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Full Length Research Paper

Improving adventitious shoot regeneration from cultured leaf explants of *Petunia hybrida* using thidiazuron

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The effect of various concentrations of thidiazuron (TDZ) with or without 2.7 µM of α-naphthalene acetic acid (NAA) on adventitious shoot formation of two *Petunia hybrida* cultivars was studied. Seeds from 'Daddy Blue' and 'Dreams White' cultivars were germinated *in vitro*. Expanded leaves from both seedlings and regenerated shoots were taken as explants. Explants were placed on MS media supplied with different concentrations and combinations of the growth regulators. Cultures were incubated under dark conditions for three weeks and then moved under light condition. The ability to form shoots varied depending on the growth regulator combination. The highest shoot percentage (87.5 to 90%) with both explant sources was obtained when TDZ was used at levels between 0.5 and 8 µM in both cultivars. Regeneration was reduced significantly when TDZ was combined with 2.7 µM NAA. A similar trend of average number of shoots produced was observed.

Key words: Cytokinins, petunia, regeneration, thidiazuron.

INTRODUCTION

Petunia hybrida is an important floriculture ornamental crop of high commercial interest. It is considered to be the first bedding plant; in addition *Petunia* is the most common among the new developed cultivars (Gerats and Vandenbussche, 2005). The application of biotechnology in plant improvement requires an efficient regeneration system. *In vitro* shoot regeneration of *Petunia* species has been reported from several explants (George et al., 1987) including protoplast (Auer et al., 1992, 1999) and cotyledon (Dulien, 1991). *In vitro Petunia* leaf explants have been used for many sorts of studies. These include: anatomical changes (Traas et al., 1990), effect of light on regeneration (Reuveni and Evenor, 2007), inheritance of regeneration capacity (Dulien, 1991), sugar and CO_2 effects on regeneration (Qu et al., 2009), ethylene effect on shoot and root formation (Dimasi-Therious et al., 1993), nitrogen and calcium effect on regeneration (Frett and Dirr 1986) and hormonal combinations (Lu, 1993; Guang-rong et al., 2004; Ying et al., 2005; Rui-yue, 2007; Xiao-feng, 2009; Xian-chun , 2010).

A concentration of BA ranged from 0.1 to 2 mgL^{-1} and α-naphthalene acetic acid (NAA) or IBA ranged from 0.1 to 0.3 mgL^{-1} were reported as optimal for shoot regeneration of *Petunia* leaf explants. In most of the surveyed literature, BA was used as a promoting cytokinin, but without a high shoot regeneration percent. TDZ was used to improve shoot regeneration of *Petunia* leaf explants. Thirukkumaran et al. (2009) reported a maximum frequency of shoot regeneration (52.1%) from leaf explants of *Petunia* using MS medium containing 2 mgL^{-1} TDZ. TDZ has been widely used to promote shoot regeneration in many plant species with a significant effect over other cytokinins. Therefore, in this study, we described the effect of using different concentrations of TDZ on shoot regeneration ability of two *Petunia* cultivars.

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.

MATERIALS AND METHODS

Seeds of two cultivars of *Petunia hybrida*; 'Daddy Blue' and 'Dreams White' 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. Seeds were then rinsed three times with sterile distilled water for 5 min per rinse. The seeds were then placed in 25 x 150 mm culture tubes containing 10 ml of MS (Murashige and Skoog, 1962) basal medium (MSO) containing 3% sucrose, 100 mgL^{-1} myinistol and solidified with 0.8% agar. Two to three seeds were placed in each tube; the tubes were incubated in a growth chamber at 22 ± 1°C for four weeks with 16 h of photoperiod illumination of 40 µmol $m^{-2}s^{-1}$ supplied from cool white fluorescent. For shoot induction, shoots from the germinated seedlings were cultured on MS basal medium supplemented with 0.1 mgL^{-1} NAA and 0.5 mgL^{-1} BA (Abu-Qaoud et al., 2010); this medium was considered as a shoot induction medium (MSI). For regeneration experiments, actively growing four week old shoots of 2 to 3 cm length harvested from MSI medium, lower leaves were trimmed and sub cultured on MSO (MS medium without hormones) for another month before leaves were harvested from these shoots as explants for regeneration experiments.

