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Direct Adventitious Shoot Regeneration from Leaf and Internode Explants of *Dianthus caryophyllus*

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ABSTRACT

Direct adventitious shoots were regenerated in *Dianthus caryophyllus* cv. 'Tempo' from leaf and internode explants cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin, thidiazuron (TDZ), zeatin, α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) singly, or in combination. Shoot regeneration was highest with 2 mg/l TDZ and 1 mg/l IAA in both explants, whereas the number of shoots per explant was greater with 2 mg/l TDZ and 1 mg/l NAA. Shoots were elongated and multiplied on MS medium supplemented with 1 mg/l BA and solidified with 1% agar to reduce hyperhydricity. *In vitro*-raised shoots were rooted in half-strength MS medium supplemented with 1 or 2 mg/l IAA, NAA and indole-3-butyric acid (IBA). The rooted plants were hardened with 80-82% survival success in pots.

Keywords: carnation, growth regulators, hardening, *in vitro*, rooting
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **NAA**, α-naphthalene acetic acid; **TDZ**, thidiazuron

INTRODUCTION

Dianthus consists of more than 300 species and contains many important ornamental species such as carnation (*Dianthus caryophyllus* L.), which is one of the most important cut flower crops in the world (Shiba and Mii 2005). Due to its excellent keeping quality, wide range of forms, ability to withstand long distance transportation and remarkable ability to rehydrate after continuous shipping, it is preferred by growers. In the United States, and world-wide, it ranks third after roses and chrysanthemums in popularity (Xia *et al*. 2006). Carnation is widely cultivated on a large scale in many countries around the world. Vegetative propagation through stem cuttings is possible, however, plant multiplication by this method is too slow to be commercially viable. Carnation has been propagated *in vitro* by using direct plant regeneration systems from excised plant tissues such as stem (Zuker *et al*. 2001), node (Nontaswatsri *et al*. 2004), leaf (Zhang *et al*. 2005) and petal (Van Altvorst *et al*. 1996). Direct regeneration of adventitious shoots provides a fast and dependable method for the production of large quantities of uniform plantlets in a short time. This stable regeneration system is important as it provides a platform for the application of more advanced techniques of genetic transformation by introduction of foreign genes (Qu *et al*. 2000). It has also been reported that shoot regeneration in carnation is influenced by genotype, explant source and the balance of plant growth regulators (PGRs) (Frey and Janick 1991; Kallak *et al*. 1997). In the present investigation an attempt was made to regenerate adventitious shoots directly from leaf and internode explants of carnation cv. 'Tempo'.

MATERIALS AND METHODS

Explant source and preparation

Stem cuttings (8-10 cm) from the apical portion of carnation (*Dianthus caryophyllus* L.) cv. 'Tempo' were obtained from the Department of Floriculture and Landscaping, University of Horticulture and Forestry, Solan, India. Leaf (0.5 cm^2) and internode (0.8-1 cm) explants were isolated from the cuttings and treated with 0.1% carbendazim (Indofil Chemical Co., Bombay, India) solution for 10-15 min followed by washing under running tap water for 30 min. The explants were surface sterilized with 5% sodium hypochlorite solution for 10 min followed by 3-4 washings with sterilized distilled water.

Culture medium and culture conditions

The sterilized explants were cultured with abaxial surface down on solidified MS (Murashige and Skoog 1962) medium containing 30 g/l (w/v) sucrose, 1 and 2 mg/l 6-benzyladenine (BA), kinetin (Kn), thidiazuron (TDZ) and zeatin (Zn) and 1 mg/l α-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) alone or in combination. The pH of the medium was adjusted with 1 N HCl and/or 1 N NaOH to 5.8 prior to adding 0.8% (w/v) Difco bacto agar. The medium was dispensed in 30 ml aliquots into 100 ml Erlenmeyer flasks (Borosil, Bombay, India), which were plugged with non-absorbent cotton plugs. Medium was autoclaved at 1.1 kg/cm^2 for 15 min at 121°C. The cultures were incubated in the dark for 2 weeks and subsequently transferred to16-hr light (50-60 µmol/m^2/s) provided with white, cool fluorescent tubes (40 W each, Philips) at 24 ± 2°C. The cultures were transferred at 4-week intervals to fresh medium with the same composition. The *in vitro*-raised shoots (2.5-3 cm) were separated from the explants and cultured on MS medium solidified with 1% agar and supplemented with 1 mg/l BA for elongation and multiplication. The number of explants producing shoots and number of shoots per explant were recorded after four weeks of culture.

