In vitro REGENERATION AND SOMACLONAL VARIATION OF *Petunia hybrida*

Hassan Abu-Qaoud[1], Anas Abu-Rayya[1] and Sami Yaish[2]

[1]Faculty of Agriculture, Department of Plant Production and Protection
An Najah National University, P.O. Box 707. Nablus, Palestinian Authority
[2]Faculty of Science, Department of Biology and Biotechnology
An Najah National University, P.O. Box 707. Nablus, Palestinian Authority

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A B S T R A C T

The effect of different levels of naphthaleneacetic acid (NAA) and benzyladenine (BA) on shoot multiplication and regeneration of *Petunia hybrida* was studied. Regenerated shoots from leaf explants were grown *ex vitro* for analysis of somaclonal variation. Seeds of *Petunia hybrida* were germinated *in vitro* on MS basal medium. The seedlings were used as a source of explants for the multiplication and regeneration experiments. For the shoot multiplication experiment, shoot explants taken from germinated seedlings were cultured on MS basal media supplemented with different concentrations of BA (0.1, 0.4, 0.8 mg l^{-1}) and NAA at concentration of 0.1 mg l^{-1}. Highest number of axillary shoot was obtained on medium supplemented with 0.8 mg l^{-1} BA and 0.1 mg l^{-1}NAA. For regeneration experiments, leaf sections taken from germinated seedlings were cultured onto MS media supplemented with three levels of BA (0.5, 1.0 and 2.0 mg l^{-1}) and 0.5 mg l^{-1}NAA. The highest shoot regeneration rate (45%) was observed in MS medium supplemented with 2 mg l^{-1} BA. For the evaluation of somaclonal variation, lateral bud explants were taken from pink colored petunia plants that were grown in the greenhouse. The buds were disinfected and cultured on MS basal media supplied with 30 mg l^{-1} gentamycin sulfate and 30 mg l^{-1} Benlate. After shoots grew, leaf sections were then taken from them shoots and cultured onto shoot regeneration medium (MS medium supplemented with 2 mg l^{-1} BA). The regenerated adventitious shoots were cultured on MS medium without growth regulator. These shoots were then rooted, acclimatized and transferred to the greenhouse for evaluation. Two forms of leaf shape (orbicular and elliptic) and three flower colors (violet, purple and light pink) appeared on the plantlets.

Key words: *Petunia hybrida*, tissue culture, somaclonal variation

INTRODUCTION

Petunia hybrida is an economically important oramental plant species (Mol et al., 1985; Quattrocchio et al., 1998; Huits et al., 1994; Solano et al., 1995; Bradley et al., 1989; Davies et al., 1998). It is greatly diversified and available in a range of colors (Christopher, 1994). Ornamental plants are produced exclusively for their esthetic values. The improvement of quality attributes such as flower color and longevity, plant shape, architecture, and creation of novel variation are important economic goals (Burchi et al., 1995). Further growth and success depends on the development of new technologies, such as tissue culture (Geneve et al., 1997). Different environmental factors were found to affect the *in vitro* organogenesis of petunia plant. Shoot organogenesis in *Petunia hybrida* leaf discs is affected by explant size, configuration and duration of exposure to benzyladenine (BA) (Beck and Camper, 1991). Also, studies on anatomical changes (Traas et al., 1990), effect of light (Reuveni and Evenor, 2007), sugar and CO_2, nitrogen and calcium (Frett and Dirr, 1986; Qu et al., 2007), and ethylene (Dimasi-Therion et al., 1993) on shoot and root regeneration in the petunia plant, were done.

Mutation, breeding and tissue culture technique can be utilized for the improvement of ornamental characteristics (Hutchinson et al., 1992). The variability associated with tissue culture has provided a pool of variation upon which selection pressure has imposed to isolate unique forms of clones. This variation known as somaclonal variation has become an important tool for plant improvement (Skirvin et al., 1993). Several studies on somaclonal in *in vitro Petunia hybrida* cultures were conducted. The evaluated characters were: leaf morphology, flower color, flower shape, and leaf variation (Jain and De Klerk, 1998). The aim of this investigation was to search for valuable somaclonal variants of the *Petunia hybrida* plant.