Shoot regeneration studies were performed in Petri dishes containing 30 ml of MSO medium which contained 3% sucrose, 100 mgL^{-1} myo-inositol and solidified with 0.8% agar; the media were supplied with 0.0, 0.5, 1.0, 2.0, 4.0 and 8 µM TDZ. Each TDZ concentration was added alone or combined with 2.7 µM NAA. The pH of all media was adjusted from 5.6 to 5.7. Two experiments were conducted; in the first experiment, fully expanded leaves from *in vitro* germinated shoots grown on MSO were used, and on the other hand, regenerated shoots from this experiment that were excised and sub-cultured on MSO medium two times were used in the second experiment. Each treatment consisted of five explants (half leaf) per Petri dish with 4 replicates for each treatment. Leaves were cut transversely into two pieces and were cultured with the abaxial side in contact with the media. All cultures were incubated under dark conditions for three weeks; they were then transferred under similar conditions of the germination and multiplication cultures.

Regenerated shoots were excised and cultured on MSO medium and sub cultured two to three times. For rooting and acclimatizing, 3 to 4 cm regenerated growing shoots were excised and basally treated with IBA at 8000 ppm (talc), the treated shoots were transferred *ex vitro* to plastic pots (10 cm diameter) containing sterile medium of 1:2 vermiculite and peat moss. 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 described above. After one week, the plastic sheet was removed gradually, the rooted shoots were then gently transferred into larger pots and kept in a greenhouse under partially shaded conditions, and the seedlings were maintained until flowering. Each experiment was repeated three times and the average are presented in this article. All collected data were sorted by cultivar and analyzed using the SAS software (SAS, 1990). One way analysis of variance of the factorial treatments was conducted for each experiment followed by means separation using the least significant difference (LSD) at 5% probability level.

RESULTS

The analysis of variance (ANOVA) indicated different growth regulator combination effects and interactions between the two Petunia cultivars (Tables 1 and 2). Shoot induction was easily achieved on shoot induction medium (Figure 1C). Direct shoot regeneration was obtained on leaf's explants of both cultivars (Figures 1A and B). Regarding experiment 1 with seedling explants, the result showed highly significant effect of all factors on shoot regeneration with both cultivars (Tables 1 and 2). Direct shoot regeneration was obtained on leaf's explants of both cultivars (Figures 1A and B). In experiment 2, TDZ and NAA showed a highly significant effect on shoot formation percentage except for NAA with 'Daddy Blue' and a non significant interaction effect of TDZ and NAA on the average shoot number with 'Dreams White' cultivar (Tables 3 and 4).

The effect of TDZ and NAA on the shoot regeneration ability of petunia seedling explants is shown in Tables 1 and 2. For Daddy Blue cultivar, maximum regeneration frequency was obtained when TDZ was used at 2, 4 and 8 µM; the highest regeneration percentage was obtained in explants cultured onto medium contained 8 µM TDZ only (Table 1). Regarding the average shoot number, maximum number (9 and 8.5) of shoot per explant of cv. Daddy Blue was obtained when 4 and 8 µM TDZ were used in the media, respectively (Table 1). Up to 90% shoot regeneration was observed when TDZ was used at 0.5 µM alone in Dreams White cultivar, however, this percentage was under the same level of significance with that obtained in media supplied with 4 and 8 µM TDZ only (87.5%) (Table 2). Shoot regeneration was highly reduced when TDZ was combined with 2.7 µM NAA (Table 2).

Shoot regeneration of explants obtained from regenerated shoots (R1) of both *Petunia* cultivars is shown in Tables 3 and 4. Regenerated shoots were observed in leaf section of 'Daddy Blue' in media supplied with all concentrations of TDZ with and without the addition of NAA except at 0.5 µM TDZ without NAA. Maximum regeneration percentage (70%) was observed under 2.7 and 2 µM NAA and TDZ, respectively, however, this percentage did not differ significantly from that obtained with (0, 4), (0, 8) and (2.7, 4) µM NAA and TDZ, respectively (Table 3). Maximum average shoot number per explant (10.25) was observed under 4 µM TDZ with NAA but did not differ significantly from numbers obtained on media supplied with 2 µM TDZ with and without NAA (Table 3).

