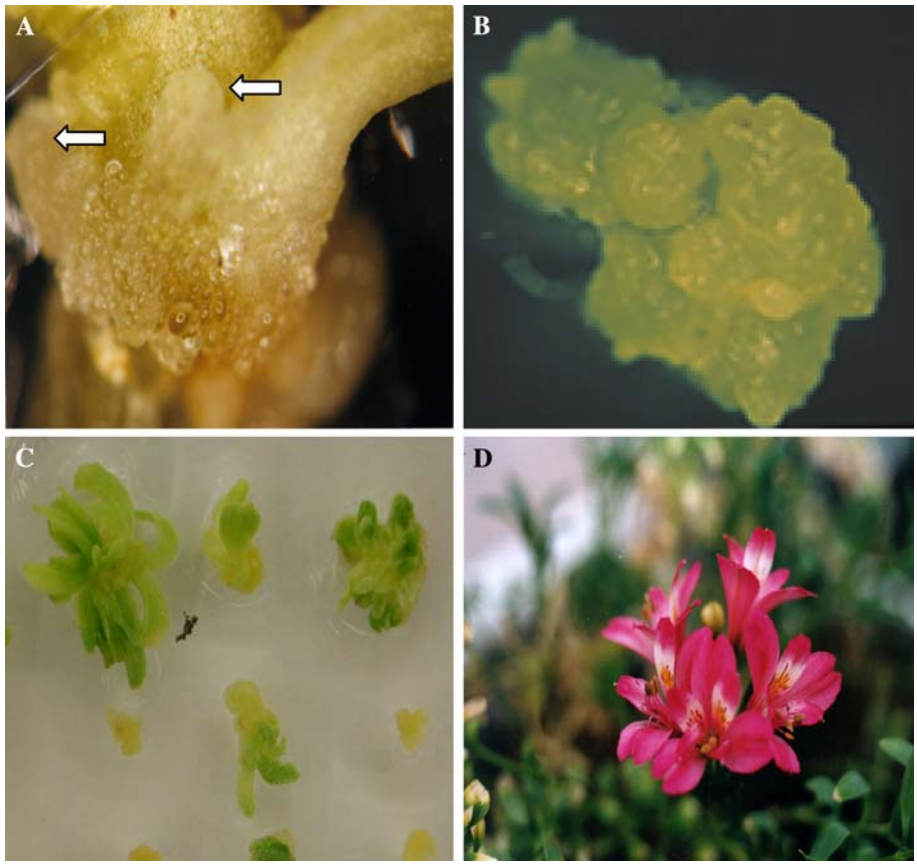

Fig. 1 Somatic embryogenesis from nodes with axil tissue in *Alstroemeria* (**A**) friable embryogenic callus (FEC) induced from nodes with axil tissue (arrows) (**B**) Yellow compact embryogenic callus (CEC) (**C**) regeneration of somatic embryos from FEC (**D**) flower formation in plant regenerated from FEC

Table 1 Effect of different growth regulators combinations on callus induction and somatic embryo (SE) development in *Alstroemeria* genotype VV024

Hormone combination[1]	% CEC[2]	% FEC[2]	# of SE/100 units of CEC[3]	# of SEs/100 units of FEC[3]
9.1 μM 2,4-d + 2.2 μM BA	37.4 ± 3.4a[4]	19.5 ± 4.2a	132.8 ± 18.4a	85.5 ± 8.9a
8.3 μM picloram + 2.2 μM BA	19.8 ± 4.4b	12.0 ± 5.4ab	46.8 ± 6.1c	55.7 ± 3.5b
9.1 μM 2,4-d	31.4 ± 2.5ab	15.4 ± 3.3a	98.8 ± 9.3b	47.3 ± 6.3b
8.3 μM picloram	18.5 ± 4.7b	5.3 ± 3.8b	20.5 ± 5.0d	23.4 ± 4.1c

[1]SH medium used as a basal medium and solidified with 7.5 g l^{-1} Microagar (pH 6.0)

[2]Three replicates (40 explants) per treatment and data collected after eight weeks of culture

[3]CEC and FEC cultures were transferred to PM (MS medium with 2.2 μM BA, 25 g l^{-1} sucrose, solidified with 2.75 g l^{-1} Gelrite (pH 6.0)

[4]Means in a column followed by the same letter are not significantly different at the 5% level as determined by LSD

For the FEC lines, two to three weeks more were required for the germination of somatic embryos into shoots (Fig. 1C). This is due to the development of FEC into CEC before following the procedures outlined above. In general, 35% of FEC and 74% of CEC-derived somatic embryos

regenerated into vigorously growing shoots. When they had reached 2–4 cm in length, they were transferred to rooting medium. Plants with a size of 4–6 cm and with 2–4 roots were transferred to the greenhouse. Rhizomes were also formed after 4–6 weeks of culture. Approximately 90–95% of the plants survived and flowered normally 3–4 months later (Fig. 1D).

Discussion

Zygotic embryos (Hutchinson et al. 1994; Gonzalez-Benito and Alderson 1992; Van Schaik et al. 1996) have previously been used for the initiation of CEC in *Alstroemeria*. Lin et al. (2000a) and Akutsu and Sato (2002) obtained FEC from stem and ovule tissues of seedlings, respectively. In *Alstroemeria*, both CEC and FEC cultures have been used for genetic modification. In the case of CEC, only chimeric transgenic plants were produced at a very low frequency (Van Schaik et al. 2000). FEC, combined with genetic modification, resulted in completely genetically modified plants (Lin et al. 2000b). Unfortunately, FEC derived from seedling tissue cannot be used for the improvement of an existing cultivar via genetic modification because *Alstroemeria* is a heterozygous and vegetatively propagated crop. To our knowledge, this is the first report, showing that tissue taken from adult, vegetatively propagated *Alstroemeria* plants has the capacity to form CEC and FEC. VV024-6 was selected from a cross because of its high multiplication rate (Lin et al. 1998) and has been vegetatively propagated in vitro a number of years before it was used in the experiments described here.

Generally, there are two methods to obtain FEC lines using nodes with axil tissue for *Alstroemeria*. The first one is directly from nodes with axil tissue without formation of CEC formation. The second method is indirect: FEC is induced from CEC cultured on CPM. Indirectly induced FEC generally shows better callus growth, somatic embryo yield and germination than directly induced FEC (results not shown).

In conclusion, the results described here demonstrate that embryogenic callus formation was induced with high efficiency on SH medium supplemented with 9.1 μM 2,4-d, 2.2 μM BA, 30 g l^{-1} sucrose, and solidified with 2.75 g l^{-1} Gelrite, at pH 6.0 following ten days of culture on SIM. Two embryogenic callus types, CEC and FEC, can be obtained from vegetatively propagated plants within three months of culture. Both callus types were able to regenerate somatic embryos and subsequently plants at a high frequency. In total, more than 500 plants with healthy roots were produced from the selected CEC and FEC lines. Particularly, the FEC lines may be superior material for use in genetic modification as was shown by Lin et al. (2000b).

Acknowledgments We thank Royal van Zanten (the Netherlands) who kindly provided in vitro plants of the genotype VV024. We thank Dirkjan Huigen and Bert Essenstam for taking care of the plants in the greenhouse. This research was partly financed by the Ministry of Education, Republic of Korea.

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