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Induction of Embryogenic Callus and Plant Regeneration in Carnation (*Dianthus caryophyllus* L.)

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Abstract: In this study, efficient plant regeneration through somatic embryogenesis is achieved in four cultivars of carnation (Nelson, Sagres, Spirit and Impulse). Embryogenic calli were induced on petal explants only, all the calli established on leaf, sepal, receptacle and style explants were not embryogenic. Embryogenic calli were obtained on Morashige and Skoog basal medium (1962) containing sucrose 9%, 2.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), 0.2 mg L^{-1} 6-benzyladenine (BA). After transfer of embryogenic calli to growth regulator free MS medium or medium containing low amount of 2, 4-D (0.2-0.4 mm L^{-1}) resulted in successful somatic embryogenesis. A high frequency (80-85%) of Somatic embryo germination and development into normal plantlets was observed on half-strength MS medium supplement with 3% sucrose without growth regulator. About 95% of somatic embryo-derived plantlets were acclimatized in the greenhouse conditions.

Key words: Emberyogenic calli, somatic embryo, callus, regeneration and carnation

INTRODUCTION

Carnation (*Dianthus caryopyllus* L.) is one of the major floriculture crops in many countries all over the world with high ornamental and commercial interest[4]. In Iran 20% of the greenhouses destined of ornamental, are occupied by this crop. Carnation breeders constantly seek new varieties with improved horticultural traits such as disease and pest resistance and long vase-life. Since carnation is highly heterozygous in nature, the ability of the breeder is restricted to satisfy these demands, besides; the alteration of individual traits by means of conventional procedures is difficult. Genetic engineering based on tissue culture technology provides an alternative way to overcome these problems.

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 scale-up in liquid suspension cultures, the use of bio reactors and somatic synthetic seed technologies and the fact that embryogenic cultures are suitable target tissues for gene transfer[16].

Efficient plant regeneration systems based on embryogenic callus has been developed for numerous species including rose[14], Kentucky bluegrass Paspalum[5], Astragalus[13], liliaceous[21], *Hylomecon vernali*[11]. Although, the regeneration of carnation via somatic embryogenesis has previously been reported[8,18,19,23] but indirect (callus pathway) somatic embryogenesis in this plant is not well documented[23] and induction of pro-embryos not reported.

In this study, an efficient procedure of somatic embryogenesis and plant regeneration in carnation is described.

MATERIALS AND METHODS

Four carnation cultivars member, Impulse, Nelson, Sagres and Spirit were detected. Immature flower buds (1 to 1.5 cm long) and young leaves (first and second pair shoot apex) were dissected from greenhouse-grown plants. Samples were surface sterilized using 70% ethanol for 30 sec and 2% sodium hypochlorite solution for 20 min followed by three rinses with sterilized double distilled water. Explants from leaf, sepal, receptacle, style and petal segments (each approximately 5×3 mm) were excised from immature buds and leaves for callus induction.

Explants were placed on MS medium containing 9% sucrose, 0.5, 1, 2, 3, 4 and 6 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2, 4-D) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) alone and combination with different concentrations of 6-benzladenine (BA) (0.1, 0.2, 0.5 and mg L^{-1}) for callus induction. The number of explants producing embryogenic calli were recorded after 9 weeks. For induction of somatic embryos, calli were dissected into

small pieces (30±5 mg Fresh Weight (FW) per piece) and cultured on MS medium with no growth regulators or supplanted with MS medium containing 0.2, 0.4, 0.6 and 1 mg L^{-1} 2, 4-D. The number of embryos induced on embryogenic calli were recorded after 6 weeks. For plant regeneration, somatic embryos were transferred onto half-strength MS medium with no growth regulator but containing 3% sucrose. The number of plantlet regenerated were recorded after 4 weeks. All cultures were grown in growth chamber at 24 °C and 30 30 µmol m^{-2} sec^{-1} illumination was provided by cool white fluorescent lamps with 16-h photoperiod. All culture media were adjusted to pH 5.8 with NaOH (1 N) before adding gelling agent (Agar-Agar, Merck) and autoclaved at 121°C for 15 min.