Rooting and hardening

In vitro-raised shoots were rooted on half-strength MS medium supplemented with 1 and 2 mg/l IAA, indole-3-butyric acid (IBA) and NAA (LOBA Chemie, Bombay). 0.2% activated charcoal (s d Fine-Chem Ltd., Bombay) was added to the medium. After four weeks of culture, rooted shoots were removed from the culture vessels, washed thoroughly and dipped in 0.01% carbendazim

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Original Research Paper

Table 1 Effect of plant growth regulators on explants producing direct adventitious shoots.*

Treatment (mg/l)						Leaf	Internode	Mean
BA	Kn	TDZ	Zeatin	NAA	IAA			
0	0	0	0	0	0	0 (0)	0 (0)	0 (0)
1	0	0	0	0	0	0 (0)	0 (0)	0 (0)
2	0	0	0	0	0	0 (0)	66.60 (54.40)	33.30 (34.20)
0	1	0	0	0	0	0 (0)	0 (0)	0 (0)
0	2	0	0	0	0	0 (0)	0 (0)	0 (0)
0	0	1	0	0	0	0 (0)	0 (0)	0 (0)
0	0	2	0	0	0	54.20 (47.40)	51.60 (46.10)	52.90 (46.60)
0	0	0	1	0	0	0 (0)	0 (0)	0 (0)
0	0	0	2	0	0	0 (0)	0 (0)	0 (0)
0	0	0	0	1	0	0 (0)	0 (0)	0 (0)
0	0	0	0	0	1	0 (0)	0 (0)	0 (0)
1	0	0	0	1	0	0 (0)	0 (0)	0 (0)
1	0	0	0	0	1	0 (0)	0 (0)	0 (0)
2	0	0	0	1	0	0 (0)	74.00 (59.40)	37.00 (50.40)
0	1	0	0	1	0	0 (0)	0 (0)	0 (0)
0	1	0	0	0	1	0 (0)	0 (0)	0 (0)
0	2	0	0	1	0	0 (0)	0 (0)	0 (0)
0	2	0	0	0	1	0 (0)	0 (0)	0 (0)
0	0	1	0	1	0	0 (0)	0 (0)	0 (0)
0	0	1	0	0	1	0 (0)	0 (0)	0 (0)
0	0	2	0	1	0	72.30 (58.30)	64.50 (53.50)	68.40 (56.20)
0	0	2	0	0	1	66.00 (54.40)	80.50 (63.80)	73.80 (59.20)
0	0	0	1	1	0	0 (0)	0 (0)	0 (0)
0	0	0	1	0	1	0 (0)	0 (0)	0 (0)
0	0	0	2	1	0	0 (0)	0 (0)	0 (0)
0	0	0	2	0	1	0 (0)	0 (0)	0 (0)
Mean						7.10 (10.40)	12.40 (20.50)	9.80 (13.20)

$LSD_{0.05}$ Treatment (A) = (1.17); Explant (B) = (0.51); A x B = (1.84)
*Mean of three replications
Figures in parentheses are arc sine transformed values

solution for 15-20 min. The plantlets were hardened and transferred to earthen pots (10 cm diameter) containing sand: soil: FYM (farm yard manure) mixed in a 1: 1: 1 ratio. The plants were maintained under a glasshouse at 24 ± 2°C and 80% relative humidity and observed for their survival until 6 weeks of transfer to pots. The data were recorded on shoots forming roots and number of roots per shoot after four weeks of culture.

Statistical analysis

Three replications with 15 explants in each replication (45 explants) were maintained for each treatment and the data were analyzed statistically using a factorial completely randomized design (Gomez and Gomez 1984). The statistical analysis based on mean values per treatment was made using ANOVA. The comparative LSD multiple range test ($P \leq 0.05$) was used to determine differences between the treatments.

RESULTS AND DISCUSSION

Explants failed to regenerate adventitious shoots in PGR-free medium or when the medium was supplemented with 1 mg/l each of BA, TDZ, IAA or NAA and 1 and 2 mg/l each of Zn or Kn alone (**Table 1**). Shoots were produced when the medium was supplemented with 2 mg/l BA or TDZ (**Fig. 1A, 1B**). About 73.8% of the explants regenerated shoots when 2 mg/l TDZ was used in combination with 1 mg/l IAA, which differed significantly from other treatments. It was observed that out of 27 PGR combinations used, only a very few responded. Frey and Janick (1991) also reported that the explant source and the balance of PGRs influenced shoot regeneration in carnation cultivars 'Scania', 'White Sim' and 'Sandra'. Shoot regeneration was significantly higher in the internode explants (12.4%) than leaf explants (7.1%). TDZ was more effective than BA or Zn in inducing shoots. Similar results in which TDZ was found to be more effective than BA in inducing shoot formation were reported by Sankhla *et al*. (1995). The interaction effect of treatment × explant revealed that 2 mg/l TDZ in combination with 1 mg/l NAA or 1 mg/l IAA resulted in a higher percentage of explants producing shoots in leaf and internode explants.

