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Adventitious shoot formation and plant regeneration from leaf explants of carnation (*Dianthus caryophyllus* L.)

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Shoot multiplication and regeneration system was developed for two carnation (*Dianthus caryophyllus* L.) cultivars. For shoot multiplication, shoots of White Sim cultivar were cultivated onto MS media containing different levels of 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA). High multiplication frequency was obtained on media containing 0.54 µM NAA combined with 4.4 or 8.8 µM BA. For regeneration study, leaf explants from White Sim and Red Sim cultivars were cultured onto media containing different concentrations of N-1,2,3-thiadiazol-5-y-N'-N'-phenyl urea (TDZ) combined with different concentrations of NAA. Shoot regeneration was obtained only on media containing high concentrations of the cytokinin, up to 85% shoot regeneration was obtained with 20 µM TDZ and 2.7 µM NAA in 'White Sim' and 75% in 'Red Sim'. Similar trend was observed with shoot number in both cultivars. High root regeneration was obtained when the explants were cultured on medium with NAA only. Shoot regeneration was associated with callus formation. Regenerated shoots were rooted *ex vitro,* acclimatized and grown normally in the greenhouse.

Key words: Cytokinins, carnation, multiplication, regeneration, thidiazuron.

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the most important floriculture commercial crops in the world. *In vitro* micropropagation technique is widely used in ornamental crops. In carnation, an efficient micropropagation system is still needed (Seo et al., 2007). Plant regeneration via adventitious shooting has been described from different carnation's explants including, petals (Frey and Janick, 1991; Nugent et al., 1991; Messeguer et al., 1993; Fisher and Vainstein 1993; Nakano et al., 1994), leaves (Van Altvorst et al., 1992, 1993; Messeguer et al., 1993; Jain et al., 2001; Kantia and Kathari 2002; Iantcheva et al., 2005) and nodal stem (Miller et al., 1991; Nugent et al., 1991; Van Altvorst et al., 1992; Watad et al., 1996; Thakur et al., 2002). On the other hand, rege-neration via somatic embryogenesis was also reported from different carnation's explants including petals (Nakano et al., 1994; Karami et al., 2006; Karami and Kordestani, 2007), leaves (Iantcheva et al., 2005; Pareek and Kothari, 2003) and roots (Seo et al., 2007). Regeneration from leaf explants in the above studies, indicated non satisfactory regeneration percentages. On the other hand, regeneration from petal explants were much higher, how-ever, establishing cultures from petals may in many cases be encountered with contamination difficulties, therefore, using leaves as

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Abbreviations: TDZ, N-1,2,3-Thiadiazol-5-yl-N'-phenylurea (thidiazuron); **NAA,** α-naphthalene acetic acid; **IBA,** indole-3-butyric acid; **BA,** benzyl amino purine.

in vitro explants could be much easier.

An efficient regeneration system in carnation is necessary for genetic transformation; therefore, the aim of this study was to establish an efficient regeneration system from leaf explants of carnation using TDZ.

MATERIALS AND METHODS

Plant material and cultural environment

Nodal explants were taken from green house grown carnation plant of both White Sim and Red Sim cultivars, the explants were disinfested for 15 min in a 0.2% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20 as a wetting agent. Nodal segments were then rinsed three times with sterile distilled water for 5 min per rinse. The buds were then placed in 25 x 150 mm culture tubes containing 10 ml of MS (Murashige and Skoog 1962) (Sigma -Aldrich) basal medium (MSO) supplied with 3% sucrose, 100 mg l^{-1} *myo-inistol* and solidified with 0.8% agar (Sigma). The tubes were incubated in a growth chamber at 22 ± 1°C for four weeks with 16 h photoperiod illumination of 40 µ mol $m^{-2}s^{-1}$ irradiance supplied from cool white fluorescent.

