Influence of growth regulators and explants on shoot regeneration in carnation

J. K. Kanwar, S. Kumar

Department of Biotechnology, University of Horticulture & Forestry, Solan, India

ABSTRACT: The influence of growth regulators, explants and their interactions on *in vitro* shoot bud formation from callus was studied in *Dianthus caryophyllus* L. The leaf and internode explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of growth regulators. The highest callus induction was observed with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg/l benzyl adenine (BA). Out of twenty seven shoot regeneration media tested, only 2 mg/l thidiazuron (TDZ) and zeatin alone or in combination with naphthalene acetic acid (NAA) and/or indole acetic acid (IAA) could differentiate calli. The highest average number of shoots was observed with 2 mg/l TDZ and 1 mg/l IAA. Significant differences were observed in calli producing shoots and number of shoots per callus in the explants of leaf and internode. The shoots were elongated and multiplied on MS medium supplemented with 1 mg/l BA and solidified with 1% agar. The shoots were rooted and hardened with 76% survival success in pots after six weeks of transfer to the pots.

Keywords: callus; carnation; growth regulators; hardening; Indios; *in vitro*; rooting; shoot regeneration

Carnation is one of the most important commercial cut flowers of the world. 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 to roses and chrysanthemums in several flower-exporting countries. In the United States, it ranks next to rose in popularity (Laurie et al. 1968; Staby et al. 1978). Because of its popularity, it is widely cultivated on a large scale in Italy, Spain, Columbia, Kenya, Sri Lanka, Canary Islands, France, Holland and Germany.

The genetic variability within carnation is relatively limited, therefore, breeding potential for new flower colours and patterns as well as resistance to biotic and abiotic stresses is also limited (Bhatt 1989). Carnation is a vegetatively propagated plant, which further limits its available genetic pool. Numerous studies have been made to regenerate carnation *in vitro* using different explants through callus phase (Thakur et al. 2002; Kumar et al. 2004; Ahmad et al. 2006; Wankhede et al. 2006). Efficient regeneration protocols for *in vitro* propagation of carnation via somatic embryogenesis have also been reported (Karami et al. 2007; Ali et al. 2008). However, Shiba and Mii (2005) reported difficulty in regenerating plants from callus due to their highly dedifferentiated nature. Shoot regeneration in carnation is influenced by genotype, explant source and the balance of plant growth regulators (Frey, Janick 1991; Kallak et al. 1997). Despite of an increase in number of reports on successful micropropagation through callus phase, more research is still needed in this area. The present investigation was undertaken to study the influence of growth regulators, explants and their interactions on regeneration potential of callus in carnation.

MATERIAL AND METHODS

Preparation of material

Stem cuttings of carnation *(Dianthus caryophyllus* L.) cultivar Indios were obtained from the Department of Floriculture and Landscaping, University of Horticulture & Forestry, Solan (H.P.), India. Leaf (0.5 cm^2) and internode (0.8–1 cm) segments were isolated from cuttings and treated with 0.1% carbendazim (Indofil, Bombay, India) solution for 10–15 min followed by washing under running tap water for 30 min. The explants were surface sterilized with 0.5% sodium hypochlorite solution for 15 min followed by 3–4 washings with sterilized distilled water.

Cultural conditions

The sterilized explants were inoculated on Murashige and Skoog (1962; MS) medium supplemented with 8 g/l agar (w/v), 30 g/l sucrose and 0.5, 1, 1.5 and 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5, 1, 1.5 and 2 mg/l naphthalene acetic acid (NAA), 1 mg/l benzyl adenine (BA) and 1 mg/l kinetin alone or in combinations for callus induction. The cultures without growth regulators served as control. The explants were cultured in 100 ml Erlenmeyer flasks (Borosil) containing 30 ml of medium. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C, at the pressure of 1.1 kg/cm^2 for 15 min. After four weeks, the callus was subcultured on the same medium for callus proliferation. After four weeks of proliferation, small pieces of callus (0.8–1 cm^2) were transferred to MS medium supplemented with 1 and 2 mg/l BA, kinetin or thidiazuron (TDZ) and 1 mg/l NAA or indole acetic acid (IAA) alone or in combinations for shoot bud induction. All the cultures were incubated in a room with controlled conditions of 24 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 to 60 µmol/m^2/s.

Data were recorded on explant producing calli, calli forming shoots and number of shoots per callus after four weeks of inoculation.

