PROLIFERATION, SHOOT ORGANOGENESIS AND SOMATIC EMBRYOGENESIS IN EMBRYOGENIC CALLUS OF CARNATION

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A B S T R A C T

The influence of 2,4-D, picloram and NAA on proliferation of embryogenic callus of two cultivars of carnation ('Impulse' and 'Sagres') was evaluated. Proliferations of embryogenic calli were observed on MS basal medium containing different concentrations of picloram (0.8, 2, 4, 8, 16 and 24 µM). Maximum of proliferation of calli was obtained with 8 and 16 µM picloram. Organogenesis was achieved following transfer of primary callus to MS basal media containing TDZ, BA and kinetin. Maximum organogenesis was obtained with 4.5 and 9 µM TDZ. Transfer of proliferated calli to growth regulator-free MS medium supplemented with 0.08, 0.16, 0.32, 0.49 and 0.65 M sorbitol resulted in successful somatic embryogenesis and maximum embryogenesis was obtained with 0.49 and 0.65 M sorbitol. Cotyledonary somatic embryos and regenerated shoots were converted into plantlets. Plantlets continued to grow under greenhouse condition.

Key words: carnation, embryogenic calli, shoot organogenesis, somatic embryogenesis

INTRODUCTION

Carnation (*Dianthus caryophyllus*) is an important floricultural crop with high commercial interest worldwide (Burchi et al., 1996). Application of biotechnology in plant breeding programs requires efficient *in vitro* regeneration procedures. Somatic embryogenesis shows several advantages as compared to other *in vitro* propagation systems, including its high multiplication rates, possibility of cryopreservation of embryogenic callus, the potential for scaling-up the process in liquid suspension

cultures, synthetic seed technologies, and the fact that embryogenic cultures are suitable target tissues for gene transfer (Merkle and Dean, 2000).

The proliferation stage in any micropropagation procedure often determines the usefulness of the protocol. In somatic embryogenesis, once embryogenic cells have been formed, they continue to proliferate, forming embryogenic callus. Proliferation of embryogenic calli is one of the most powerful aspects of somatic embryogenesis for such application as mass propagation and gene transfer (Merkle et al., 1995). Induction of embryogenic calli in carnation has been reported on cultivars 'Impulse' and 'Sagres' (Karami et al., 2006), but proliferation of such callus is still to be worked out.

From the fundamental aspects, particular attention is paid to the callus-mediated organogenesis, which is a unique pathway of plant morphogenesis (Kallak et al., 1997). Shoot organo-genesis of carnation via callus has previously been reported (Kallak et al., 1997) but shoot organogenesis via embryogenic callus has not.

In this paper the method for proliferation and shoot organogenesis of embryogenic calli and recovery of typical plantlets in carnation is described.

MATERIAL AND METHODS

Proliferation and shoot organogenesis in embryogenic callus

Two cultivars of carnation: 'Impulse' and 'Sagres' were selected. Immature flower buds (1 to 1.5 cm long) were harvested from green-house-grown plants and stored at 4°C for 3-4 weeks. They were then surface-sterilized for 30 sec. in 70% ethanol and for 20 min. in 2% sodium hypochlorite, followed by three rinses with sterilized distilled water. Sepals and receptacles were removed from the buds and the pieces of about 4 mm in length excided from the basal part of petals and placed on the callus induction medium. Primary embryogenic calli was induced on MS (Murashige and Skoog, 1962) medium containing 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8 µM 6-benzyl-aminopurine (BA) and 0.26 M sucrose as described by Karami et al. (2006). To proliferation, primary embryogenic calli were transferred onto culture media MS supplemented with 30 g 1^{-1} sucrose and different concentrations of 2,4-D (1.12, 2.25 4.5, 9, 18 and 27 µM), 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram) (0.8, 2, 4, 8, 16 and 24 µM) and α-naphthylacetic acid (NAA) (1, 2.7, 5.3, 10.6, 21 and 26,3 µM). Thirty callus clumps (each 30-40 mg) were taken for each treatment and the experiments were conducted in six replicates. Percentage of proliferating callus clumps were recorded after 4 weeks culture. Differences between means were scored with Duncan's multiple range test. Number of embryos were calculated after 5 weeks.

Calli proliferation was done on MS medium containing 8 µM picloram and was repeated in four successive cycles. To initiate shoot organogenesis, primary embryogenic

calli were transferred onto culture media containing MS supplemented with 0.08 M sucrose and different concentrations of thidiazuron (TDZ) (2.2, 4.5 and 9 µM), BA (1.6, 3.2 and 6.2 µM) and kinetin (2.3, 4.6 and 9.2 µM). About 600 mg of embryogenic callus were taken for each treatment and the experiments were conducted in six replicates. Number of shoots were recorded 5 weeks after culture. Differences between means were scored with Duncan's multiple range test.