In Dreams White cultivar, shoot regeneration occurred on the media supplied with TDZ alone and under media supplied with 4 and 8 µM TDZ combined with 2.7 µM NAA (Table 4). The higher regeneration percentage (90%) was observed under the higher TDZ level, but with the same significant level of that percentage was obtained under 4 µM TDZ (75%). Similar effect of TDZ was shown on the average shoot number per regenerated explant (Table 4). Higher average number (6.75 and 7.25) was observed under high concentrations of TDZ (4 and 8 µM without NAA). Regenerated shoots were easily rooted and acclimatized before transferred to the greenhouse (Figure 1D).

DISCUSSION

The data presented in this study shows that the two

Table 1. Effect of TDZ and NAA on adventitious shoot regeneration frequency and average number of shoots from leaf explants of cv. Daddy Blue (seedling explants).

Growth regulators concentration (µM)		Shoot regeneration (%)	Average number of shoots per explant
NAA	**TDZ**		
0.0	0.0	0.0^{d}	0.0^{c}
	0.5	45.8^{bc}	3.5^{b}
	1.0	50.0^{bc}	6.25^{c}
	2.0	70.0^{ab}	3.0^{b}
	4.0	67.9^{abc}	9.0^{a}
	8.0	82.2^{a}	8.5^{a}
2.7	0.0	0.0^{d}	0.0^{c}
	0.5	29.2^{cd}	0.75^{c}
	1.0	7.0^{d}	0.5^{c}
	2.0	48.3^{bc}	3.8^{b}
	4.0	27.8^{d}	2.5^{b}
	8.0	30.0^{cd}	3.5^{b}
Significance			
NAA	-	*	*
TDZ	-	***	**
NAA*TDZ	-	*	ns

Percentage of explants showing adventitious shoot regeneration. The value represents the mean of three independent experiments. Means in the same column followed by the same letter are not significantly different (p< 0.05; least significant difference test LSD). ns, not significant; *,**,*** significant at p<.05,.01or at .001,,respectively.

Table 2. Effect of TDZ and NAA on adventitious shoot regeneration frequency and average number of shoots from leaf explants of cv. Dreams White (seedling explants).

Growth regulators concentration (µM)		Shoot regeneration (%)	Average number of shoots per explant
NAA	**TDZ**		
0.0	0.0	0.0^{c}	0.0^{c}
	0.5	90.0^{a}	19.5^{a}
	1.0	50.0^{b}	11.25^{b}
	2.0	52.5^{b}	8.75^{b}
	4.0	87.5^{a}	16.25^{a}
	8.0	87.5^{a}	11.25^{b}
2.7	0.0	0.0^{c}	0.0^{c}
	0.5	0.0^{c}	0.0^{c}
	1.0	0.0^{c}	0.0^{c}
	2.0	35.0^{b}	3.0^{c}
	4.0	0.0^{c}	0.0^{c}
	8.0	0.0^{c}	0.0^{c}
Significance	-		
NAA	-	***	***
TDZ	-	***	***
NAA*TDZ	-	***	***

Percentage of explants showing adventitious shoot regeneration. The value represents the mean of three independent experiments. Means in the same column followed by the same letter are not significantly different (p< 0.05; Least Significant Difference test LSD). ns, not significant; *,**,*** significant at p<.05,.01or at .001,,respectively.

Figure 1. A and B, *Petunia* leaf explants of Daddy Blue cultivar with adventitious regenerated shoots. C, *Petunia* shoots growing on shoot induction medium(MSI). D, *Petunia* rooted shoots under acclimatization conditions.

Petunia cultivars respond to TDZ and NAA differently. Such findings is similar to that obtained by other researchers (Ying et al., 2005; Xiao-feng, 2009 and Xian-chun, 2010). Differences were found in the organo-genesis response of the two *Petunia* genotypes (St40 and TLV1). Auer et al. (1992) found that cytokinin oxidase activity was continuously increased in respond to BA, but with a larger increase with St40, therefore, he suggested that differences in BA uptake and metabolism subsequently affected the accumulation of isoprenoid cytokinins and the activity of cytokinin oxidase in early stage of shoot development.