MATERIAL AND METHODS

Seed germination

"Mixed Colors" petunia seeds from Royal Flower Company were used in these experiments. Seeds were enclosed in a white mesh, surface sterilized by immersing in 10% Chlorax solution (0.52% sodium hypochlorite) and rinsed three times for five minutes in sterile distilled water. Sterilized seeds were planted in 60 test tubes (25 x 180 mm) each containing 10-ml of Murashige and Skoog (MS) basal medium. Three seeds were planted in each test tube. The tubes were incubated for four weeks at 22 °C under cool light at 50μ mol m^{-2} s^{-1} photon flux density and 16 h photoperiod

Shoot multiplication

MS basal media supplemented with different 3 levels of BA (0.2, 0.4, 0.8 mg l^{-1}) and NAA 0.1 mg l^{-1} and were used in this experiment; the 8 combinations were considered as treatments. Each treatment was replicated 25 times. Each replicate was one test tube with one shoot per test tube. The experiment was arranged in a completely randomized design.

Shoots of about 2 cm in length excised from the seedlings germinated as above were transferred into media supplemented with various hormonal combinations (one shoot per test tube). These shoots were incubated under 16 h day light (cool light) at 22 °C in the incubator. After four weeks, the number of axillary shoots, their length and number of leaves in each test tube were recorded. Optimal hormonal combination was determined based on the result of these variables.

Shoot regeneration

Fully expanded leaves taken from shoots grown on multiplication media were excised, cut transversely into two pieces and cultured in Petri dishes on MS basal medium supplemented with BA (0.5, 1.0 and 2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). The eight combinations of both growth regulators represented the treatments. Each treatment consisted of five explants (half leaf) per Petri dish with 4 replicates for each treatment. Leaves were and were cultured with the abaxial side in contact with the media. All cultured leaves were incubated in darkness for 3 weeks. The leaves were then transferred under conditions similar to those of the germination and multiplication cultures. Regenerated shoots were excised and cultured on MS basal medium and sub-cultured 2 times.

Each experiment was repeated twice and the average shoot regeneration rate was evaluated. Data were analyzed using the SAS software program. Analysis of variance followed by mean separation was conducted.

Somaclonal variation experiment

Petunia hybrida seeds of the same source as above were planted in pots in the greenhouse. The plants were irrigated every other day for 2 months until sufficient growth was achieved. Lateral buds with a small piece of the stem were excised from of 10 pink colored young plants (Fig. 2C) and washed thoroughly with running tap water for 30 minutes. The buds were then placed in 250 ml of 10% Chlorax containing 2 drops of Tween 20 for 20 minutes and next washed 3 times with sterile distilled water; each time for 5 minutes. Then the buds were immersed in 96% ethanol for few seconds and left to dry.

The buds were trimmed to remove bleached damaged tissues and transferred to test tubes containing MS basal media supplemented with 30 mg l^{-1} Benlate (fungicide) and 30 mg l^{-1} Gentamycin sulfate (Antibiotic). The test tubes were transferred to the growth chamber at 25 °C (±1 °C) and 16 h cool day light. After one month, clean growing shoots 2 cm in length were transferred to new test tubes containing 10 ml of shoot multiplication media (MS media supplemented with 0.8 mg l^{-1} BA). They were incubated in the growth room under 16 h cool day light at 25°C for four weeks. The shoots which had multiplied were then transferred onto MS basal media and kept for three weeks. Then the leaf explants were taken from them and cultured on regeneration media (MS supplemented with 2 mg l^{-1} BA).

