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In vitro Preliminary Study on *Petunia hybrida* breeding under Sodium Chloride Stress Conditions

[1]Sara E. Gomaa and [2]Naglaa M. Esmaiel

[1]Veg., Medicinal and Aromatic Plants Breeding Dept. Horticulture Research Institute, Agricultural Research Center, Alexandria, Egypt; P.O. Box 21523.
[2]Floriculture Dept., Horticulture Research Institute, Agricultural Research Center, Alexandria, Egypt; P.O. Box 21523. E-mail: nglsml@yahoo.com

ABSTRACT

Using low quality or salty water in medicinal and ornamental plants irrigation is expected in the near future because of the diminishing fresh water sources and the rapid growth of populations. One of the major abiotic stresses is salinity, which leads to alterations in morphological, physiological, biochemical, and molecular features and consequently affects plant development and profitability negatively in arid and semiarid regions. In the coming 25 years, it is predicted that salinization will decrease cultivated lands to 30% and probably to 50% by 2050. This study was performed in the tissue culture lab of Antoniades Gardens, Alexandria; HRI- ARC in 2013-2015 to investigate the *in vitro* regeneration capability of *Petunia hybrida* on four medium protocols using three different explants(leaf base, internode and cotyledon), in order to enhance the *in vitro* tolerance of petunia plants to salt stress using NaCl. The explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators (BAP and NAA). Results showed that the highest percentage of callus induction (100%) from internodal segments cultured on MS supplemented with 0.5 mg/l BAP. The *in vitro* derived calli subcultured for shoot regeneration; whereas, MS with 2.0 mg/lBAP gave the highest frequency of shootlets (37.0) compared to the intermodal- derived calli. In addition, an *in vitro* selection procedure was developed to enhance petunia salt resistance. NaCl was added directly to the above - mentioned medium at eight different concentrations (20, 40, 60, 80, 100, 120, 140 and 160 mM). However, doses above 80 mM were found to be lethal. All morphogenetic characters were inversely-proportional to the salt concentration except proline content which was directly proportional with salt concentration as an indication for increasing *P. hybrida* salt resistance. The *in vitro* raised shootlets were subcultured on half strength MS augmented with 1.0 mg/l IBA for root formation. Rooted plantlets were transferred to the greenhouse for acclimatization, then to the field until flowering. In conclusion, the best *in vitro* propagation protocol of *Petunia hybrida* could be done by culturing internodes (E_2) on MS + 0.5 mg/l BAP (M_2). Since it showed good tolerance towards salinity up to 80 mM via inducing 6 folds more of proline than the control treatment. It is recommended to plant *Petunia hybrida* as an ornamental plant in coastal areas using ground water, that has a high content of NaCl, as a source of irrigation and by that we are preserving more water that can be used elsewhere. This study can be used in further programs for petunia improvement.

Key words: *Petunia hybrida*, Tissue culture, Salt stress, NaCl, proline.

Introduction

Water preservation is one of the main environmental concerns (Gonzalo, 2011). Using low quality or salty water in medicinal and ornamental plants irrigation is expected in the near future because of the diminishing fresh water sources and the rapid growth of populations (Niu and Rodriguez, 2006). One of the major abiotic stresses is salinity, which leads to alterations in morphological, physiological, biochemical, and molecular features and consequently affects plant development and profitability negatively in arid and semiarid regions (Wang *et al.,* 2001). Around 20% of world soils have high levels of salts, either in the dirt itself or in the watering system (Niu and Rodriguez, 2006), which causes damage to the national economy and the surroundings (Rengasamy, 2010; Yang *et al.,* 2010). In the coming 25 years, it is predicted that salinization will decrease cultivated lands to 30% and probably to 50% by 2050 (Rozema, 2008). Generally, decorative plants are easily affected by salt accumulation. Inducing salt tolerant plants has been attempted by traditional breeding programmers, but they did not achieve the results needed (Rai *et al.,* 2011). As a result, it is crucial to find more rapid and successful programs to enhance and select salt tolerant plants. Subsequently, methods of plant tissue culture could offer a chance to begin the process of producing good decorative plants with desired characters. Generally, the *in vitro* selection pressure technique is the most common technique for abiotic tolerant genotypes

Corresponding Author: Sara E. Gomaa, Veg., Medicinal and Aromatic Plants Breeding Dept., Horticulture Research Institute, Agricultural Research Center, Alexandria, Egypt; P.O. Box 21523.
E-mail: sara_gomaa@hotmail.com

selection. It, basically, depends on the *in vitro* culturing of plant cells, tissues or organs using a medium supplemented with the selective agents, permitting selection and regeneration of plants with desirable traits. The *in vitro* pressure technique has been successfully used to develop salt stress tolerance in plants, using salts as a selective agent, permitting the favored survival and development of desirable traits. This technique has been conducted using different explants, such as (callus, suspension, somatic embryos, shoot cultures, etc.), which have been monitored to find any difference in their capacities to tolerate high salt media. Mostly, Sodium Chloride (NaCl) was the common type of salt used in such experiments (Woodward and Bennett, 2005).