Shoot multiplication experiment

In vitro clean growing shoots of White Sim cultivar were excised and transferred to the multiplication media (MSO) supplemented with different combination levels of both naphthalene acetic acid (NAA) and benzyl amino purine (BA) (Sigma) (Table 1 and Figure 1D). At the end of the experiment, shoot number, shoot length and number of leaves were recorded, the experiment was conducted two times, and data presented were the average values of the two experiments.

Regeneration experiment

Leaf explants were taken from *in vitro* multiplied shoots of both White Sim and Red Sim cultivars from the multiplication experiment; before leaf excision, the shoots were grown on MSO media without hormones. Leaves were divided transversely and placed with the abaxial side down on the regeneration media and ten explants were cultured in each Petri dish filled with 30 ml of the regeneration media. Regeneration media consisted of MSO medium supplied with 3% sucrose, 100 mg l^{-1} *myo-inositol* and solidified with 0.8% agar (Sigma), the media were supplied with different combinations of NAA and TDZ (Sigma). The following regeneration experiments were conducted:

Experiment one

In this experiment, MSO was supplied with 0.0, 2.7 and 5.4 µM NAA and 0.0, 0.5, 1.0, 2.0, 4.0 and 8 µM TDZ.

Experiment two

In the second experiment, higher levels of TDZ (10, 15, 20 µM) were combined with 0.0, 2.7 and 5.4 µM NAA. The combinations of NAA and TDZ in each experiment were considered as treatments; each treatment was replicated four times in a completely randomized arrangement. Each experiment was repeated two times. Regenerated shoots were excised and cultured on MSO medium; the shoots were subcultured 2to 3 times on this medium. For rooting and acclimatization, elongated shoots of 3 to 4 cm length were excised and basally treated with T8 (commercial IBA rooting powder), the treated shoots were transferred *ex vitro* to plastic pots (10 cm diameter) containing sterile substrate of 1: 2 vermiculite and peat moss, the pots were put in a plastic pan. The cultures were irrigated with sterile water and sealed with a thin layer of plastic sheet, they were then kept in a growth room under the same conditions. After one week, the plastic sheet was removed gradually, the rooted shoots were then gently transferred into larger pots (30 cm diameter) and kept in a greenhouse under partially shaded conditions (Figure 1F).

Data collection and analysis

Various parameters were collected from both multiplication and regeneration experiments (Tables 1 and 2). The significance of treatment effects on the different parameters was determined using analysis of variance (ANOVA) using the SAS software (SAS 1990). If treatments were found to be significant, then their comparative performance was tested using Duncan's Multiple Range test (DMR) at 5% probability level.

RESULTS AND DISCUSSION

Shoot multiplication experiment

Notable significant differences were observed in the average number of shoots regenerated per explants and the average shoot length among the different treatments, however, no significant differences were obtained with the number of leaves per regenerated shoot (Table 1). Higher shoot number was obtained on media supplied with 4.4 µM BA only, or 0.54 µM NAA and 8.8 µM BA, with 5.0 and 5.8 shoots per tube, respectively. However, a significant low shoot number was obtained with other combinations. Regarding the average shoot length, all combinations except 0.0 and 8.8 µM NAA and BA gave significantly the same shoot length that ranges between 2.49 and 4.17 cm. BA at 8.8 µM gave the lower shoot length (1.31 cm). The average leaf number per shoots ranges between 6.4 and 12.67 but without significant difference among the different combinations.

The results of this study are in agreement with that reported by Kharrazi et al. (2011) who found that BA at 4.4 to 8.8 µM and NAA at 0.54 µM was optimum for normal shoot multiplication of two carnation cultivars. In addition, maximum number of shoots was obtained with MS media supplied with 4.4 µM BA for nodal explants (Ali et al., 2008).

