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Direct shoot regeneration of three Petunia cultivars

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All relevant data are within the paper and its Supporting Information files.

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Abstract: A tissue culture system for acquiring high-efficiency regeneration of Petunia was optimized. Leaf explants of Alvan, Large Flower Alvan (LF Alvan) and Mahalat cultivars of *Petunia hybrida* were cultured separately on MS medium including various concentrations of TDZ and BA without auxin in order to assess direct shoot regeneration. Alvan showed the highest frequency of shoot regeneration (100%) and the highest mean number of shoots per explant (25.33) on MS containing 2 mg/l TDZ. For LF Alvan cultivar the highest percentage of shoot organogenesis (100%) and the highest mean number of shoots per explant (18.20) were observed when MS medium containing 1 mg/l BA was used. With the Mahalat cultivar the maximum rate of direct regeneration was obtained on MS supplemented with 0.5 and 1 mg/l BA (80%). The mean number of shoots per explant (9.63) was obtained when 2 mg/l TDZ was used. Regenerated shoots were successfully elongated (2 to 3 cm in length) and transferred into half-strength MS as the rooting medium supplemented with 0.1 mg/l NAA. The shoots were successfully rooted, acclimatized and transferred to the greenhouse.

1. Introduction

Petunia (*Petunia hybrida)* is well known as an economically important ornamental plant and is grown worldwide for its beautiful and fragrant flowers. Propagation techniques with modern approaches intend to give a hand to scientists to provide demands of ornamental industry (Rout *et al*., 2006). An efficient plant regeneration system is necessary for the successful genetic transformation (Ntui *et al*., 2010). There are several reports for in vitro shoot regeneration of *Petunia hybrida* species from several explants including leaf (Preece, 2000; Ntui *et al*., 2010; Abu-Qaoud *et al*., 2010; Khan *et al*., 2011; Abu-Qaoud, 2012; Burbulis *et al*., 2015), somatic cells (Rao *et al*., 1973) cotyledon (Dulien, 1991), embryo (Dimasi-Theriou *et al*., 1993), protoplast (Auer *et al*., 1992; Auer *et al*., 1999; Abu-Qaoud *et al*., 2010), petal (Razdan, 2003), and microspore (Li *et al*., 2013). Various factors could affect organogenesis in *P. hybrida* such as light (Reuveni and Evenor, 2007), sugar and CO_2 (Qu *et al*., 2007), ethylene

(Dimasi-Theriou *et al*., 1993), nitrogen and calcium (Frett and Dirr, 1996) and also hormonal combinations (Ying *et al*., 2005; Xiao-Feng *et al*., 2009; Xian-Chun, 2010) Petunia regeneration happens directly and indirectly by combinations of auxins and cytokinins in medium culture (Michalczuk and Michalczuk, 2000; Ziv *et al*., 2005).

Adventitious bud formation from somatic cells of *P. hybrida* was induced by exogenous cytokinins such as BA (6-benzyladenine), Zeatin, Kinetin and TDZ (Thidiazuron) (Rao *et al*., 1973; Thirukkumaran *et al*., 2009). It is reported that TDZ acted different from traditional cytokinins and was able to accomplish both the cytokinin and auxin requirements of different plant species for regeneration (Murthy *et al*., 1998; Sanikhani *et al*., 2006). The highest frequency of direct shoot organogenesis of Daady Blue and White Dreams cultivars of *P. hybrida* was obtained on MS medium supplemented with different concentration of TDZ (Abu-Qaoud, 2012). Also, TDZ alone provided the highest percentage of shoot organogenesis and mean number of shoot per explant of *P. hybrida* cv. Mitchell (Thirukkumaran *et al*., 2009). It is also reported that exogenous cytokinin especially BA could control the commitment of Petunia leaf explants to induce shoots in tissue culture (Auer *et al*., 1992; Abu-Qaoud *et al*., 2010). Therefore in this study, we investigated the effect of TDZ and BA as well as genotype on direct shoot regeneration of three Petunia cultivars. This efficient regeneration system is very useful in genetic transformation projects of *P. hybrida*.