Petunia hybrida is one of the *Solanaceae* family members (Quattrocchio *et al.,* 1998), which includes around 30 subspecies. Petunia species include annual and perennial herbaceous plants (Stehmann *et al.,* 2009). Among the new developed plants, *Petunia hybrida* is the most well-known species, which is a hybrid of *Petunia axillaris* x *Petunia integrifolia,* generally known as the garden Petunia (Gerats and Vandenbussche, 2005). It is mostly used as bedding plant and it comes in a wide variety of colors (Christopher, 1994). *P. hybrida* is economically significant due to its use globally as a decorative plant (Lorenz-Lemke *et al.*, 2006; Dell'Olivo *et al.*, 2011). It is produced from seeds as an annual plant for outside decorative purposes. Some ornamental growers develop them as pendulous growth flowering habit, which makes them suitable for developing as blooming hanging plants in window boxes. Moreover, *P. hybrid* is thought to be a minor plant model-system, because of different desired biological features and the availability of technical tools for genomic, biochemical, cytogenetic and functional analyses (Angenent *et al.,* 1993).In addition, it has been considered as an example to study flavonoid biosynthesis, botanical improvement, and self- incompatibility (Winkel-Shirley, 2001; Souer *et al.*, 1996; Farshad *et al.*, 2013).

The present study was conducted to investigate the *in vitro* regeneration capability of *Petunia hybrida* on four medium protocols using three different explants, and to enhance the *in vitro* tolerance of *P. hybrida* plants to NaCl salt so that it can be possible to cultivate these plants in coastal regions, where plants suffer from high content of salt in air, land and irrigation water.

Materials and Methods

This study was conducted in collaboration between Antoniades Gardens tissue culture lab, Floriculture Department and the Vegetable, Medicinal and Aromatic Plants Breeding Department, Sabhia Research Station, Alexandria; under the Horticulture Research Institute (HRI), Agricultural Research Center (ARC), during the period 2013-2015.

Petunia hybrida seeds were obtained from Semillasfito` Co. (Carrer de la Selva de Mar, 111, 08019 Barcelona, Spain). Seeds were surface sterilized according to Khlifa (2008), then germinated and planted under aseptic conditions. Small plantlets containing 6 - 8 leaves were used as a source of explants [leaf base (E_1), internode (E_2) and cotyledon (E_3)]. Explants were then cultured on Murashige and Skoog (1962) medium supplemented with 0.1 mg/l naphthalene acetic acid (NAA) + 0.4 mg/l (BAP) [M_1] and MS with different concentrations of 6 - benzyl amino purine (BAP) (0.5, 1.0 and 2.0 mg/l) [M_2, M_3 and M_4 respectively] to study their effect on callus induction (%),callus weight (g) and shoot number. Light conditions were 16/8 hrs (light /dark cycle) through cooling fluorescent white lamps with light intensity of 3000 Lux.

Pure and desiccated sodium chloride was added directly to the culture media before autoclaving at the final concentrations of 0, 20, 40, 60, 80, 100, 120, 140 and 160 mM, knowing that 1mM of NaCl = 58.44 ppm (Villarino and Mattson, 2011). *P. hybrida* cultures were transferred to the above mentioned NaCl prepared media concentrations (2 explants /jar), 20 replicates for each concentration, then regenerated shoots were transferred to jars containing½ MS + 1.0 mg/l of indole butyric acid (IBA) for root induction. After development of shoots and roots, regenerated plants were, gently, washed with tap water to remove agar from the roots and transplanted to small pots filled with moistened potting mix of peat moss, perlite and sand (1:1:1 v/v/v), then covered with clear polyethylene covers. After a week, these covers were perforated with small pin to allow gradual acclimatization of the plants. A week later, polyethylene covers were removed and pots were incubated in the greenhouse for another week, then they were transferred to the open field for normal growth and flowering.

Proline content was determined according to Bates *et al*. (1973) to test the ability of plants to tolerate salinity.

Obtained data for *P. hybrida* morphogenetic response in form of shoot number, shoot length, leaves number and root length were, statistically, analyzed as a factorial (two factors) experiment [Media protocols and explants] in a Randomized Complete Block Design (RCBD) with twenty replicates. Callus induction, Embryogenic callus were subjected to arcsine transformation and shoot number was subjected to square root transformation prior to statistical analysis according to Steel and Torrie (1980). Comparisons among means were made using the least significant difference test (L.S.D.), means of the same letters are not significant at 0.01 probability level, whereas, for the second experiment made to enhance petunia salt tolerance, the following model was used to analyze data obtained of the experiment:

$Yij = \mu + \alpha i + e ij$

Where Yij = the observation of the explant; μ = overall mean; α i = the effect of NaCl concentration, andeij = random error assumed to be independently and randomly distributed. F-test and least significant difference (L.S.D) procedures were available within the SAS software package (version 9.13, 2008).