The average number of shoots per explant ranged from 1.08 to 4.50 depending on the treatment. The highest average number of shoots/explant (4.50) was observed with a combination of 2 mg/l TDZ and 1 mg /l NAA, which differed significantly from the other treatments (**Table 2**). Kumar *et al*. (2006) obtained 27 shoot buds per callus on MS medium supplemented with 0.6 mg/l TDZ and 1.2 mg/l Zn in carnation cv. 'Candy'. Ahmed *et al*. (2006) reported 5 µM TDZ as the optimum concentration for the induction of maximum number of shoot buds and shoots per explant in carnation 'Finest Mix'. Mujib *et al.* (1993) obtained highest number of adventitious shoots with 0.5 mg/l BA in carnation cv. 'William Sim'. The number of shoots did not differ statistically in both explants. The treatment × explant interaction revealed best response when 1 mg/l NAA was used in combination with 2 mg/l TDZ in both explants.

The *in vitro*-raised shoots were transferred to MS medium supplemented with 1 mg/l BA and solidified with 1% agar for elongation and multiplication (**Fig. 1C**). In the present study, the problem of hyperhydricity was observed in the shoots derived from leaf or internode explants. The problem was more acute when cultures were maintained continuously on TDZ-containing medium. Therefore, shoots were transferred to a medium solidified with 1% agar and supplemented with BA (1 mg/l) to reduce the problem of hyperhydricity. Ahmed *et al.* (2006) reported similar results and observed that 10 days without exposure to TDZ-containing medium was optimum for shoot bud induction. Hakkaart and Versluijs (1983) used 8 or 10 g/l agar to solidify the medium in order to reduce the glassiness of shoots in cv. 'Sam's Pride'.

After elongation, shoots were transferred to rooting medium consisting of half-strength MS salts, 1 and 2 mg/l IAA, IBA or NAA. Shoots did not root in PGR-free medium whereas 100% rooting was achieved with any of the auxins used (**Fig. 1D**). The highest number of roots/shoot (8.33) was observed with 2 mg/l IBA followed by 1 mg/l IBA (**Table 3**). IAA and NAA produced a lower res-

Table 2 Effect of plant growth regulators on number of shoots per explant.

Treatment (mg/l)						Leaf	Internode	Mean
BA	Kn	TDZ	Zeatin	NAA	IAA			
0	0	0	0	0	0	0 (1.00)	0 (1.00)	0 (1.00)
1	0	0	0	0	0	0 (1.00)	0 (1.00)	0 (1.00)
2	0	0	0	0	0	0 (1.00)	2.16 (1.75)	1.08 (1.38)
0	1	0	0	0	0	0 (1.00)	0 (1.00)	0 (1.00)
0	2	0	0	0	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	1	0	0	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	2	0	0	0	2.66 (1.90)	2.33 (1.82)	2.50 (1.86)
0	0	0	1	0	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	2	0	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	0	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	0	0	1	0 (1.00)	0 (1.00)	0 (1.00)
1	0	0	0	1	0	0 (1.00)	0 (1.00)	0 (1.00)
1	0	0	0	0	1	0 (1.00)	0 (1.00)	0 (1.00)
2	0	0	0	1	0	0 (1.00)	2.33 (1.82)	1.16 (1.41)
0	1	0	0	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	1	0	0	0	1	0 (1.00)	0 (1.00)	0 (1.00)
0	2	0	0	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	2	0	0	0	1	0 (1.00)	0 (1.00)	0 (1.00)
0	0	1	0	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	1	0	0	1	0 (1.00)	0 (1.00)	0 (1.00)
0	0	2	0	1	0	4.66 (2.36)	4.33 (2.30)	4.50 (2.34)
0	0	2	0	0	1	2.33 (1.79)	1.66 (1.63)	2.00 (1.71)
0	0	0	1	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	1	0	1	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	2	1	0	0 (1.00)	0 (1.00)	0 (1.00)
0	0	0	2	0	1	0 (1.00)	0 (1.00)	0 (1.00)
Mean						0.36 (1.11)	0.47 (1.16)	0.41 (1.13)