Induction of somatic embryos

Proliferating embryogenic calli were transferred to hormone-free MS medium containing 0.08 M sucrose in combination with different concentrations of sorbitol (0.08, 0.16, 0.32, 0.49 and 0.65 M) to initiate somatic embryogenesis. About 250 mg embryogenic callus was used for each treatment and the experimental treatments consisted of six replicates. Number of embryos was counted after 5 weeks. Differences between means were scored with Duncan's multiple range test.

Plantlet formation and plants acclimatization

To initiate maturation and germination, somatic embryos were isolated from the culture mass and placed onto the half-strength MS medium without plant growth regulators and supplemented with 0.08 M sucrose. To induce root formation, adventitious shoots were isolated from callus and transferred onto half-strength MS medium containing 0.08 M sucrose and 5.3 µM NAA. Germinated somatic embryos and rooted shoots were transferred into plastic pots containing an autoclaved mixture of soil, sand, and compost (1:1:1 v/v) and kept for 2 weeks, then transplanted into plastic pots containing garden soil and grown in the growth room (18 ± 2°C, 16-h photoperiod under 25 µmol m² s irradiation). Acclimatization of plants was finally carried out for 3 weeks in a greenhouse at 28°C followed by transferring to a greenhouse without temperature control.

All cultures were incubated at 24°C and 16-h photoperiod under 30 µmol m² s irradiation provided by cool white fluorescent lamps in a growth room. pH of the culture media were adjusted to 5.8 using 1N NaOH before adding gelling agent (Agar-Agar, Merck). All culture media were sterilized in autoclave at 121°C for 15 minutes.

RESULTS AND DISCUSSION

Proliferation of embryogenic callus

All embryogenic calli produced somatic embryos on MS medium without growth regulators. In the media containing 2,4-D and NAA, embryogenic calli converted to somatic embryos and succulent non-embryogenic calli. In the media containing picloram embryogenic calli converted to somatic embryos and formed new embryogenic calli. Of different treatments tested, embryogenic calli proliferated only on media supplemented with picloram. The highest rate of proliferation (about 40%) was achieved on media containing 8 and 16 µM of this growth regulator (Tab. 1).

Table 1. Effect of different concentrations of picloram on proliferation of embryogenic callus in two cultivars of carnation after 4 weeks

Percentage of proliferating embryogenic callus		Picloram concentration [µM]
'Sagres'	'Impulse'	
6 e	12 d*	0.8
15 d	20 c	2
20 c	31 b	4
33 a	44 a	8
31 ab	41 a	16
27 b	30 b	24

*Means in columns having the same letter are not significantly different according to Duncan's multiple range test ($P<0.05$)

Table 2. Effect of repeated cycles on proliferation of embryogenic callus in two cultivars of carnation on MS medium containing 8 µM picloram

Percentage of proliferating embryogenic callus		Repeated cycles
'Sagres'	'Impulse'	
33 d	44 d*	Cycle 1
68 c	60 c	Cycle 2
89 b	80 b	Cycle 3
100 a	97 a	Cycle 4

*Explanations, see Table 1

On medium containing picloram, proliferation of embryogenic callus occurred in successive cycles in which the percentage of proliferated callus clumps increased significantly in subsequent cycles (Tab. 2). In the 4th cycle, the proliferation rate 97% was achieved. These calli did not convert to somatic embryos (Fig. 1A). Similar results have also been reported in secondary somatic embryogenesis of carnation (Karami et al., 2007).

The most broadly documented factor associated with proliferation of embryogenic calli is auxin (Merkle et al., 1995). Picloram is an auxin, which was used for proliferation of embryogenic cultures of ornamental *Alstroemeria* (Lin et al., 2000), peanut (Little et al., 2000) and cassava (Groll et al., 2001). This is the first report that defines the culture conditions necessary to proliferate embryogenic calli of two carnation cultivars.