In vitro rooting and hardening

In vitro raised shoots (2.5–3 cm) were separated from the callus for their elongation and multiplica-

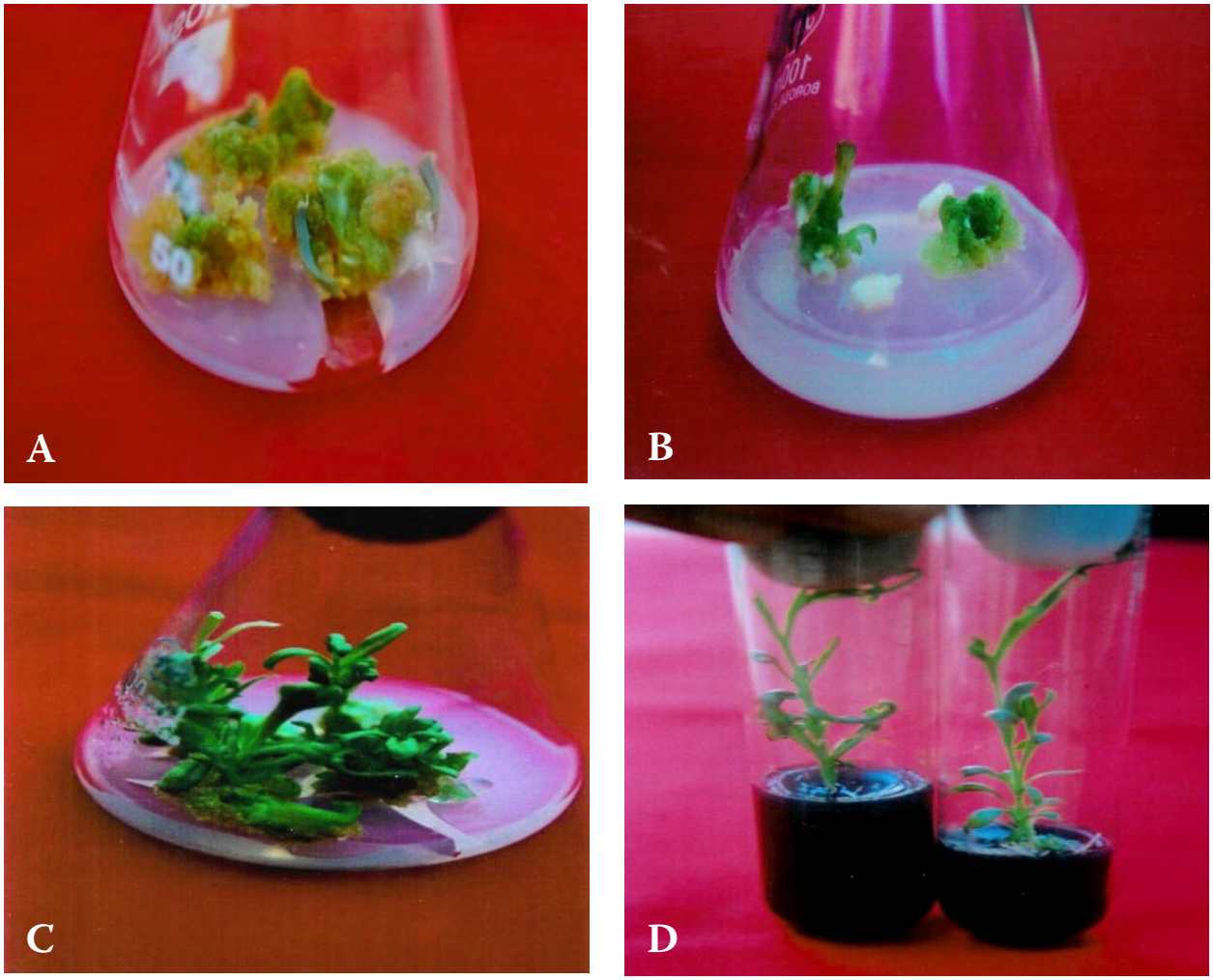

Fig. 1. *In vitro* plant regeneration in carnation. Callus formation from leaf explants with 2 mg/l 2,4-D and 1 mg/l BA after four weeks of culture (A), shoot regeneration from internode derived calli with 2 mg/l zeatin and 1 mg/l IAA after four weeks of culture (B), shoot regeneration from leaf derived calli with 2 mg/l zeatin and 1 mg/l IAA after four weeks of culture (C) and rooting of *in vitro* raised shoots on 1/2 MS medium supplemented with 2 mg/l IBA and 0.2% activated charcoal after four weeks of culture (D)

Table 1. Effect of growth regulators on the callus induction (%) after four weeks of culture

