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Plant Regeneration from Shoot Tip-Derived Embryogenic Callus of *Dianthus superbus*

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The highest degree of callus formation was obtained from the shoot tips of *Dianthus superbus* when cultured on the MS medium supplemented with 2.0 mg/L NAA and 0.5 mg/L BAP. Embryogenic calluses were obtained from the seperated friable calluses on MS medium containing 2.0 mg/L 2,4-D after 7-8 wk of culture. For plant regeneration, embryogenic calluses were selected and cultured on the proliferation medium. After 3 wk, somatic embryos appeared on MSK medium (0.5 mg/L NAA, 2.0 mg/L kinetin) and N_6 medium (2.0 mg/L kinetin, 0.1 mg/L NAA, 0.1 mg/L 2,4-D and 2.0 g/L casein hydrolysate). When these somatic embryos were kept under continuous illumination, shoots were successfully regenerated on the both media. The shoots were rooted on MS medium supplemented with 2.0 mg/L NAA.

Keywords : *Dianthus superbus* L., embryogenic callus, somatic embryo, plant regeneration

The study of plant morphogenesis has one research area with which tissue culture has been associated and another in which the use of the *in vitro* technology has made significant contributions to both fundamental knowledge and application (Thorpe, 1990). In particular, understanding of the processes of somatic embryogenesis has been clearly enhanced through the use of tissue cultures. As an approach to plantlet regeneration, somatic embryogenesis has several advantages. The advantages include the efficiency of the process, the potential for the production of much higher number of plantlets, and the morphological and cytological uniformity of the plantlets.

Auxin was known to regulate the induction and development of embryogenesis (Fujimura and Komamine, 1979). 2,4-D-induced suppression of embryo maturation may be mediated through endogenous ethylene production (Wochok and Wetherell, 1971). Ethephon releases embryos without an appreciable reduction in the growth and multiplication of the embryogenic clumps in suspension cultures of carrot. Moreover, it has been shown that the auxin-grown tissue cultures of carrot produce more ethylene than auxin-free cultures (Pinfield *et al.*, 1991). High ethylene content would result in enhanced activity of cellulase, pectinase or both causing breakdown of the clumps before polarity is established in the proembryos for further organized development. Thus in 2,4-D contained medium tissue multiplication goes on but mature embryos do not appear. Besides auxin, the form of nitrogen in the medium and cell-to-cell interaction significantly affects *in vitro* embryogenesis (Halperin and Wetherell, 1965).

Micropropagation methods are currently used on a commercial scale for herbaceous, ornamental and horticultural plants. The genus *Dianthus* contains a large number of commercially important ornamentals such as carnation, indian carpet, chinese pink, *Dianthus superbus*. But among them methods of cell, tissue and organ culture have been developed for carnation only. In case of carnation, adventitious shoots formation from petals (Kakehi, 1979; Gimelli *et al.*, 1984; Frey and Janick, 1991; Lu *et al.*, 1991), anthers (Villalobos, 1981), stem segments (Roest and Bokelmann, 1981; Radojevic *et al.*, 1990; Frey and Janick, 1991) and axillary bud explants (Miller *et*

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al., 1991) have been reported.

This report describes the condition for the effective shoot formation from embryogenic calluses by using explants of *Dinathus superbus*. We have studied the effect of plant growth regulators and culture conditions on the regeneration of the plant. In this respect, the work reported here establishes the first protocols for embryogenesis and micropropagation of this species and this information is of interest to those involved in studies of differentiation and determination at the cell, tissue and organ levels.

MATERIALS AND METHODS

Plant materials

Seeds of *Dianthus superbus* L. were obtained from the Alpine Experiment Station, RDA, Korea. Seeds were surface sterilized in 4% sodium hypochlorite with two drops of Triton-X 100 per liter for 30 min, then rinsed 5 times with sterile distilled water. They were germinated in the light on solid medium without growth regulators. After germinated *in vitro*, differentiated plantlets were used for subsequent experiments.

Callus induction

Shoot tip explants trimmed to 0.5-0.7 cm height and leaf segments cut in 0.5-0.7 cm^2 were used to callus induction. All explants were placed in petri dishes 8.5 cm in diameter with 20 mL of culture medium containing MS basal salt mixture (Sigma M5524). The effect of different concentrations of 2,4-D, NAA and BAP on the formation or induction of callus was tested. Cultures for callus induction were maintained in the dark at 27℃. The petridishes containing explants were sealed with parafilm. Each experiment was repeated more than 5 times.