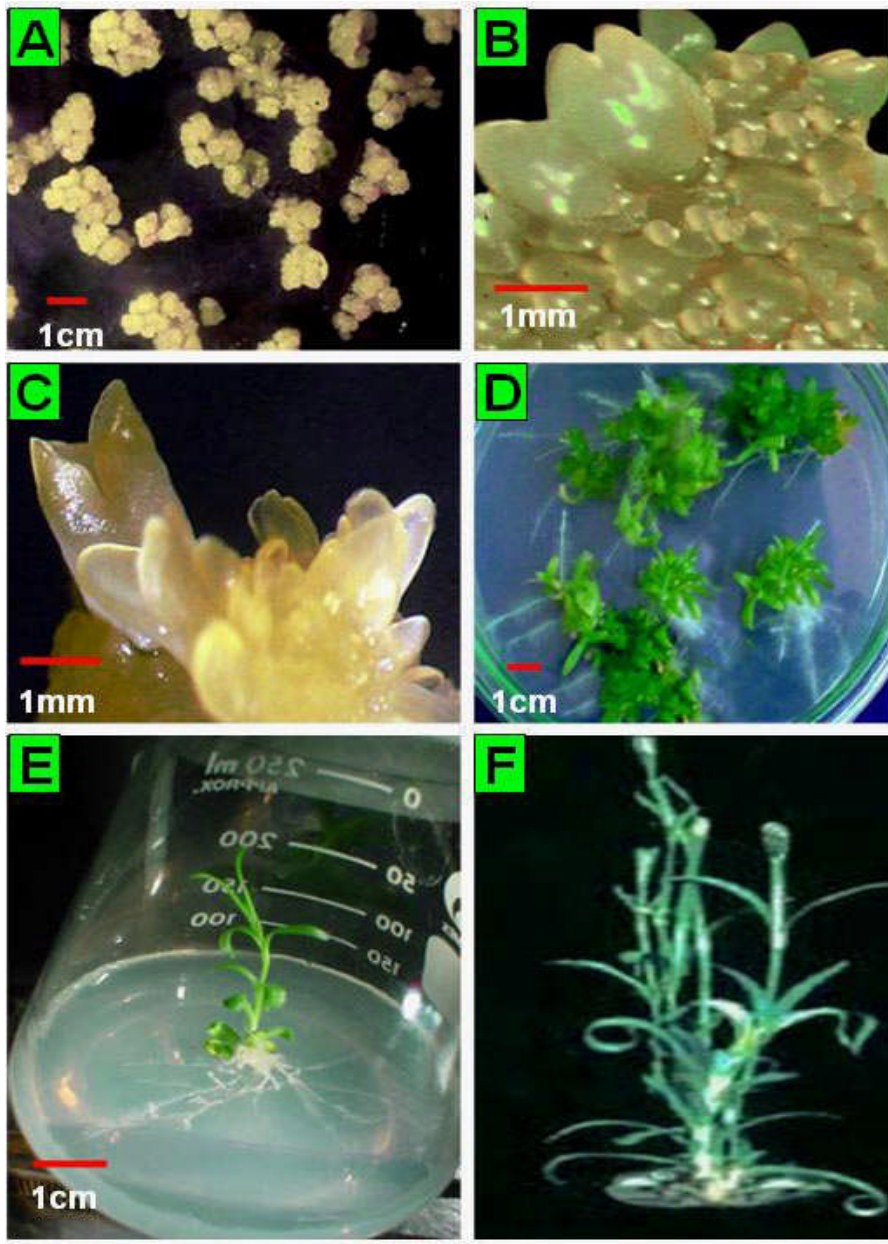

Figure 1. A: Proliferation of embryogenic callus on MS medium containing 8 µM picloram in the 4 t^{he} cycle. **B**: Somatic embryogenesis at globular stage on medium containing 0.08 M sucrose and 0.49 M sorbitol after 1 week. **C**: Somatic embryogenesis at cotyledon stage on medium containing 0.08 M sucrose and 0.49 M sorbitol after 3 weeks. **D**: Formation of roots from shoots on half-strength MS medium containing 5.3 µM NAA after 2 weeks **E**: Plantlet regenerated from a somatic embryo cultured on half-strength MS medium after 4 weeks. **F**: A potted plant in greenhouse.

Shoot organogenesis from embryogenic callus

Following transfer of embryogenic primary callus to media containing TDZ, BA or kinetin, the initiation of adventitious shoots was observed. After one week, a few abnormal somatic embryos were observed as well, but after next three weeks they all converted to shoots.

A highest number of shoots (155 and 151 on 600 mg callus) was obtained on media containing 3.2 and 6.2 µM TDZ, respectively (Tab. 3). A higher effectiveness of TDZ than of other cytokinins on direct shoot organogenesis from petal (Nakano et al., 1994) and node (Nontaswatsri et al., 2002) explants have been reported in carnation previously.

Direct shoot organogenesis from different explants has been reported in carnation but functional plant regeneration via organogenesis from callus culture is difficult to obtain because of an accompanying hyperhydricity (Jain et al., 2001). In this paper, high frequency of shoot organogenesis from embryogenic calli is shown.

Induction of somatic embryos from embryogenic callus

Globular shaped somatic embryos were induced within 1-2 weeks from proliferating embryogenic calli after transfer to growth regulator-free medium containing 0.08 M sucrose in combination with 0.08, 0.16, 0.32, 0.49 and 0.65 M sorbitol (Fig. 1B). After 2 – 3 weeks, globular embryos further developed into torpedo and cotyledonary-shaped embryos (Fig. 1C).