Results and Discussion

***In vitro* Morphogenesis of *Petunia hybrida*:**

Establishing reliable *in vitro* plant regeneration protocol is a prerequisite step before conducting any *in vitro* selection experiment. In case of a particular plant species showed no competence for *in vitro* regeneration, the chances of regeneration from useful variant cell lines in the same species may also be unsuccessful (Barakat and El-Sammak; 2011 and Naglaa *et al.,* 2013). Once an organogenesis of tissue culture protocol has been established, the next step is to apply the developed protocol to *in vitro* selection studies. Having this as a principle, the present work was initiated with the aim of finding a tissue culture protocol competent to regenerate plant from cultured tissues of *Petunia hybrida*. However, callus induction response showed no significant difference with different media protocols, also no significant difference was found in case of tested different explants. But, the interaction between them showed significance, so that internodes (E_2) gave the highest callus induction (100 %) with MS + 0.5 mg/l BAP (M_2) and MS + 2.0 mg/l BAP (M_4) media protocols. On the other hand, the same explant showed a completely opposite trend with the lowest average of callus induction (70%) when cultured on MS + 0.4 mg/l BAP + 0.1 mg/l NAA (M_1) media protocol as mentioned in table (1).

Table 1: Means(*)of callus induction (%), embryogenic callus (%), callus weight (g) and shoot number influenced by four medium protocols, three explants and their interaction for *Petunia hybrida*.

Factor	Traits			
	Callus induction	Embryogenic callus	Callus weight	Shoot No.
	Medium protocols (M)			
M_1 (MS + 0.4 mg/l BAP + 0.1 mg/l NAA)	86.67 a	63.33 b	1.00 b	6.80 c
M_2 (MS + 0.5 mg/l BAP)	88.33 a	80.00 a	1.14 ab	28.70 a
M_3 (MS + 1.0 mg/l BAP)	85.00 a	63.33 b	1.35 a	8.77 c
M_4 (MS + 2.0 mg/l BAP)	85.00 a	86.67 a	1.02 b	14.97 b
	Explant (E)			
E_1 (Leaf base)	85.00 a	76.50 a	0.87 c	15.60 a
E_2 (Internodes)	86.25 a	77.63 a	1.13 b	17.58 a
E_3 (Cotyledon)	87.50 a	78.75 a	1.39 a	11.25 b
	Medium protocols (M) * Explant (E)			
M_1E_1	95.00 b	50.00 bc	0.85 de	9.50 cde
M_1E_2	70.00 f	45.00 c	0.69 ef	4.70 e
M_1E_3	95.00 b	95.00 a	1.47 ab	6.20 de
M_2E_1	85.00 c	90.00 a	1.09 cd	25.2 b
M_2E_2	100.0 a	60.00 bc	1.10bcd	37.0 a
M_3E_3	80.00 d	90.00 a	1.24 bc	23.9 b
M_3E_1	85.00 c	55.00 bc	1.16 bcd	13.7 c
M_3E_2	75.00 e	95.00 a	1.27 abc	8.00 de
M_3E_3	95.00 b	40.00 c	1.62 a	4.60 e
M_4E_1	75.00 e	75.00 ab	0.38 f	14.0 c
M_4E_2	100.0 a	90.00 a	1.45 abc	20.6b
M_4E_3	80.00 d	95.00 a	1.23 bc	10.3 cd

**Means of the same letters are not significant at 0.01 probability level while means of different letters are significant at 0.05 probability level.*

Respecting, embryogenic callus (%), data presented in table (1) clearly showed that there were no significant differences between the tested explants. On the other hand, MS + 0.5 mg/l BAP (M_2) and MS + 2.0 mg/l BAP (M_4) recorded the highest percentage of embryogenic callus obtained with an average of 80% and 86%, respectively. The interactions between medium protocol and explants was, also, highly significant. The cotyledon (E_3) gave the highest mean value of embryogenic callus (95 %) when cultured on either MS + 0.4 mg/l BAP + 0.1 mg/l NAA (M_1) or MS + 2.0 mg/l of BAP (M_4). The same explant showed an opposite trend and recorded the lowest mean value (40 %) when cultured on MS + 1.0 mg/l BAP (M_3).

Also, highest values for embryonic callus were obtained when internodes (E_2) were cultured on MS + 1.0 mg/l BAP (M_3) with an average percentage of (95%).