Regeneration experiment

Shoot regeneration from leaf explants was compared on media containing different combinations of NAA and TDZ in two separated experiments:

Experiment 1

The result of this experiment is shown in Tables 2 and 3. Shoot regeneration in 'White Sim' occurred only on media supplied with two combinations of NAA and TDZ (2.7 and 2.0 µM) and (5.4 and 8.0 µM), the regeneration

Table 1. Effect of different levels of NAA and BA on shoot multiplication of White Sim carnation cultivar.

Growth regulator (µM)		Average shoots number	Average shoot length (cm)	Average number of leaves/shoot
NAA	BA			
0.0	0.0	1.30[b]	4.17[a]	10.33
0.0	4.4	5.00[a]	2.59[ab]	10.67
0.0	8.8	3.00[b]	1.31[b]	8.13
0.54	4.4	2.00[b]	3.05[ab]	10.30
0.54	8.8	5.80[a]	3.20[ab]	6.40
Sig. level (p-Value)		0.0003	0.0499	NS

Means in the same column followed by the same letter are not significantly different (p< 0.05; DMR test).

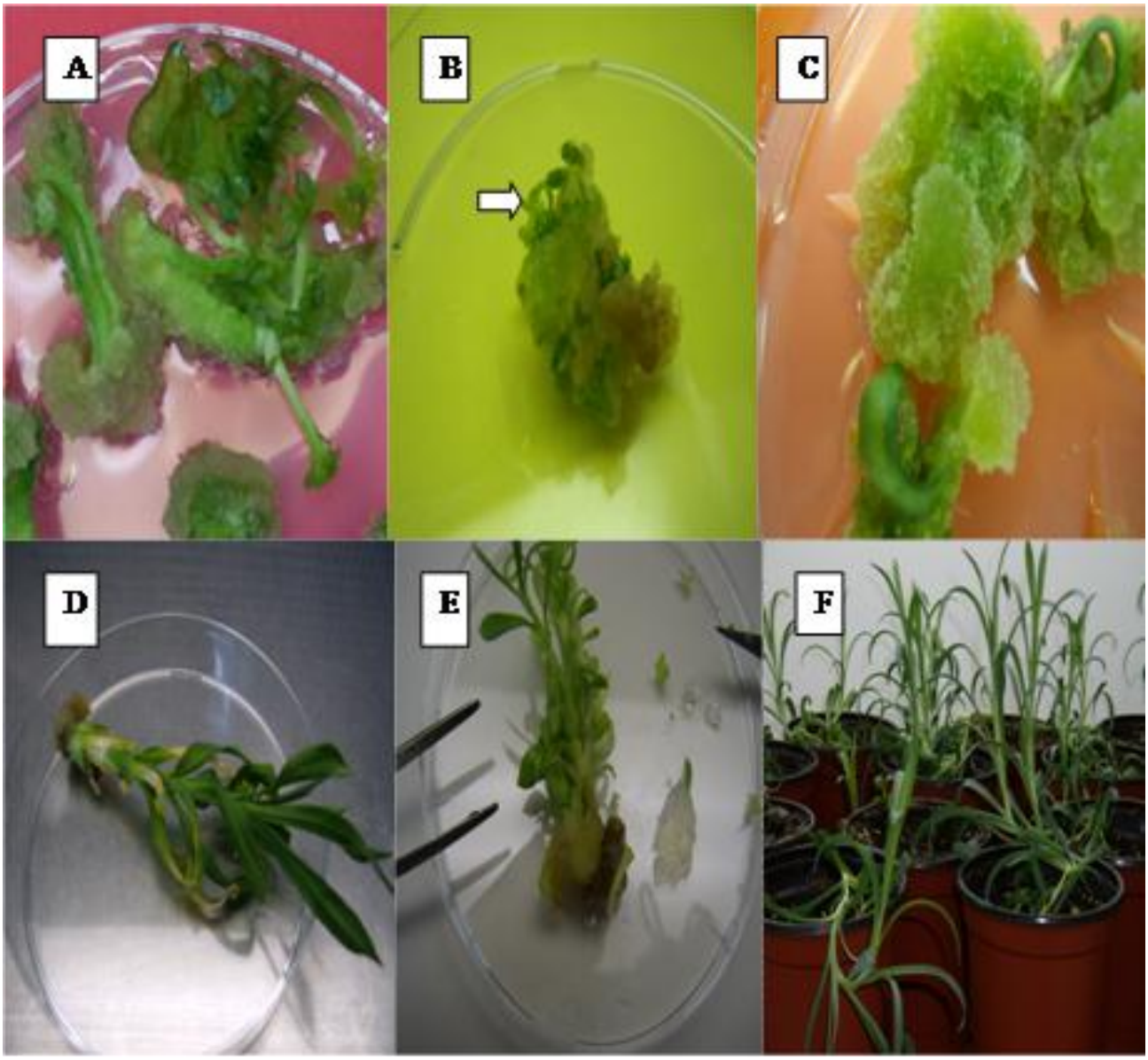

Figure 1. A, Adventitious shoot regenerated form leaf explants (White Sim); B, regenerated shoot associated with callus (off white color); C, non regenerated callus (firm and white-green color); D, established shoot of carnation from nodal bud onto MSO medium (Red Sim); E, shoots from multiplication medium (White Sim); F, Regenerated plants grown in the green house.