The germinated somatic embryos were transferred into 7.5 cm plastic pots containing autoclaved mixture of soil, sand and compost in the ratio 1:1:1 (w/v) and kept in greenhouse for acclimatization prior to field transferee.

For histological investigation, calli were fixed in FAA (formalin- acetic acid- ethanol, 2:1:17 v/v) for 24 h, dehydrated in serial grads of alcohol and then embedded in paraffin. Serial sections (7 µm thick) were cut and stained with hematoxylin.

The experiments were set up as factorial design with concentration of BA and 2, 4-D and genotype as main factors. In each experiment, 20 explants or 200±30 mg callus were placed in one Petri-dish with three dishes (replicates) for each treatment. The ANOVA was performed for data from each experiment and means were compared using Duncan's multiple range test ($p< 0.05$).

RESULTS AND DISCUSSION

In all cultivars, two types of callus could be recognized according to color, texture and time of callus initiation. Type I callus was soft, succulent and yellowish-green (Fig. 1A) and in all media callus initiation started on the cut edges of the leaf, sepal, receptacle, style and petal explants within 2-3 weeks. These callus was rapidly and high-frequently (95-100%) produced on all types of explants. Histological examination showed that cells in Type I callus were irregular and the relative sizes of nucleus are smaller (Fig. 1C). Apparently cells were non-embryogenic calli. Type II calli were hard, creamy-white and nodular in texture (Fig. 1B) with slow growing habit and callus initiation was observed either on the edges of the petal explants or on Type I callus within 6- 8 weeks.

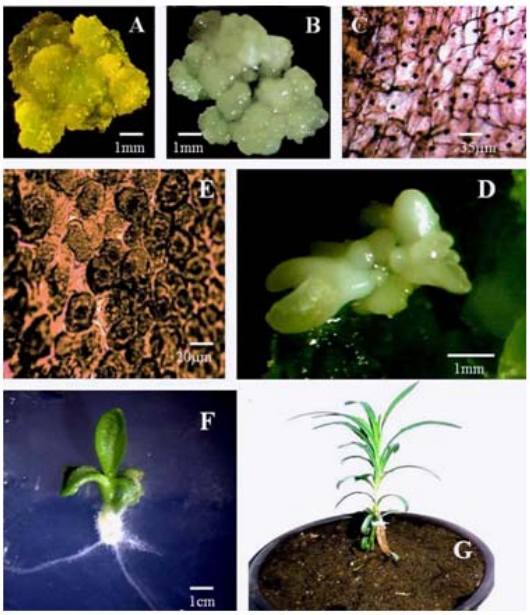

Fig. 1: Somatic embryogenesis and plant regeneration in D. caryopyllus. (A): Type I callus from petal explant on MS medium containing 2.0 mg L^{-1} 2, 4-D, 0.2 mg L^{-1} BA and sucrose 9% after 4 weeks. (B): Type II callus on MS medium containing 2.0 mg L^{-1} 2, 4-D, 0.2 mg L^{-1} BA and sucrose 9% after 9 weeks. (C): Histological section of Type I callus. (D): Histological section Type II callus. (E): Somatic embryogenesis at different developmental stages on medium MS medium containing 0.2 mg L^{-1} 2,4-D and sucrose 3% after 4 weeks. (F): Regenerated plantlet from the development of somatic embryo on half-strength MS medium and sucrose 3% with no growth regulators after 2 weeks. (G): A potted plant in greenhouse

Histological examination showed that cells in the Type II callus, known as embryogenic calli had dense cytoplasm, thick cell walls and relatively bigger nucleus (Fig. 1E). These characteristics of cells in embryogenic callus have also been reported for *Allium ampeloprasum*.[3] *Gladiolus hort*[20] and *Agapanthus praecox* ssp.[21] but embryogenic calli (type II callus) was apparently a mass of pro-embryos not reported in the carnation.