Shoots regenerated from leaf explants were transferred onto MS basal medium for three weeks. The shoots which had grown to 2-cm length were excised and immersed in indole-3-butyric acid (IBA) powder of 2500 ppm concentration and transferred into pots containing sterile rooting media (2:1 mixture of sterile sphagnum peat and vermiculite). Shoots were irrigated by sterile distilled water and incubated for three weeks in the growth room under 16 h day light at 22 °C (±1 °C). Three weeks later, the number of shoots which produce roots was counted.

Rooted shoots were transferred gently to larger pots containing a mixture of sphagnum peat and vermiculite (2:1, v/v). The pots were then transferred to the greenhouse and irrigated every other day for morphological evaluation. Leaf shape and flower color were compared to that of the original plant.

RESULTS AND DISCUSSION

Seed germination

After 3 weeks, all seeds germinated successfully onto the basal medium. They continued to grow on this media for one month. All seedlings were clean without any contamination. This result was in agreement with a previous work of Dixon (1985) who reported that a high seed germination percentage of petunia was achieved on agar medium without growth regulators.

Shoot multiplication

Both BA and NAA exhibited a significant effect on the average number of produced shoots, shoot length and leaf number (Tab. 1). The highest number of axillary shoots (7.8) was obtained on the medium containing 0.8 mg l^{-1} BA. (Fig. 1 A, C). However, other combinations of NAA and BA produced similar shoot number except medium with 0.1 NAA and 0.8 mg l^{-1} BA. The lowest average shoot number (2.89) was obtained in the control. The highest shoot length (4.63) was obtained on medium containing 0.1 mg l^{-1} NAA (Tab. The similar effects were observed on media containing 0.2, and 0 and 0.4 and 0 mg l^{-1} of BA and NAA, respectively. The lowest average shoot length was obtained on medium containing 0.1 mg l^{-1} NAA and 0.2 mg l^{-1} of and BA respectively. A similar effect of both BA and NAA was exhibited on the average leaf number per test tube. The highest leaf number (25) per test tube was obtained on the medium containing 0.8 mg l^{-1} BA (Tab. 1).

Shoot regeneration

NAA did not affect significantly shoot regeneration. BA however, showed a significant effect on shoot regeneration with a significant interaction effect of both growth regulators (Tab. 2). When BA was used at 1.0 and 2.0 mg l^{-1}, maximum regeneration percentage was observed (35 and 45%, respectively). A similar effect was observed with number of the regenerated shoots (Tab. 2).

Table 1. The effect of different levels of BA and NAA on axillary shoot number, length and leaf number of micropropagated petunia shoots

BA [mg l^{-1}]	NAA [mg l^{-1}]					
	0.0			0.1		
	shoot number	shoot length [cm]	leaf number	shoot number	shoot length [cm]	leaf number
0.0	2.8 b*	3.0 bc	15.2 b	2.9 b	4.6a	9.0cd
0.2	5.2 b	4.0 a	16.4 b	3.9 b	2.3 c	5.7 d
0.4	3.9 b	4.6 a	12.4 bc	4.5 b	3.2 c	6.8 d
0.8	7.8 a	3.8 ab	25.0 a	6.2 a	3.4 b	5.6 d

*Number in each column followed by the same letter or letters are not significantly different at $p \leq 0.5$ according to (Duncan's Multiple Range test) (DMRT) for each variable

Table 2. Effect of BA and NAA on adventitious shoot regeneration from leaf explants of *Petunia hybrida*

Growth regulators concentration [mg l^{-1}]		Shoot regeneration [%]	Average number of shoots per explant
NAA	BA		
0.0	0.0	0.0 b[1]	0.0 b
	0.5	0.0 b	0.0 b
	1.0	35.0 a	2.0 ab
	2.0	45.0 a	7.5 a
0.5	0.0	0.0 b	0.0 b
	0.5	0.0 b	0.0 b
	1.0	0.0 b	0.0 b
	2.0	30.0 a	1.5 ab
Significance[2]			
NAA		ns	ns
BA		***	**
NAA*BA		***	**