Table 2. Effect of different levels of NAA and TDZ on regeneration of White Sim carnation cultivar.

Growth regulator (µM)		Shoot regeneration percentage	Average number of shoot	Root regeneration percentage	Average root number	Callus rate (1-5 scale)
NAA	TDZ					
0.0	0.0	0.0[b]	0.0	40.0[b]	1.75[b]	0.0
0.0	0.5	0.0[b]	0.0	0.0[c]	0.0[b]	1
0.0	1.0	0.0[b]	0.0	0.0[c]	0.0[b]	2
0.0	2.0	0.0[b]	0.0	0.0[c]	0.0[b]	3
0.0	4.0	0.0[b]	0.0	0.0[c]	0.0[b]	4
0.0	8.0	0.0[b]	0.0	0.0[c]	0.0[b]	5
2.7	0.0	0.0[b]	0.0	50.0[b]	2.75[b]	2
2.7	0.5	0.0[b]	0.0	0.0[c]	0.0[b]	3
2.7	1.0	0.0[b]	0.0	0.0[c]	0.0[b]	3
2.7	2.0	20.8[a]	3.75	0.0[c]	0.0[b]	4
2.7	4.0	0.0[b]	0.0	0.0[c]	0.0[b]	4
2.7	8.0	0.0[b]	0.0	0.0[c]	0.0[b]	3
5.4	0.0	0.0[b]	0.0	100.0[a]	6.25[a]	0
5.4	0.5	0.0[b]	0.0	0.0[c]	0.0[b]	5
5.4	1.0	0.0[b]	0.0	0.0[c]	0.0[b]	4
5.4	2.0	0.0[b]	0.0	0.0[c]	0.0[b]	4
5.4	4.0	0.0[b]	0.0	0.0[c]	0.0[b]	5
5.4	8.0	25.8[a]	3.3	0.0[c]	0.0[b]	4
Sig. level (p-Value)		<0.0001	0.1706	<0.0001	<0.0001	
NAA* TDZ effect		Sig	NS	Sig	NS	

Means in the same column followed by the same letter are not significantly different (p< 0.05; DMR test).

Table 3. Effect of different levels of NAA and TDZ on regeneration of Red Sim carnation cultivar.