The result of the present study confirms the strong TDZ activity on adventitious shoot induction in *Petunia* plant. High number of shoot per explant was obtained with media supplied with TDZ. This result is in agreement with Thirukkumaran et al. (2009) who obtained a maximum frequency of shoot regeneration (52.1%) from leaf explants of *Petunia*. The regeneration frequency obtained in this study was higher than for other researchers (87.5 to 90%). TDZ has been demonstrated to have a high cytokinin metablosim on shoot regeneration of different plant species including; *Nicotiana tabacum, Phaseolus lunatus, Actinidia chinensis, Vitis vinifera, Pyrus communis, Dianthus caryophyllus, Rhododendrons* and *Impatiens walleriaba* (Chevreau et al., 1989; Nakano et al., 1994; Tomsone et al., 2004; Subotic et al., 2008). The result of the study also shows that the regeneration ability of the two *Petunia* cultivars was significantly reduced when NAA was included in the media. *In vitro* regeneration is mainly regulated by the balance and the interaction between the provided hormones in the medium and those endogenously produced by the explants (Subotic, 2008). Therefore, elevating the

Table 3. Effect of TDZ and NAA on adventitious shoot regeneration frequency and average number of shoots from leaf explants of cv. Daddy Blue (explants from regenerated shoots).

Growth regulators concentration (µM)		Shoot regeneration (%)	Average number of shoots per explant
NAA	**TDZ**		
0.0	0.0	0.0^{e}	0.0^{c}
	0.5	0.0^{e}	0.0^{c}
	1.0	25.0^{cde}	5.5^{b}
	2.0	45.0^{bcd}	9.0^{a}
	4.0	50.0^{abc}	6.5^{b}
	8.0	65.0^{ab}	5.0^{c}
2.7	0.0	0.0^{e}	0.0^{c}
	0.5	25.0^{cde}	5.0^{b}
	1.0	35.0^{cd}	5.25^{b}
	2.0	70.0^{a}	9.25^{a}
	4.0	65.0^{ab}	10.25^{a}
	8.0	25.0^{cde}	1.25^{c}
Significance			
NAA	-	ns	ns
TDZ	-	***	***
NAA*TDZ	-	***	*

Percentage of explants showing adventitious shoot regeneration. The value represents the mean of three independent experiments. Means in the same column followed by the same letter are not significantly different (p< 0.05; Least Significant Difference test LSD). ns, not significant; *,**,*** significant at p<.05,.01or at .001,,respectively.

Table 4. Effect of TDZ and NAA on adventitious shoot regeneration frequency and average number of shoots from leaf explants of cv. Dreams White (explants from regenerated shoots).

Growth regulators concentration (µM)		Shoot regeneration (%)	Average number of shoots per explant
NAA	**TDZ**		
0.0	0.0	0.0^{d}	0.0^{c}
	0.5	30.0^{c}	4.0^{b}
	1.0	20.0^{c}	3.5b
	2.0	65.0^{b}	3.0^{b}
	4.0	75.0^{ab}	6.75^{ab}
	8.0	90.0^{a}	7.25^{a}
2.7	0.0	0.0^{c}	0.0^{c}
	0.5	0.0^{c}	0.0^{c}
	1.0	0.0^{c}	0.0^{c}
	2.0	0.0^{c}	0.0^{c}
	4.0	40.0^{c}	2.5^{c}
	8.0	15.0^{cd}	3.0^{c}
Significance			
NAA	-	***	**
TDZ	-	***	***
NAA*TDZ	-	***	ns

Percentage of explants showing adventitious shoot regeneration. The value represents the mean of three independent experiments. Means in the same column followed by the same letter are not significantly different (p< 0.05; Least Significant Difference test LSD). ns, not significant; *,**,*** significant at p<.05,.01or at .001,,respectively.

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). It also indicates that the regeneration ability did not change using explants from the regenerated shoots (R1). In both explants sources (seedlings and R1), adventitious shoots were directly developed with minimum callus formation (Figure 1A), therefore, it is possible that no variation was associated with this protocol, in addition, acclimatized plantlets that grew successfully in the greenhouse of each cultivar did not exhibit any morphological changes in leaf shape or flower color. Variation in leaf morphology and flower color was associated with the *in vitro* system of several ornamental plants (Bouman and De kelerk, 1996).

Conclusion

In conclusion, the present study provides an efficient shoot regeneration system in petunia using leaf explant without any apparent variation in the regenerates that may be suited to the development of an efficient gene transfer system.

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