To ensure that only pure callus used in the regeneration experiment, necrotic tissues were discarded and only the soft and friable calluses formed on the basal part of the cultures were selected and used in the subsequent experiments.

Induction of embryogenic callus

In order to investigate induction of embryogenic calluses, these collected calluses were designed 0.5× 0.5 cm size and then transferred to a fresh MS medium supplemented with 2,4-D (0, 0.1, 0.5, 1.0, 2.0 mg/L). Four callus pieces were used per treatment. The calluses were subcultured 2-3 times every 30 d. Cultures were maintained at 27℃ in the dark.

Proliferation of somatic embryos

After 7-8 wk of subculture, embryogenic calluses were selected and placed on many combinated media for the proliferation of somatic embryos. The effect of various concentrations of NAA in combination with BAP or kinetin was investigated. N_6 medium containing only N_6 basal salt mixture (Sigma C1416), MS modified vitamin powder (Sigma M68 96), various plant growth regulators and casein hydrorysate (2.0 g/L) was also tested.

Primordial shoots and plant regeneration

The proliferated somatic embryos were transferred to the regeneration medium and kept at 27℃ under continuous illumination (21.5 $\mu E \cdot m^{-2} \cdot s^{-1}$). Differentiated shoots, 5 mm in height, were seperated from the calluses and cultured on MS medium with or without 2.0 mg/L NAA for rooting. Regenerated plants with established root systems were acclimatized themselves to water, and then transferred to pots.

RESULTS AND DISCUSSION

Callus induction

The effect of plant growth regulators on callus induction from leaf segments and shoot tip of *D. superbus* was investigated. Calluses were induced when the leaf segments and the shoot tips of *D. superbus* were explanted on a revised MS medium supplemented with 2,4-D, NAA and BAP (Table 1).

The combination of NAA and BAP was more effective than that of 2,4-D alone in the formation of calluses. The calluses were formed on the basal part of the explants within a month and they were soft in texture and friable in structure.

As a result, callus formation was the best on the MS medium containing 2.0 mg/L NAA and 0.5 mg/L BAP from shoot tip of *D. superbus* but this condition also induced formation of adventitious roots around the marginal surface. Therefore, for the

Table 1. Effect of plant growth regulators on callus induction from leaf and shoot tip in *Dianthus superbus*

Plant growth regulator (mg/L)			Callus induction[a]	
2,4-D	NAA	BAP	Leaf segment	Shoot tip
0.1	0	0	−	−
0.5	0	0	−	+
1.0	0	0	+	++
2.0	0	0	+	++
3.0	0	0	+	+
0	1.0	0.5	+	+
0	1.0	1.0	+	+
0	1.0	2.0	−	−
0	2.0	0.5	+	+++
0	2.0	1.0	+	++
0	2.0	2.0	+	++
0	3.0	0.5	+	++
0	3.0	1.0	++	++
0	3.0	2.0	++	++

[a]−, none; +, poor; ++, moderate; +++, plentiful.

purpose of collecting only pure calluses, the soft and friable calluses formed on the basal part of cultures were selected. In the case of this study, a combination of BAP with NAA and shoot tip as an explant was found suitable for stimulating callus induction.

Induction of embryogenic callus

To examine the effect of 2,4-D on embryogenic callus induction, collected pure calluses were subcultured on revised MS medium supplemented with 0.1-2.0 mg/L 2,4-D in the dark at 27℃. They grew rapidly and satisfactorily (Fig. 1A) on MS medium containing 2.0 mg/L 2,4-D. Proliferated calluses of 4-5 wk culture were structually indistinguishable from that initially induced from the shoot tip explants. After 7-8 wk of culture, nodular, mucilaginous, pale-yellow and knobby calluses, and friable and translucent calluses proliferated all over the surface such as that shown in Fig. 1B, which were consited two distinguished types. The first one was recognized as embryogenic callus due to its potential to develop into shoots when transferred onto the regeneration medium in a subsequent experiment, whereas the other as nonembryogenic calluses because they did not undergo regeneration on whatever medium they were cultured. The result that embryogenic calluses were induced at higher proportions in 2,4-D treatment conformed the view that the calluses formed