Growth regulator (µM)		Shoot regeneration percentage	Average number of shoot	Root regeneration percentage	Average root number	Callus rate (1-5 scale)
NAA	TDZ					
0.0	0.0	0.0	0.0	55.0[b]	1.75[b]	0.0
0.0	0.5	0.0	0.0	0.0[c]	0.0[c]	1
0.0	1.0	0.0	0.0	0.0[c]	0.0[c]	2
0.0	2.0	1.6	0.5	0.0[c]	0.0[c]	4
0.0	4.0	0.0	0.0	0.0[c]	0.0[c]	4
0.0	8.0	0.0	0.0	0.0[c]	0.0[c]	5
2.7	0.0	0.0	0.0	85.0[a]	4.0[b]	1
2.7	0.5	0.0	0.0	0.0[c]	0.0[c]	2
2.7	1.0	0.0	0.0	0.0[c]	0.0[c]	4
2.7	2.0	0.5	0.25	0.0[c]	0.0[c]	4
2.7	4.0	0.0	0.0	0.0[c]	0.0[c]	4
2.7	8.0	0.0	0.0	0.0[c]	0.0[c]	3
5.4	0.0	0.0	0.0	100.0[a]	8.0[a]	0
5.4	0.5	0.0	0.0	0.0[c]	0.0[c]	5
5.4	1.0	0.0	0.0	0.0[c]	0.0[c]	5
5.4	2.0	0.0	0.0	0.0[c]	0.0[c]	4
5.4	4.0	0.0	0.0	0.0[c]	0.0[c]	5
5.4	8.0	0,0	0.0	0.0[c]	0.0[c]	4
Sig. level (p-Value)		0.4553	0.4553	<0.0001	<0.0001	
NAA* TDZ effect		NS	NS	Sig	NS	

Means in the same column followed by the same letter are not significantly different (p< 0.05; DMR test).

Table 4. Effect of different levels of NAA and TDZ on regeneration of White Sim carnation cultivar.

Growth regulator (µM)		Shoot regeneration percentage	Average number of shoot	Root regeneration percentage	Average root number	Callus rate (1-5 scale)
NAA	TDZ					
0.0	10.0	0.0[c]	0.0[b]	0.0	0.0	1
0.0	15.0	0.0[c]	0.0[b]	0.0	0.0	1
0.0	20.0	0.0[c]	0.0[b]	0.0	0.0	1
2.7	10.0	30.0[b]	3.0[a]	0.0	0.0	3
2.7	15.0	45.0[b]	4.0[a]	0.0	0.0	4
2.7	20.0	85.0[a]	3.0[a]	0.0	0.0	5
5.4	10.0	0.0[c]	0.0[b]	0.0	0.0	5
5.4	15.0	0.0[c]	0.0[b]	0.0	0.0	3
5.4	20.0	0.0[c]	0.0[b]	0.0	0.0	3
Sig. level (p-value)		<0.0001	<0.0001	NS	NS	
NAA* TDZ effect		Sig	Sig	NS	NS	

Means in the same column followed by the same letter are not significantly different (p< 0.05; DMR test).

Table 5. Effect of different levels of NAA and TDZ on regeneration of Red Sim carnation cultivar.

Growth regulator (µM)		Shoot regeneration percentage	Average number of shoot	Root regeneration percentage	Average root number	Callus rate (1-5 scale)
NAA	TDZ					
0.0	10.0	0.0 c	0.0 b	0.0	0.0	1
0.0	15.0	0.0 c	0.0 b	0.0	0.0	1
0.0	20.0	0.0 c	0.0 b	0.0	0.0	1
2.7	10.0	35.0 b	1.75 a	0.0	0.0	4
2.7	15.0	35.0 b	1.75 a	0.0	0.0	4
2.7	20.0	75.0 a	3.75 a	0.0	0.0	4
5.4	10.0	0.0 c	0.0 b	0.0	0.0	3
5.4	15.0	0.0 c	0.0 b	0.0	0.0	3
5.4	20.0	0.0 c	0.0 b	0.0	0.0	3
Sig. level (p-value)		0.0003	<0.0001	NS	NS	
NAA* TDZ effect		Sig	Sig	NS	NS	

Means in the same column followed by the same letter are not significantly different (p< 0.05; DMR test).