Treatment (mg/l)	Explant		
	leaf	internode	mean
Control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
2,4-D (0.5)	51.67 (45.96)	9.17 (17.54)	30.42 (31.75)
2,4-D (1.0)	53.00 (46.72)	15.17 (22.89)	34.08 (34.81)
2,4-D (1.5)	64.67 (53.54)	22.83 (28.49)	43.75 (41.01)
2,4-D (2.0)	74.00 (59.35)	15.67 (19.33)	44.83 (39.34)
NAA (0.5)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
NAA (1.0)	11.17 (19.50)	0.00 (0.00)	5.58 (9.75)
NAA (1.5)	15.33 (23.04)	0.00 (0.00)	7.67 (11.52)
NAA (2.0)	22.33 (28.18)	0.00 (0.00)	11.17 (14.09)
BA (1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Kinetin (1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
2,4-D + BA (0.5 + 1.0)	52.33 (46.34)	12.33 (20.51)	32.33 (33.43)
2,4-D + kinetin (0.5 + 1.0)	47.33 (43.47)	13.17 (21.27)	30.25 (32.37)
2,4-D + BA (1.0 + 1.0)	57.33 (49.22)	11.17 (19.50)	34.25 (34.36)
2,4-D + kinetin (1.0 + 1.0)	54.00 (47.30)	7.80 (16.11)	30.90 (31.71)
2,4-D + BA (1.5 + 1.0)	64.50 (53.45)	17.83 (24.97)	41.17 (39.21)
2,4-D + kinetin (1.5 + 1.0)	70.17 (57.06)	11.83 (20.11)	41.00 (38.59)
2,4-D + BA (2.0 + 1.0)	75.17 (60.17)	23.33 (28.85)	49.25 (44.51)
2,4-D + kinetin (2.0 + 1.0)	66.00 (54.35)	18.17 (25.22)	42.08 (39.79)
NAA + BA (0.5 + 1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
NAA + kinetin (0.5 + 1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
NAA + BA (1.0 + 1.0)	16.33 (23.81)	0.00 (0.00)	8.17 (11.93)
NAA + kinetin (1.0 + 1.0)	12.50 (20.67)	0.00 (0.00)	6.25 (10.33)
NAA + BA (1.5 + 1.0)	21.50 (27.61)	0.00 (0.00)	10.75 (13.81)
NAA + kinetin (1.5 + 1.0)	14.33 (22.49)	0.00 (0.00)	7.17 (11.10)
NAA + BA (2.0 +1.0)	21.33 (27.49)	0.00 (0.00)	10.67 (13.75)
NAA + kinetin (2.0 + 1.0)	21.33 (27.50)	0.00 (0.00)	10.67 (13.75)
Mean	32.82 (30.99)	6.61 (9.80)	19.71 (20.40)

LSD (P = 0.05), treatment (A) = (1.16), explant (B) = (0.30), A × B = (1.63); figures in parentheses are arc sine transformed values

tion on MS medium supplemented with 1 mg/l BA and solidified with 1% agar (w/v) to lessen the production of vitrified shoots. The shoots were rooted on half strength MS medium supplemented with 2 mg/l indole butyric acid (IBA) and 0.2% activated charcoal. After four weeks of culture, the rooted shoots were removed from the culture vessels, washed thoroughly and dipped in 0.01% cabendazim (Bavistin) solution for 15–20 min. The plantlets were hardened and transferred to earthen pots (10 cm diameter) filled with sand, soil and farm yard manure (FYM) mixed in 1:1:1 ratio. The plants were maintained under glasshouse and observed for their survival till six weeks of transfer to pots.

Table 2. Effect of growth regulators on calli producing shoots (%) after four weeks of culture

Treatment (mg/l)	Explant		
	leaf	internode	mean
Control	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA (2.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin (2.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ (2.0)	12.67 (3.68)	11.00 (3.43)	11.83 (3.55)
Zeatin (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Zeatin (2.0)	10.33 (3.33)	15.17(3.99)	12.75 (3.66)
NAA (1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
IAA (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + IAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + NAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + IAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + IAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + NAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + IAA (2.0 + 1.0)	19.67 (4.53)	17.67 (4.29)	15.67 (4.41)
TDZ + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ + IAA (1.0 + 1.0)	12.33 (3.64)	10.33 (3.34)	11.33 (3.49)
TDZ + NAA (2.0 +1.0)	18.33 (4.39)	11.00 (3.45)	14.67 (3.92)
TDZ + IAA (2.0 + 1.0)	12.67 (3.69)	21.00 (4.67)	16.83 (4.18)
Zeatin + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Zeatin + IAA (1.0 +1.0)	5.33 (2.45)	3.00 (1.95)	4.17 (2.20)
Zeatin + NAA (2.0 +1.0)	15.00 (3.97)	10.67 (3.41)	12.83 (3.69)
Zeatin + IAA (2.0 + 1.0)	21.67 (4.75)	21.00 (4.67)	21.33 (4.71)
Mean	4.74 (1.94)	4.47 (1.89)	4.60 (1.92)