Table 4 shows the number of embryos produced on proliferated embryogenic calli on growth regulator-free medium containing different concentrations of sorbitol. Increasing sorbitol concentration from 0.08 to 0.49 M significantly increased the rate somatic embryogenesis. The highest number of embryos (about 180) was achieved on media containing 0.49 and 0.65 M sorbitol. Karami et al. (2006) reported the increase in the efficiency of somatic embryogenesis in carnation callus at high sucrose concentration. It is a common knowledge that the role of high sugar concentration in somatic embryogenesis is linked with high osmolarity (Paiva et al., 2003). This positive effect could mimic the changes in osmolarity that occur in the tissues surrounding the embryo within the seed (Merkle et al., 1995). Therefore, the impact of sorbitol in this study could possibly be due to its osmotic potential.

Plantlet formation and plants acclimatization

Cotyledonary somatic embryos transferred to half-strength MS medium without growth regulators germinated into shoot and root initials within 3 – 4 weeks producing efficiently (80-85%) entire plantlets in both cultivars (Fig. 1E). The effectiveness of adventitious shoot rooting on NAA containing medium (5.3 µM NAA) was as high as 95% (Fig. 1D). A high percentage (approximately 85%) of plantlets were successfully transferred to soil (Fig. 1F) and developed to mature plants in the greenhouse with 95%

Table 3. The effect BA, TDZ and kinetin on mean number of shoots formed on primary embryogenic callus of two cultivars of carnation after 5 weeks of culturing

Number of shoots induced on 600 mg of embryogenic callus		Kind and concentration [µM] of growth regulator		
'Sagres'	'Impulse'	kinetin	TDZ	BA
109 c	117 c*			2.2
106 c	119 c			4.5
102 d	120 c			9
144 b	130 b		1.6	
169 a	155 a		3.2	
173 a	151 a		6.2	
83 e	54 f	2.3		
99 d	78 d	4.6		
107 c	64 e	9.2		

*Explanations, see Table 1

Table 4. Effect of different concentrations of sorbitol on somatic embryo induction from proliferating embryogenic callus in two cultivars of carnation after 5 weeks of culturing

Number of embryos induced on proliferating embryogenic callus		Sorbitol concentration (M)
'Sagres'	'Impulse'	
70 d	61 d*	0.08
93 c	82 c	0.16
137 b	116 b	0.32
165 a	185 a	0.49
168 a	178 a	0.65

*Explanations, see Table 1

survival rate. All acclimatized plants were transferred to field conditions and grew normally in the natural environment.

CONCLUSION

This study showed that callus proliferation, somatic embryogenesis and direct shoot organogenesis from embryogenic calli of carnation is largely related to kind of hormone in the culture media. Using techniques described here it is possible to obtain high percentages of somatic embryos or adventitious shoots which could successfully be regenerated into mature plants. Establishment of conditions required for the high frequency of plant regeneration can find use in mass propagation of carnation and would facilitate genetic transformation of this species.

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PROLIFERACJA, ORGANOGENEZA PĘDU I SOMATYCZNA EMBRIOGENEZA W EMBRIOGENNYM KALUSIE GOŹDZIKA

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S T R E S Z C Z E N I E

Badano wpływ 2,4-D, pikloramu i NAA na proliferację embriogennego kalusa dwóch odmian goździka (‘Impulse’ i ‘Sagres’). Proliferacja embriogennego kalusa zachodziła na podstawowej pożywce MS zawierającej różne stężenia pikloramu (0,8, 2, 4, 8, 16 i 24 µM). Maksymalna proliferacja kalusa następowała w stężeniach pikloramu 8 i 16 µM. Organogeneza miała miejsce po przeniesieniu pierwotnego kalusa na pożywkę podstawową MS zawierającą TDZ, BA i kinetinę. Maksymalna organogeneza zachodziła przy stężeniu TDZ 4,5 i 9 µM. Przeniesienie proliferującego kalusa na pożywkę MS bez regulatorów wzrostu i uzupełnioną sorbitolem w stężeniach 0,08; 0,16; 0,32; 0,49 i 0,65 M powodowało somatyczną embriogenezę, przy czym najwyższa embriogeneza następowała przy stężeniach sorbitu 0,49 i 0,65 M. Liścieniowe somatyczne zarodki i regenerujące pędy przekształciły się w rośliny, które rozwijały się dalej w warunkach szklarniowych.

Słowa kluczowe: goździk, kalus embriogenny, organogeneza pędu, somatyczna embriogeneza