2. Materials and Methods

Seed germination

Seeds of three local cultivars of Petunia, Alvan, Large Flower Alvan (LF Alvan) and Mahalat, were sterilized with 70% ethanol for 30s, and sodium hypochlorite solution 1% for 10 minutes. They rinsed 3 times with sterilized water and cultured on MS medium. Seeds were grown under 25 ± 2 °C with 16/8 hour photoperiod, under fluorescent illuminations (40 µmol $m^{-2}s^{-1}$).

Organogenesis

The newly formed leaves were cut 6-8 mm in length, and then cultivated on 5 modified MSmedia: MS medium without hormones (MS_1), MS + 0.5 mg/l BA (MS_2) [Sigma-Aldrich, Steinheim, Germany], MS + 1 mg/l BA (MS_3), MS + 1 mg/l TDZ (MS_4) [Sigma-Aldrich, Steinheim, Germany] and MS + 2 mg/l TDZ (MS_5). Abu-Qaoud *et al*., (2010) got more regeneration when they used 0.8 mg/l BA. Therefore we selected 0, 0.5 and 1 mg/l BA to better estimate BA effect. Also as Thirukkumaran *et al*., (2009) reported more regeneration with 2 mg/l TDZ, we selected 0, 1 and 2 mg/l TDZ to investigate its effect. Moreover, the MS was supplemented with 30 g/l sucrose and solidified with 7 g/l agar [Duchefa, Haarlem and The Netherlands]. The optimum pH of all culture media was considered 5.8 which adjusted with 1N NaOH before sterilization. Then all media were sterilized using autoclave at 121°C for 20 min. Explants were placed on regeneration medium with the adaxial side upward. The cultures were incubated at 25±2°C, with a light to dark period of 16/8 hours under cool-white fluorescent light at 40 µmol m^{-2} s^{-1}. Explants were sub-cultured every two weeks. They were investigated using binocular Stereo Microscope, regarding to the mean number of explants inducing shoots and the mean number of induced shoots and buds per explants after 4-5 weeks on regeneration medium.

Rooting and acclimatizing

Regenerated shoots were transferred into half-strength MS supplemented with 30 g/l sucrose, 0.1 mg/l NAA [Duchefa, Haarlem, and The Netherlands] and solidified with 7 g/l agar. The rooted plantlets rinsed under tap water and planted on the plastic pots with combination of sterile peat moss and perlite mixture (2:1). They kept in greenhouse conditions.

Statistical analysis

The experiment was done based on completely randomized design with three replications and 10 leaf explants in each replication. Data were normalized through arcsin (√x) and (√x+0.5) transformation in SPSS. The normalized data were analyzed using SAS statistical analysis package and were compared via Duncan's multiple range test at $P \leq 0.01$ and $P \leq 0.05$.

3. Results

Effect of BA on organogenesis

Direct shoot formation was obtained in all three cultivars after 4-5 weeks. No regeneration occurred on MS_1 medium which means hormones are necessary to induce shooting (Tables 1, 2). When 0.5 mg/l BA (MS_2) was used no differences in frequency of regeneration was observed among cultivars. The low

Table 1 - Effect of modified MS medium supplemented with different concentration of BA on shoot regeneration from leaf explants of *P. hybrid*

MS Media	Frequency of regeneration			The mean number of shoots per explant		
	Cultivars			Cultivars		
MS_1	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 e	0.00 ± 0.00 e	0.00 ± 0.00 e
MS_2	83.33 ± 2.8 b	83.33 ± 1.8 b	80.00 ± 3.1 b	6.21 ± 0.12 d	13.31 ± 0.85 b	5.05 ± 1.00 d
MS_3	80.00 ± 3.7 b	100.00 ±0.00 a	80.00 ± 1.1 b	10.12 ± 0.41 bc	18.20 ± 0.85 a	7.76 ± 0.56 cd

The values represent the mean ± standard error of three replicates. Different letters are showing considerable differences at P≤0.05.