LSD (P = 0.05), treatment (A) = (0.15), explant (B) = (0.04), A × B = (0.20); figures in parentheses are square root transformed values

Statistical analysis

Three replications with 10 explants in each replication (30 explants) were maintained for each treatment and the data were analyzed statistically using factorial completely randomized design (Gomez, Gomez 1984). The statistical analysis based on mean value per treatment was done using the technique of analysis of variance. The comparative LSD multiple range test (P = 0.05) was used to determine differences between treatments.

RESULTS AND DISCUSSION

Table 1 summarizes the range of responses after four weeks of culture. The isolated segments of leaf and internode explants initiated callus from the cut

Table 3. Effect of growth regulators on number of shoots per callus after four weeks of culture

Treatment (mg/l)	Explant		
	leaf	internode	mean
Control	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA (2.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin (2.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ (2.0)	4.17 (2.27)	3.17 (2.04)	3.67 (2.15)
Zeatin (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Zeatin (2.0)	2.83 (1.95)	2.33 (1.82)	2.58 (1.89)
NAA (1.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
IAA (1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + IAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + NAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
BA + IAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + IAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + NAA (2.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Kinetin + IAA (2.0 + 1.0)	3.43 (2.10)	3.17 (2.04)	3.30 (2.07)
TDZ + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
TDZ + IAA (1.0 + 1.0)	4.17 (3.64)	3.50 (2.11)	3.83 (2.89)
TDZ + NAA (2.0 +1.0)	5.67 (2.58)	4.67 (2.38)	5.17 (2.48)
TDZ + IAA (2.0 + 1.0)	6.50 (2.74)	6.17 (2.68)	6.33 (2.70)
Zeatin + NAA (1.0 + 1.0)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Zeatin + IAA (1.0 +1.0)	2.17 (1.78)	1.83 (1.68)	2.00 (1.73)
Zeatin + NAA (2.0 +1.0)	3.50 (2.12)	2.50 (1.68)	3.00 (1.99)
Zeatin + IAA (2.0 + 1.0)	4.17 (2.27)	3.17 (2.04)	3.67 (2.15)
Mean	1.35 (1.41)	1.13 (1.36)	1.24 (1.38)

LSD (P = 0.05), treatment (A) = (0.04), explant (B) = (0.01), A × B = (0.07); figures in parentheses are square root transformed values

ends after one or two weeks and fully developed callus was achieved after one month (Fig. 1A). The explants failed to produce callus in the presence or absence of 0.5 mg/l NAA, 1 mg/l BA or kinetin alone or together. All other treatments induced callus in about 6–49% of the explants. The highest callus induction (49.25%) was achieved on MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BA followed by 2 mg/l 2,4-D. These results differ significantly from the rest of treatments and were in conformity with Kiss and Mandy (2003) who reported callus induction on MS medium supplemented with different concentrations of 2,4-D and BA. Choudhary and Chin (1995) obtained callus from carnation cv. White Sim with 5 or 10μM 2,4-D. In the present study, callus was initiated on all the media containing 2,4-D whereas poor growth followed by lower callus induction was observed on

the media containing NAA and kinetin, indicating that 2,4-D is an important factor for callus initiation in carnation. Kumar et al. (2006) obtained callus induction with 1 mg/l 2,4-D, 2.2 mg/l TDZ and 0.12 mg/l zeatin from leaf segments of carnation.

The interaction between treatment and explant revealed the highest response with 2 mg/l 2,4-D and 1 mg/l BA in both the explants. About 75.17% explants of leaf and 23.33% explants of internode produced callus and differed significantly from each other (Table 1).