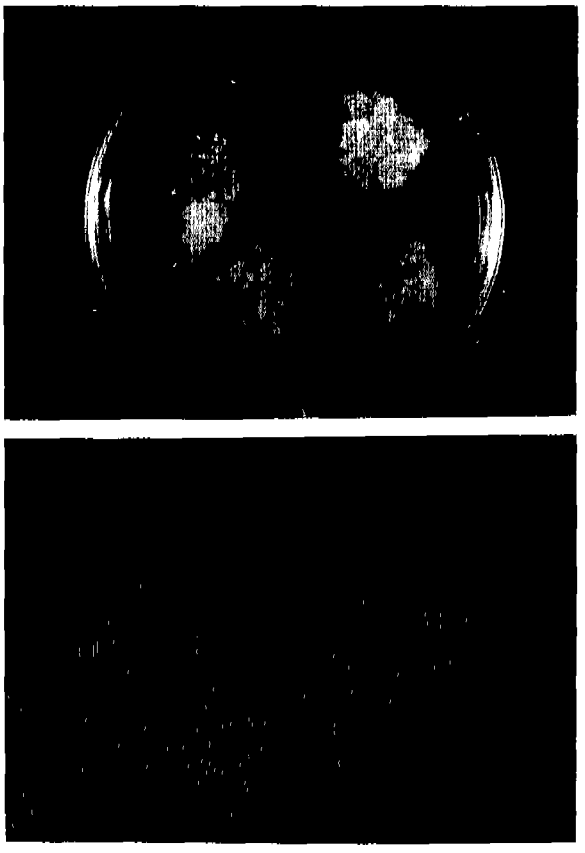

Fig. 1. Formation of embryogenic callus from the shoot tip-derived callus in *Dianthus superbus*. A, Proliferation of pure calluses on MS medium containing 2.0 mg/L 2,4-D. Bar=5 mm; B, Embryogenic callus (EC) and nonembryogenic callus (NEC) formed on MS medium containing 2.0 mg/L 2,4-D after 7-8 wk of culture. Bar=1 mm.

on a medium containing 2,4-D were adventageous for embryogenesis (Lazzeri *et al.*, 1987).

Proliferation of somatic embryos

Auxin is the most important factor for regulation of induction and development of embryogenesis (Fujimura and Komamine, 1979). All the successful cases of somatic embryogenesis, a rather high auxin concentration is required for the formation of the embryogenic callus, whereas a lower auxin concentration favours the development of somatic embryo from embryogenic callus (Fujimura and Komamine, 1979; Chen *et al.*, 1985; Kawahara and Komamine, 1991). *In vitro* development of somatic embryos in carrot was a two step process, each requiring a different medium. The callus was initiated and multiplied in an auxin-rich medium as groups of meristemic

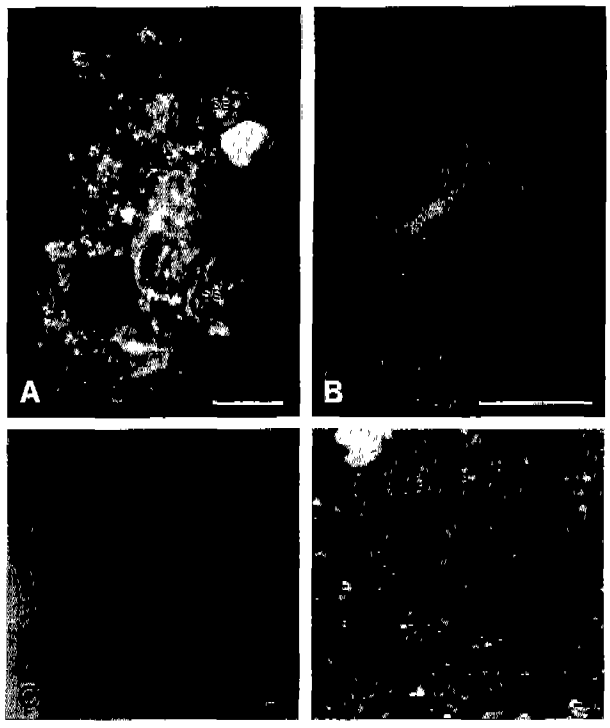

Fig. 2. Regeneration of shoot and plantlet from embryogenic callus in *Dianthus superbus*. A, Somatic embryos (SE) derived from embryogenic callus on N_6 medium supplemented with 2.0 mg/L kinetin, 0.1 mg/L NAA, 0.1 mg/L 2,4-D and 2.0 g/L casein hydrolysate. Bar=1 mm; B, Shoot regeneration under continuous illumination. Bar=1 mm; C, Regenerated plantlet with leaves and root in MS basal medium supplemented with 2.0 mg/L NAA. Bar=5 mm; D, Regenerated plantlet transferred to soil. Bar=5 mm.