The induction of embryogeneic callus was usually promoted by a relatively higher concentration of auxins, especially 2, 4-D[1] and it has been suggested that the formation of an embryogenic cell is related to nuclear DNA hypermethylation in the presence of high concentration of 2,4-D[12]. Type II callus (this study)

were only induced on petal explants in MS medium supplemented with 2.0 mg L^{-1} 2, 4-D and 0.2 mg L^{-1} BA. The induction of Type II callus was suppressed on medium containing lower (0.5 and 1 mg L^{-1}) or higher (4 and 6 mg L^{-1}) concentrations of 2, 4-D. Many factors, including choice of growth regulators and that of explants are responsible for successful somatic embryogenesis. Although somatic embryogenesis has been observed in many plants from various explants[22], the choice of explant is crucial to induce embryogenic callus in *D. caryophyllus*. of all five explants, only petals segments produced embryogenic callus on the media tested, supporting the conclusion that the internal state of explant cells is of prime importance in the expression of somatic embryogenesis, with other condition such as exogenous growth regulators being simply "permissive" for expression of this intrinsically determined pattern of development[22,24]. In previous protocols of somatic embryogenesis of carnation explants of leaf[18] and stem[8] for induction of callus has been used, but using of petal is not reported.

Table 1 and 2 shows the number of explants on the media containing different BA concentrations, which have significant differences ($p<0.05$) in Type II calli formation. Maximum Type II callus was induced on 0.2 mg L^{-1} BA and higher (0.5 and 1 mg L^{-1}) BA concentrations suppressed the formation of type II callus. Signification differences ($p<0.05$) were observed among cultivars. The highest formation of Type II calli was observed for cv. Sagres followed by Nelson, Impulse and Spirit. Using cytokinins in combination with auxins to induce somatic embryogenesis in callus culture has been reported for some plant species[2,9,17]. In this study, the addition of low concentration of BA to medium containing 2, 4-D has required for the embryogeneic callus induction. The requirement of BA for somatic embryogenesis may be dependent on explant source, as reported by[2].

Table 1: Effect of genotype and BA on type II callus induction in petal explants in *D. caryopyllus* after 10 weeks of culture in meduim of MS containing 2.0 mg L^{-1} 2,4-D and 9 % sucrose (w/v)[a]

	No. of explants which have type II callus (mean±SE)[b]			
	Impulse	Sagres	Nelson	Spirit
Genotype	5.33±0.33^{c}	8.33±1.00^{a}	6.44±0.52^{b}	4.33±0.33^{d}
BA (mg L^{-1})	0	0.1	0.2	0.5
	0	5.16±0.57^{b}	10.66±.1.52^{a}	2.55±0.33^{c}

[a]: Means having the same letter in row were not significantly different by Duncan's multiple range test ($p<0.05$); [b]: 20 explants/replicate, 3 replicate/treatment

Table 2: Effect of interaction between genotype and BA on type II callus induction in petal explants *D. caryopyllus* after 10 weeks of culture in MS meduim containing 2.0 mg L^{-1} 2,4-D and 9% sucrose (w/v)[a]

	No. of explants which have type II callus (mean±SE)[b]			
BA (mg L^{-1})	Impulse	Sagres	Nelson	Spirit
0	0	0	0	
0.1	5.00±1.33^{e}	7.00±0.5^{d}	5.00±0.57^{e}	3.66±0.33^{ef}
0.2	9.66±2.15^{c}	14.00±2.75^{a}	11.33±2.00^{b}	7.33±1.115^{d}
0.5	1.33±0.33^{h}	4.00±0.33^{ef}	3.00±0.33^{gf}	1.33±0.33^{gh}
1	0	0	0	0

[a]: Means having the same letter in column or row were not significantly different by Duncan's multiple range test ($p<0.05$); [b]: 20 explants/replicate, 3 replicate/treatment

Somatic embryos were induced within 1-2 weeks from type II calli (embryogenic calli) when they transferred to a hormone-free medium containing 3% sucrose or low concentrations (0.2 and 0.4 mg L^{-1}) of 2, 4-D. Globular embryos further developed into heart-shaped and torpedo-shaped embryos after 1 week. Cotyledonary somatic embryos were observed four weeks after subculturing the embryogenic calli (Fig. 1D). No embryo induction was occurred on succulent type I calli on medium similar to that used for type II calli. Non-embryogenic calli are usually not able to be development into somatic embryo[8]. This could be dependent on their developmental state and gene expression pattern of the calli. In many plant spaces has been shown that there are differences between embryogenic and non-embryogenic calli in terms of morphology, physiology, metabolism and gene expression pattern[8].