About 4–20% calli differentiated into shoots depending upon the type of the explant and growth regulators used (Figs. 1B and C). In the present investigation, out of 27 treatments used, the calli were differentiated into shoots only on the medium supplemented with 2 mg/l TDZ and zeatin alone or together with NAA and IAA and with 2 mg/l kinetin and 1 mg/l IAA. The rest of the treatments were ineffective in differentiating calli (Table 2). This might have resulted from the imbalance in exogenous cytokinin-auxin ratio or endogenous level of growth regulators. Frey and Janick (1991) reported that the explant source and the balance of plant growth regulator influenced shoot regeneration in carnation. Shiba and Mii (2005) reported the difficulty in differentiating shoots from calli in most *Dianthus* species, especially carnation (Nakano, Mii 1992). The highest callus differentiation (21.33%) was observed with 2 mg/l zeatin and 1 mg/l IAA, which differs significantly from the other treatments. Jagannatha et al. (2001) found MS medium supplemented with 10μM kinetin as the best medium for *in vitro* propagation of carnation cv. Sterile Dop. Maximum frequency of shoots from leaf segment derived calli was achieved with 0.6 mg/l TDZ and 1.2 mg/l zeatin in carnation (Kumar et al. 2006). The best interaction on callus differentiation was achieved with 2 mg/l zeatin and 1 mg/l IAA in leaf explants and with 2 mg/l zeatin or TDZ and 1 mg/l IAA in the internode explants and are statistically on a par with each other (Table 2).

An average number of 2–6 shoots per callus was observed with all the treatments applied (Table 3). The highest number of shoots (6.33) was observed with 2 mg/l TDZ and 1 mg/l IAA followed by 2 mg/l TDZ and 1 mg/l NAA. These treatments differ significantly from the rest of the treatments. Kumar et al. (2006) observed 27 shoot buds per callus mass with 0.6 mg/l TDZ and 1.2 mg/l zeatin. The difference in the results may be due to the differences in genotype used. TDZ found to be more effective than BA in inducing shoot formation and proliferation (Sankhla et al. 1995). The highest interaction between shoot number and explant was observed with 2 mg/l TDZ and 1 mg/l IAA in the explants of leaf and internode, which did not differ statistically. It was observed that the explants producing calli, calli producing shoots and number of shoots differ significantly in both the explants (Tables 1–3).

The shoots produced were elongated and multiplied on the medium supplemented with 1 mg/l BA, which was solidified with 1% agar to reduce vitrification (Thomas et al. 2000). The shoots were rooted (Fig. 1D) and hardened with 76% survival success in the pots.

From the present study it was concluded that although the callus was produced with most of the growth regulators the differentiation of callus in cv. Indios was difficult, as only a few treatments responded. Lu and Chandler (1995) reported that the regeneration systems applied widely in transformation studies have been restricted to inducing direct adventitious shoots from excised plant tissues in carnation. The callus mediated regeneration not only improves genetic variability of otherwise limited germplasm of carnation but may be utilized in carnation improvement program via *in vitro* cell selection, somaclonal variations, genetic transformation and for various biotic and abiotic stresses in this crop.

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Vliv růstových regulátorů a explantátů na regeneraci výhonů u karafiátu

ABSTRAKT: Vliv růstových regulátorů, explantátů a jejich interakce na *in vitro* tvorbu výhonových pupenů z kalusu byl studován u taxonu *Dianthus caryophyllus* L. Explantáty listu a internodia byly pěstovány na médiu Murashige a Skoog (MS), které obsahovalo různé koncentrace růstových regulátorů. Nejvyšší indukce kalusu byla pozorována v případě 2 mg/l 2,4-dichlorfenoxyoctové kyseliny (2,4-D) a 1 mg/l benzyladeninu (BA). Z dvaceti sedmi médií testovaných pro regeneraci výhonů pouze 2 mg/l thidiazuronu (TDZ) a zeatinu samostatně nebo v kombinaci s kyselinou naftalen-1-octovou (NAA) nebo kyselinou indol-3-octovou (IAA) diferencovaly kalusy. Nejvyšší průměrný počet výhonů byl pozorován v případě 2 mg/l TDZ a 1 mg/l IAA. Významné rozdíly byly zaznamenány v počtu výhonů vytvářejících kalusy a v počtu výhonů na kalus u explantátů listu a internodia. Výhony byly prodlužovány a množeny na MS médiu obohaceném o 1 mg/l BA a ztuženém 1 % agarem. Šest týdnů po přesazení do nádob zakořenilo a zesílilo 76 % výhonů.

Klíčová slova: kalus; karafiát; růstové regulátory; zesílení; Indios; *in vitro*; zakořenění; regenerace výhonů

Corresponding author:

Prof. Surinder Kumar, Ph.D., University of Horticulture & Forestry, Department of Biotechnology,
P.O. Nauni, Solan – 173 230 (H.P.), India
tel.: + 91 01792 252 310, fax: + 91 01792 252 242, e-mail: skuhf07@yahoo.co.in