Table 2 - Effect of modified MS medium supplemented with different concentration of TDZ on shoot regeneration from leaf explants of *P. hybrid*

MS Media	Frequency of regeneration			The mean number of shoots and buds per explant		
	Cultivars			Cultivars		
	Alvan	LF Alvan	Mahalat	Alvan	LF Alvan	Mahalat
MS_1	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 f	0.00 ± 0.00 f	0.00 ± 0.00 f
MS_2	83.33 ± 3.4 b	80.00 ± 1.0 b	66.66 ± 2.1 c	16.25 ± 1.00 b	12.00 ± 1.21 c	6.61 ± 0.08 e
MS_3	100.00 ± 0.00 a	83.33 ± 0.8 b	70.00 ± 1.7 c	25.33 ± 1.02 a	14.31 ± 0.96 bc	9.63.00 ± 0.11 d

The values represent the mean ± standard error of three replicates. Different letters are showing considerable differents at P≤0.05.

mean numbers of shoot per explant (5.05 and 6.21) were observed in MS_2 for Mahalat and Alvan cultivars, respectively. When 1 mg/l BA (MS_3) was used differences were observed in all three cultivars and LF Alvan cultivar showed 100% shoot regeneration (Table 2), with a mean number of 18.20 shoots per explants (Fig. 1 a).

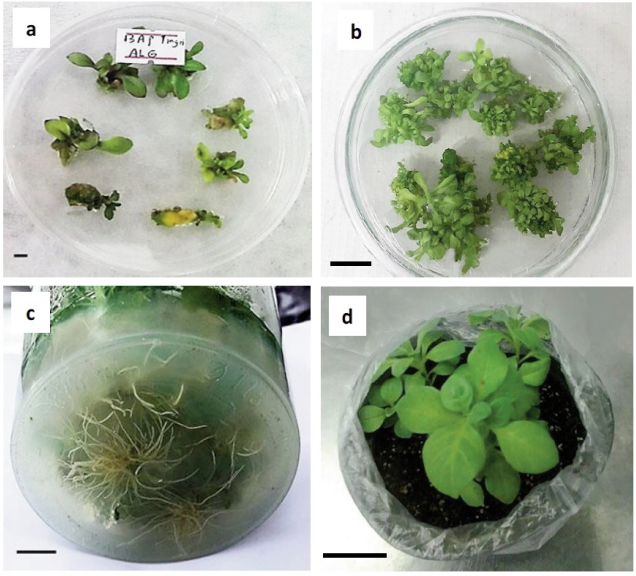

Fig. 1 Plant regeneration from leaf explants of different cultivars of *Petunia hybrida*. (a) Direct shoot regeneration of LF Alvan on MS + 1 mg/l BA (bar: 2 mm); (b) Direct shoot regeneration of Alvan on MS + 2 mg/l TDZ (bar: 4 mm); (c) Root formation after 2 weeks on rooting media (bar: 5 mm); (d) A 4 weeks old plantlet after transfer to the pot (bar: 1 cm).

Effect of TDZ on organogenesis

Significant differences were observed among three cultivars when TDZ concentration was increased (Tables 3, 4). The low shoot regeneration frequency was obtained on MS_4 and MS_5 media for Mahalat cultivar (Table 4). Alvan cultivar showed the highest percentage of shoot regeneration (100%) and mean number of shoots per explant (25.33) on MS with 2 mg/l TDZ (Fig. 1 b) and the lowest one (6.61) was belong to Mahalat cultivar on MS with 1 mg/l TDZ.