Table 3 and 4 shows the number of embryos on embryogenic callus (type II calli) at different sucrose concentration. In all cultivars, the number of somatic embryos produced from embryogenic callus was significantly increased ($p< 0.05$) by adding low concentrations of 2, 4-D to the culture media. Maximum embryos was induced on 0.2 mg L^{-1} 2,4-D. No embryos were formed on MS medium containing 0.6 and mg L^{-1} 2, 4-D. Signification differences ($p<0.05$) were observed among cultivars Sagres and/or Nelson with Impulse and/or Spirit. In general, prolonged embryo differentiation beyond globular stage and its subsequent maturation require the removal of growth regulators from the medium or there least concentrations[15]. In this report, emberyogenic calli differentiated onto somatic embryo on hormones-free medium or medium containing low concentration of 2,4-D., although medium containing more concentrations of 2, 4-D superseded differentiation of embryogenic calli.

In many plant spaces has been shown that individual genotypes within a given species vary greatly in embryogenic capacity[15]. Such genotypic differences in embryogenic capacity might reflect current differences in the ability to activate key elements in the embryogenic pathway. Present study, significant genotypic differences observed for both induction of embryogenic calli and somatic embryos.

Table 3: Effect of genotype and 2,4-D on somatic embryo induction for type II callus *D. caryopyllus* after 6 weeks of culture in MS medium containing %3 sucrose (w/v)[a]

	No. of induced from type II callus (mean±SE)[b]			
	Impulse	Sagres	Nelson	Spirit
Genotype	75.88±8.20[b]	95.88±6.33[a]	88.33±7.55[a]	77.44±4.12[b]
2, 4-D (mg L^{-1})	0	0.2	0.4	0.6
	59.58±8.20[b]	134.58±12.66[a]	59.00± 5.67[b]	0.0

[a]: Means having the same letter in row were not significantly different by Duncan's multiple range test ($p<0.05$); [b]: 200±20 mg embryogenic callus/replicate , 3 replicate/treatment

Table 4: Effect of interaction between genotype and 2,4-D on somatic embryo induction for type II callus in four cultivar *D. caryopyllus* after 6 weeks of culture in MS medium containing %3 sucrose (w/v)[a]

2, 4-D	No. embryo induced from type II callus (mean±SE)[b]			
(mg L^{-1})	Impulse	Sagres	Nelson	Spirit
0	49.00±3.80[f]	79.00±8.70[d]	44.00±5.52[f]	[de]6.50±65.66
0.2	112.33±12.22[c]	141.00±15.4[b]	160.00±15.3[a]	123.00±10.79[c]
0.4	65.66±9.7[de]	67.00±8.00[de]	60.33±6.61[fe]	43.00±7.93[f]
0.6	0	0	0	0

[a]: Means having the same letter in column and row were not significantly different by Duncan's multiple range test ($p<0.05$); [b]: 200±20 mg embryogenic callus/replicate, 3 replicate/treatment

Genotype effects in carnation have been observed in direct somatic embryogenesis culture[23] and organogenesis[10].

After 4 weeks induced somatic embryos were transferred to half-strength hormone free MS medium and they developed into perfect plantlets within 2 weeks (Fig. 1F). Germination rates of somatic embryos of all cultivars were about %80 to %85. Protocols indirect somatic embryo formation have been reported previously[8,19] but in all of them mentioned that the regeneration efficiency was quite low. Because somatic embryogenesis was asynchronous, plantlets obtained from the same culture were at different developmental stage. Roots failed to develop when somatic embryos were not separated from calli cultured on half-strength hormone free MS medium (medium suitable for rooting). A high percentage (approximately 80%) of rooted plantlets were successfully transferred to soil (Fig. 1G) and developed to normal plants in the greenhouse with 90% survival. All acclimatized plants were transferred to field conditions and grew normally in their natural environment. No morphological variant was observed among these plants.

CONCLUSION

The study describes an efficient plant regeneration system in *D. caryophyllus* through somatic embryogenesis. Establishment of some conditions required for the high frequency of somatic embryogenesis would facilitate protoplast culture, somatic hybridization, genetic transformation and artificial seed production of carnation.

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