[1]The value represents the mean of two independent experiments. Means in the same column followed by the same letter(s) are not significantly different at $p \leq 0.05$; (Multiple Range Test DMRT)
[2]ns: not significant, **, *** significant at ($p < 0.05$, 0.01 or at $p < 0.001$), respectively

The results of the present study demonstrate that BA is important for shoot initiation. Growth regulators induce the competence of tissue to respond to further developmental signals (Christianson and Warnick, 1985; 1988). Cells often respond differently at different developmental stages, and interactions between auxin and cytokinin signaling pathways may occur (Shi et al., 1994). The inclusion of cytokinin in the culture media enables callus to be induced from a wide range of plant species. More importantly cytokinin allows initiation of multi-cellular meristematic regions capable of differentiation into organized structures (Lowe et al., 1996). There was no regeneration from *Petunia hybrida* calli subcultured on low cytokinin media (Renaudin et al., 1991).

The number of adventitious buds and shoots produced by explants may be easily regulated by varying the concentration of BA in the culture medium (Pollard and Walker, 1990). The results of this research correspond with these of Auer et al. (1992) who found that the exposure of the explants to BA containing medium increased dramatically the number of petunia shoots produced per explants.

Somaclonal variation

Petunia adventitious shoots were successfully regenerated from leaf explants on the regeneration medium (Fig. 1B). These shoots were used for the evaluation of the somaclonal variation. 100 shoots were transferred on the rooting medium (Fig. 1D). After three weeks of incubation 60% of the shoots produced roots. The rooted plants continued to grow in the greenhouse.

Leaf variation

Leaves of sixty plants growing in the greenhouse were inspected for morphological variation. The results are shown in Table 3. The shape of the original plant leaves (normal) was ovate (Fig. 2A, B). Another two new forms of leaves were observed on somaclonal variants: 11 plants produced orbicular leaves and 6 plants produced elliptic leaves (Tab. 3).

Flower variation

Among 40 plants that continued their growth in the greenhouse until flowering, three new colors appeared (Fig. 2D). Two plants produced purple colored flowers, 8 were dark pink and 3 were violet, while the others were similar to the original color (pink).

In this study different types of variants were obtained in both leaf shape and flower color (28% and 32.5%) respectively.

The results demonstrated that new phenotypes from *Petunia hybrida* could be produced by somaclonal variation through *in vitro* regeneration. Variation in leaf morphology was obtained. Bouman and De Klerk (1996) examined the extent of variability of leaf shape in ornamental plants. A large increase in variability was observed after regeneration from non-organized callus. Variation was also observed in flower color.

Variation has been observed in ornamental plants with respect to leaf shape and flowers color. Plant growth regulators affect the rate of somaclonal variation indirectly by increasing the multiplication rate and inducing adventitious shoots (Bairu et al., 2006). Using amplified fragment length polymorphism (AFLP) analysis to assess genetic fidelity between primary regenerants of *Echinacea purpurea* derived from leaf organogenesis and their donor plants, it was found that only two regenerants (T2-15-2 and T2-15-3) had Jaccard's similarity coefficient value of 1 as compared to their donor. Thus, they were true-to-type to their donor T2-15-0 (Chuang et al., 2009).