4. Discussion and Conclusions

We could show that auxin is not necessary for direct shoot regeneration of three cultivars of *P. hybrida*. It is already reported that the number of shoot per explants dramatically increased when explants exposed to the medium containing BA (Auer *et al.*, 1992). The highest shoot regeneration rate (45%) and the maximum average number of shoots per explant (7.5) from Petunia leaf explants on MS with 2 mg/l BA + 0.5 mg/l NAA has also been reported (Abu-Qaoud *et al.*, 2010). In the current study the highest shoot regeneration frequency in Alvan cultivar and the mean number of shoots per explant in both Alvan and Mahalat cultivars were observed when 2 mg/l TDZ was used which is in conformity with Thirukkumaran *et al.* (2009). The importance of TDZ on regeneration and shoot induction frequency

and the mean number of shoots per explant was also investigated in Daddy blue and Dreams white genotypes (Abu-Qaoud, 2012). This study showed that a cytokinin source of TDZ or BA may be enough for direct shoot regeneration of three mentioned cultivars of *P. hybrida*. Application of TDZ instead of both auxin and cytokinin requirements for organogenesis in the wide range of plant species has been supported (Murthy *et al*., 1998). Probably TDZ tends to make balance among endogenous growth regulators that is essential for inducing specific modes of regeneration. It was found that many factors such as genotype and exogenous growth regulators have the capability to influence on biochemical pathways controlling the endogenous cytokinin content (Krikorian, 1995). In the present study a significant difference in regeneration frequency was observed among studied cultivars probably due to the different level of endogenous hormones. For LF Alvan cultivar, the maximum regeneration frequency (100%) and the highest number of shoots per explants (18.20) were obtained when BA concentration was increased from 0.5 to 1 mg/l while the other two cultivars showed less reaction. These findings confirm the report of Jamshidnia and Sayed Tabatabaei (2013), and Burbulis *et al*., (2015) on differences in shoot regeneration frequency among three different genotypes of Petunia. Here we report an efficient direct shoot regeneration system in *Petunia hybrida* using leaf explants of Alvan cultivar. This cultivar can be considered as a suitable cultivar for transformation experiments.

To conclude, the present study provided an efficient direct shoot regeneration system without auxin in Petunia using leaf explants that could be improve transformation studies.

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Table 3 - Analysis of variance of different concentrations of TDZ on shoot regeneration of *P. Hybrida*

Source of Variation	DF	Mean squares		P-value	
		Frequency of regeneration	Mean number of shoots per explant	Frequency of regeneration	Mean number of shoots per explant
TDZ	2	3.1897 **	11.4806 **	0.000	0.000
Cultivar	2	1.3250**	6.5896 **	0.009	0.004
TDZ × Cultivar	4	0. 8015*	4.2010 **	0.022	0.009
Error	18	0. 215	0.9080		
Total	26				

*, **, significant at 5% and 1% levels, respectively.

Table 4 - Effect of TDZ on shoot regeneration of *P. Hybrida* using Duncan's multiple range test

MS Media	Frequency of regeneration			The mean number of shoots and buds per explant		
	Cultivars			Cultivars		
	Alvan	LF Alvan	Mahalat	Alvan	LF Alvan	Mahalat
MS_1	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 f	0.00 ± 0.00 f	0.00 ± 0.00 f
MS_4	83.33 ± 3.4 b	80.00 ± 1.0 b	66.66 ± 2.1 c	16.25 ± 1.00 b	12.00 ± 1.21 c	6.61 ± 0.08 e
MS_5	100.00 ± 0.00 a	83.33 ± 0.8 b	70.00 ± 1.7 c	25.33 ± 1.02 a	14.31 ± 0.96 bc	9.63 ± 0.11 d

MS_1= MS medium without hormones, MS_4= MS + 1 mg/l TDZ; MS_5= MS + 2 mg/l TDZ.
Means compared using Duncan's multiple range test. The Values represent the mean ± standard error of three replicates. Different letters are showing considerable differents at P≤ 0.05.

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