cells called 'embryogenic clumps'. The auxin generally used was 2,4-D in the range 0.5-1.0 mg/L. In repeated subcultures on the proliferation medium the embryogenic clumps continue to multiply without the appearance of mature embryos (Sung and Okimoto, 1981). In this study of *D. superbus*, embryogenic calluses showed somatic embryos (Fig. 2A) after subcultures in MSK (0.5 mg/L NAA added on 2.0 mg/L kinetin) and N_6 medium (2.0 mg/L kinetin, 0.1 mg/L NAA, 0.1 mg/L 2,4-D and 2.0 g/L casein hydrolysate), which contained low concentration of auxin. And then the somatic embryos did not develop to maturity anymore. Somatic embryos incubated continuous in the dark were brown and lost the embryogenic potential. These results showed that induction and developement of somatic embryos were dependent upon the auxin concentration. And besides auxin, another factor was related with the development of the somatic embryos.

Table 2. Effect of plant growth regulators on shoot formation from embryogenic callus in *Dianthus superbus*

Plant growth regulators (mg/L)				Frequency[a] of shoot regeneration (%)
NAA	BAP	Kinetin	2,4-D	
0.1	0.5	–	–	0
0.5	1.0	–	–	0
0.5	2.0	–	–	1.3
0.1	–	0.5	–	0
0.5	–	1.0	–	6
0.5	–	2.0	–	44
0.1	–	2.0	0.1	multiple[b]

[a]Experiment were tested with five times and for each repetition 16 callus segments were used.
[b]N_6 medium: Based on N_6 salt and supplemented with 2.0 g/L casein-hydrolysate.

Primordial shoots and plant regeneration

Culture media for the primordial shoots and plant regeneration were similar to those used for somatic embryo induction. And for further development, the cultures was kept at 27℃ under continuous illumination at light intensity 21.5 $\mu E \cdot m^{-2} \cdot s^{-1}$. By manipulating the growth substances, MSK and N_6 media (Table 2) were suitable for the regeneration of primordial shoots.

As shown in Fig. 2B, after 3 wk of culture green spots appeared on somatic embryos and then they grew eventually to form shoots. It seems that light-grown cultures exhibited better embryogenic response than those maintained in the dark. That is to say, in this species, a combination of kinetin with NAA and light condition was found suitable for stimulating plant regeneration from calluses. And the kind of salt also seems to be the key factor for achieving rapid regeneration from such embryogenic calluses.

The highest frequency of roots was observed in MS basal medium supplemented with 2.0 mg/L NAA and the plantlets (Fig. 2C) with established root systems were acclimatized to water, then transferred to pots (Fig. 2D).

In conclusion we have established a plant regeneration system from explants of *D. superbus* at a comparatively high frequency. This result may be generally applicable to other species in the genus of *Dianthus*.

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술패랭이꽃(*Dianthus superbus*) 莖端分裂組織에서 由來된 胚發生 캘러스로부터 植物體 再分化

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적 요

술패랭이꽃(*Dianthus superbus* L.)의 배발생 캘러스 배양을 통하여 식물체 재분화가 이루어졌다. 캘러스는 2.0 mg/L NAA와 0.5 mg/L BAP가 첨가된 MS 기본배지에 경단분열조직을 치상하여 27℃ 암상태에서 유기하였고 이들 캘러스를 2.0 mg/L 2,4-D가 첨가된 배지에 30일 간격으로 7-8주간 계대배양하여 배발생 캘러스를 획득하였다. 배발생 캘러스는 MSK(0.5 mg/L NAA, 2.0 mg/L kinetin), N_6 (0.1 mg/L NAA, 0.1 mg/L 2,4-D, 2.0 mg/L kinetin) 배지에서 체세포배를 형성하였으며 광조건에서의 배양을 통해 체세포배의 지속적인 분화를 유도하였다. 그 결과 MSK 배지상의 체세포배의 캘러스에서는 44%의 shoot 재분화율이, 그리고 N_6 배지에서는 캘러스당 10-15개의 multiple shoot의 형성이 이룩되었다. 이들 재분화된 shoot는 2.0 mg/L NAA가 첨가된 배지에서 뿌리 분화가 유도되었으며 이들을 pot로 이식하여 재분화 개체를 획득하였다.

주요어: 술패랭이꽃, 배발생 캘러스, 체세포배, 식물체 재분화

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