LSD $_{0.05}$ Treatment (A) = (0.12); Explant (B) = (0.06); A x B = (0.18)
Figures in parentheses are square root transformed values

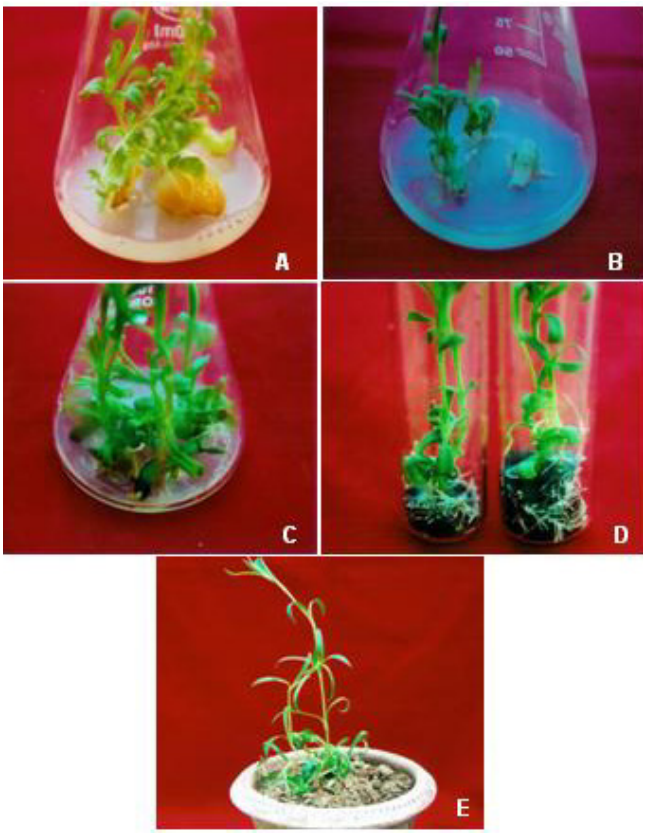

Fig. 1 Direct adventitious shoot regeneration from leaf and internode explants. Shoot regeneration from (**A**) leaf (**B**) internode explants on MS medium supplemented with 2 mg/l TDZ and 1 mg/l NAA after four weeks of culture. (**C**) Shoot multiplication on MS medium solidified with 1% agar and supplemented with 1 mg/l BA. (**D**) Rooting of *in vitro* raised shoots on half-strength MS medium supplemented with 2 mg/l IBA and 0.2% activated charcoal after four weeks of culture. (**E**) Hardened plant of carnation after six weeks of transfer to pots.

Table 3 Effect of plant growth regulators on number of roots/shoot.*

Treatment (mg/l)			Leaf	Internode	Mean
IAA	IBA	NAA			
0	0	0	0 (0)	0 (0)	0 (0)
1	0	0	6.66 (2.57)	4.66 (2.15)	5.66 (2.36)
2	0	0	7.66 (2.76)	5.23 (2.30)	6.50 (2.53)
0	1	0	8.66 (2.94)	7.66 (2.76)	8.16 (2.85)
0	2	0	8.33 (2.87)	8.33 (2.89)	8.33 (2.88)
0	0	1	4.33 (2.07)	4.33 (2.07)	4.33 (2.07)
0	0	2	5.33 (2.30)	5.33 (2.30)	5.33 (2.30)
Mean			5.85 (2.22)	5.09 (2.07)	5.47 (2.17)

LSD $_{0.05}$ Treatment (A) = (0.12); Explant (B) = (0.06); A x B = (0.18)
*Mean of three replications
Figures in parentheses are square root transformed values

ponse than IBA. Jagannatha *et al.* (2001) found 20 µM IBA as the best treatment for rooting two carnation cultivars 'Sterile Dop' and 'IAHS-22'. Wankhede *et al.* (2006) found that half-strength MS basal medium with 0.8, 1 or 0.05 mg/l IBA were the superior treatments for root initiation in carnation cv. 'Supergreen'. However, Ahmed *et al.* (2006) obtained *in vitro* rooting in elongated microshoots of carnation on MS medium supplemented with 1 µM NAA. The shoots derived from the leaf explants produced more roots per shoot than internode explants. The interaction between treatment × cultivar revealed that maximum number of roots per shoots was produced with IBA in both the explants. The rooted plantlets derived from leaf and internode explants were hardened with 80 to 82% survival after 6 weeks of transfer to pots (**Fig. 1E**). Salehi (2006) acclimatized plantlets of 13 cultivars of carnation in pots containing a mixture of sand, leaf mould and vermiculite mixed in a 1: 1: 1 ratio.

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