percentages obtained were (20.5 and 25.5%), respectively, the average number of shoots per regenerated explants was 3.75 and 3.35 shoots (Table 2). However, shoot regeneration with Red Sim cultivar was very low (1.6 and 0.5%) at 2.0 µM TDZ and 2.7 and 2.0 µM of NAA and TDZ, respectively. The average number of shoots was also very low for this cultivar, no shoot regeneration occurred on any other media (Table 3). Shoot regeneration was associated with a distinctive callus formation which was high and ranged from 4 to 5 according to the suggested scale. Therefore, indirect shoot regeneration occurred. In addition, only white and soft callus resulted in shoot regeneration (Figure 1A and B).

Root formation was recorded on both free hormone media and media supplied with auxin only, rooting trend was similar in both cultivars, up to 100% rooting was observed when NAA was used at 5.4 µM (Tables 2 and 3). Rooting percentages and the average number of roots obtained on these media were different significantly from that on other media. The average number of regenerated roots ranged between 1.75 to 6.25 and 1.75 to 8 roots in both cultivars.

Experiment 2

In this experiment, higher concentrations of TDZ were combined with the concentrations of NAA (Tables 4 and 5). The result indicated a higher significant shoot regeneration with elevating TDZ to 20 µM, up to 85% shoot regeneration was obtained with the combination of 2.7 µM NAA and 20 µM TDZ in White Sim cultivar and 75% for Red Sim cultivar. Shoot regeneration also occurred on media supplied with 2.7 µM NAA and 10.0 µM TDZ and that supplied with 2.7 µM NAA and 15 µM TDZ combinations, the obtained shoot percentages were 30 and 45 for 'White Sim' and 35 in both media with 'Red Sim' cultivar (Tables 4 and 5). No shoot regeneration occurred with other combinations. No root regeneration was observed in

this experiment.

The concentration of the cytokinin used (TDZ) has an important effect on shoot regeneration. TDZ was reported to be more effective in shoot regeneration from petal explants of carnation (Nugent et al., 1991; Karami et al., 2006; Karami and Kordestani 2007).

In contrast with petal explants, leaf explants had poor regeneration in previous reports. In this study, TDZ was effective for shoot regeneration from leaf explants (Nakano et al., 1994). Shoot regeneration was observed with higher levels of TDZ, no shoot formation was obtained with lower cytokinin levels. TDZ has been demonstrated to have a high cytokinin metabolism on shoot regeneration of different plant species (Fasolo et al., 1989; Fiola et al., 1990; Tomsone et al., 2004; Suboticet al., 2008; Abu-Qaoud, 2012). TDZ induced shoot regeneration with NAA and the high level of NAA (5.4 µM) inhibited shoot regeneration in experiment two. *In vitro* regeneration is mainly regulated by the balance and the interaction between provided hormones in the medium and those endogenously produced by the explants (Subotic et al., 2008). Therefore, elevating the endogenous level of auxin with the exogenous application might have an inhibitory effect on shoot regeneration. It was also indicated that an enhancement of auxin–cytoknin metabolism shifts the auxin cytokinin pool favoring adventitious shoot regeneration in *Rhipsalidopsis* plant (Sriskandarajah et al., 2006). In this study, the adventitious shoots produced were rooted, acclimatized easily and grew normally in the greenhouse (Figure 1F). This result was similar to that of Nugent et al. (1991) who found that shoots derived from stem segments of carnation rooted and healed faster than shoots from other explants. Majada et al. (2000) reported that ventilation of culture vessels promoted *in vitro* hardening of micropropagated carnation shoots.

In conclusion, the present study provides an efficient system for adventitious shoot regeneration in carnation using leaf explants. Moreover, this study shows that the produced rooted shoots were normal and grew normally in the greenhouse. Therefore, the plant regeneration system established in this study is efficient and suitable for the application of biotechnology methods for the improvement of carnation crop.

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