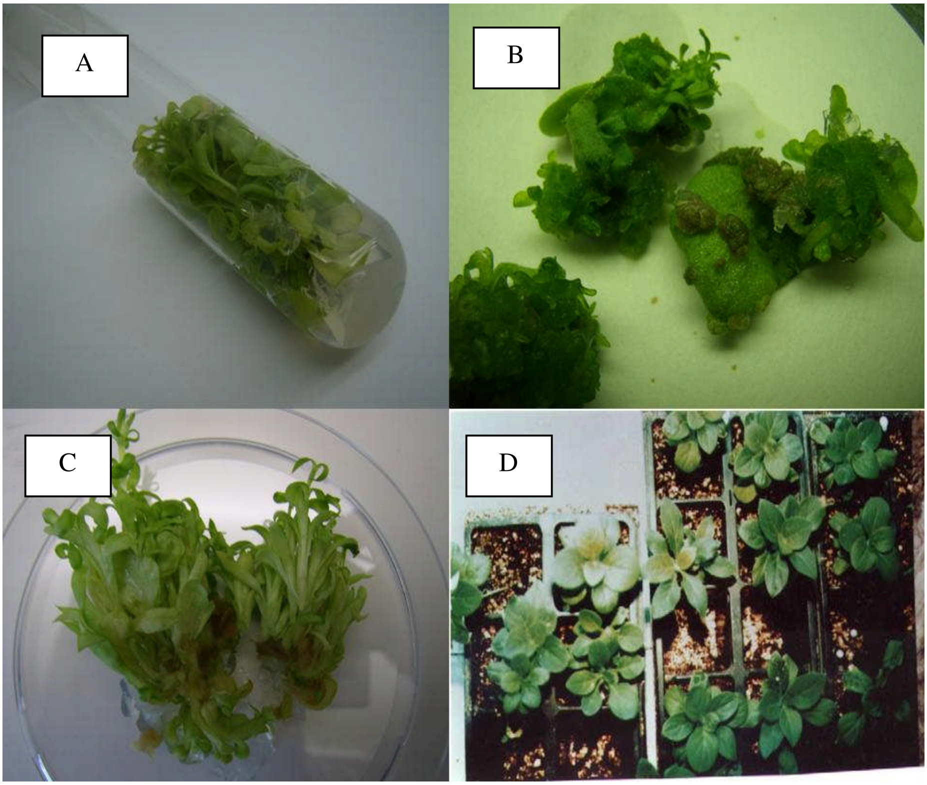

Figure 1. A and C – In vitro multiple shoots of *Petunia hybrida* on multiplication medium. B – Adventitious petunia shoots regenerated from leaf section. D – Acclimatized petunia shoots

Table 3. Number of new leaf and flower forms of petunia plants developed *in vitro*

Leaf shape			
Original (ovate)	orbicular	elliptic	total
43	11	6	60

Flower color				
Original (pink)	dark pink	violet	purple	total
27	8	3	2	40

In conclusion, the findings of this work could be useful in further study. More investigation could be done to improve the regeneration of petunia from leaf explants. In addition, the molecular technique could be used for detection and conforming somaclonal variation.

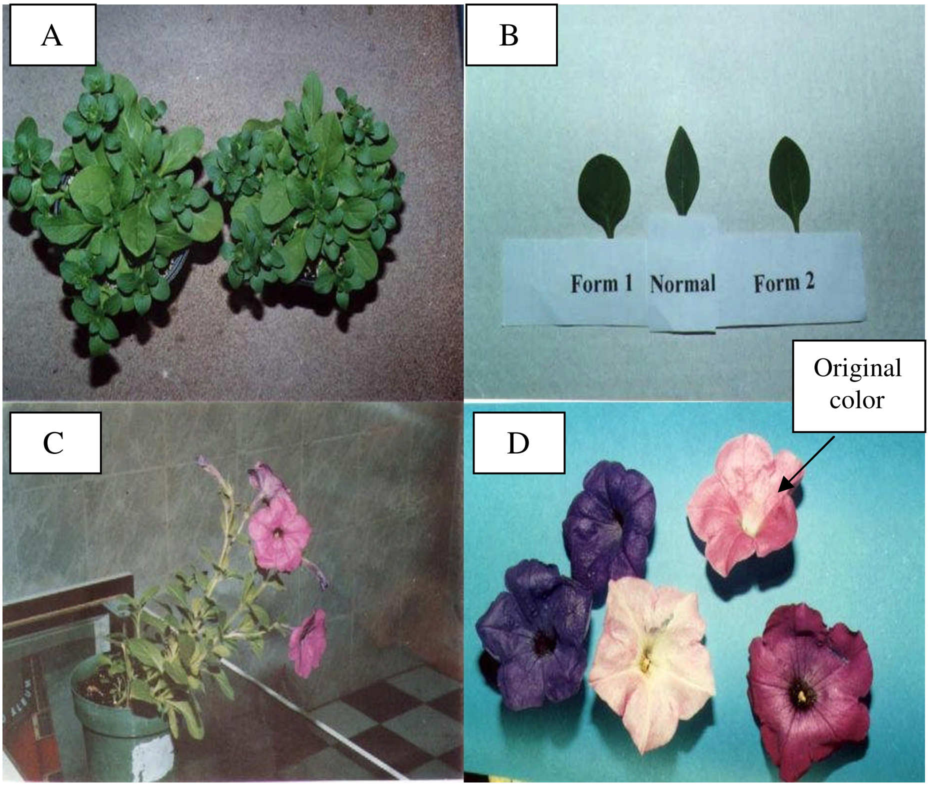

Figure 2. Somaclonal variation in petunia plants regenerated from adventitious shoots. A – Growing petunia plantlets obtained from regenerated shoots; B – New leaf forms developed on regenerated plantlets; C – Original petunia plant (pink color) used as a source of explants for somaclonal variation; D – New flower colors developed on the regenerated plantlets

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ZMIENNOŚĆ SOMAKLONALNA PODCZAS REGENERACJI *in vitro* PĘDÓW *Petunia hybrida*

Hassan Abu-Qaoud, Anas Abu-Rayya i Sami Yaish

STRESZCZENIE

Badano wpływ rożnych stężeń kwasu naftylooctowego (NAA) i benzyladeniny (BA) na regenerację i namnażanie pędów *Petunia hybryda.* Występowanie zmienności somaklonalnej analizowano podczas wzrostu *ex vitro* pędów zregenerowanych z eksplantatów liściowych. Nasiona *Petunia hybrida* kiełkowały *in vitro* na pożywce podstawowej MS. Siewki posłużyły jako źródło eksplantatów do doświadczeń nad mikrorozmnażaniem i regeneracją pędów. W doświadczeniach nad mikrorozmnażaniem eksplantaty pędowe pobrane z siewek były wyszczepiane na pożywkę MS z dodatkiem różnych stężeń BA (0,1, 0,4, 0,8 mg l^{-1}) i NAA w stężeniu 0,1 mg l^{-1}. Największą liczbę pędów kątowych uzyskano na pożywce z BA w stężeniu 0,8 mg l^{-1} i NAA 0,1 mg l^{-1}. Do doświadczeń nad regeneracją pobierano liście z siewek, które następnie przecinano na pół i wszczepiano na pożywkę MS z dodatkiem BA w stężeniach 0,5, 1,0 i 2,0 mg l^{-1} oraz NAA w stężeniu 0,5 mg l^{-1}. Największą efektywność regeneracji pędów (45%) stwierdzono na pożywce MS wzbogaconej w 2 mg l^{-1} BA. Do oceny zmienności somaklonalnej pobierano eksplantaty w postaci pąków bocznych z rosnących w szklarni roślin petunii o różowych kwiatach. Pąki były dezynfekowane i wszczepiane na pożywkę MS z dodatkiem 30 mg l^{-1} siarczanu gentamycyny i 30 mg l^{-1} Benlate. Gdy z pąków rozwinęły się pędy, pobierano z nich eksplantaty liściowe i wyszczepiano na pożywkę do regeneracji pędów (MS z dodatkiem 2 mg l^{-1} BA). Zregenerowane pędy przybyszowe były ukorzeniane, aklimatyzowane i wysadzone w szklarni dla oceny zmienności. W stosunku do formy wyjściowej, rośliny otrzymane z pędów przybyszowych miały dwie nowe formy liści (kołowe i eliptyczne) oraz trzy nowe kolory kwiatów (fioletowe, purpurowe i jasnoróżowe).

Słowa kluczowe: *Petunia hybrida*, kultury tkankowe, zmienność somaklonalna