With regard to callus weight (g); results in table (1) showed that callus weight was, significantly, influenced by differences between the tested medium protocols, the used explants and the interaction between them. The used M_2 and M_3 medium protocols, led to the highest mean values of callus weight (1.14 and 1.35 g, respectively). It was evident from table (1) that the cotyledons (E_3) recorded the highest significant value of callus weight 1.39 g, because cotyledon explant, also known as storage leaves, is a highly active part of the seedling contains the most active substances (proteins, enzymes.. etc),; whereas, leaf bases gave the lowest mean value (0.87 g). The highest response of callus weight was obtained when cotyledon (E_3) was cultured on MS + 1.0 mg/l BAP (M_3) media with an average weight of 1.62 g/explant. On the other side, leaf base (E_1), brought about the lowest callus weight (0.38 g/explant) when cultured on MS+ 0.4 mg/l BAP + 0.1mg/l NAA (M_1).

Regarding shoot number; data presented in table (1) clearly showed that MS + 0.5 mg/l BAP (M_2) protocol used, resulted in the significantly highest value of shoot number 28.7, followed by (M_4). The least mean value of shoots number was produced on (M_3) and (M_1) with an average mean of 8.8 and 6.8. The superior explants which recorded the highest mean of shoot number, were recorded from the leaf base (E_1) and internode (E_2) of 15.6 and 17.58, each in turn, with no significant difference between them, followed by cotyledons (E_3). The interaction between the used medium protocols and tested explants showed that internode (E_2) resulted in the highest mean of shoot number (37.0) when cultured on MS + 0.5 mg/l BAP (M_2). In an opposite trend, the internode (E_2) recorded the lowest mean value of shoot number (4.7) when cultured on MS + 0.4 mg/l BAP + 0.1 mg/l NAA (M_1), as well as cotyledons (E_3), when cultured on (M_3) medium protocol it giving an average mean of (4.6).

***In vitro* selection:**

According to the previous results; internodes (E_2) and MS medium supplemented with 0.5 mg/l BAP (M_2) were used to examine the effect of NaCl concentrations; because they gave the highest number of shoots from the previous mentioned experiment. NaCl was added to the medium in the concentrations (0, 20, 40, 60, 80, 100, and 120, 140 and 160 mM). Results of shoot number, shoot length, leaf number, root length and proline content are shown in table (2).

As for morphogenetic response; data presented in table (2) and figure (1) showed that, there was no significant difference between the control treatment (0.0 mM) and 20 mM NaCl respectively in shoot number, shoot length and leaf number. On the contrary, with the rest of the concentrations, these characters were, significantly, in an inverse proportionate; as, character decreased, significantly, when NaCl concentrations viz. 40, 60 and 80 mM, increased in the above - mentioned medium (M_2). Root length was, significantly, affected when cultured on different concentrations of NaCl.

Table 2: *In vitro* morphogenetic response and proline content of *Petunia hybrida* towards different concentrations of NaCl.

NaCl concentration	Morphogenetic response				Proline content (µml)
	Shoot No.	Shoot length (cm)	Leaf No.	Root length (cm)	
0 mM	34.25 a	3.18 a	29.95 a	1.61 a	1.3955 e
20 mM	28.30 a	2.59 a	26.80 a	0.85 b	2.2969d
40 mM	13.95 b	2.50 b	16.10 b	0.56 c	3.8571 c
60 mM	5.30 c	1.21 c	9.90 c	0.34 cd	5.7279 b
80 mM	1.55 d	0.52 d	3.30 d	0.27 d	6.2147 a

**Means of the same letters are not significant at 0.01 probability level while means of different letters are significant at 0.05 probability level.*

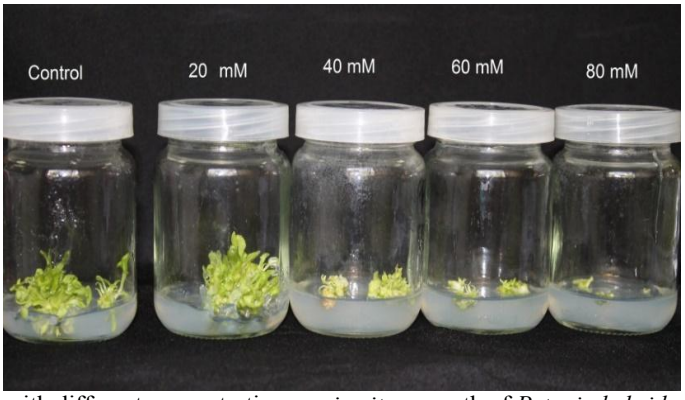

Fig. 1: Effect of NaCl with different concentrations on *in vitro* growth of *Petunia hybrida.*

In total, it was obvious that all morphogenetic characters were inversely proportional to the salt concentration. Results obtained after adding NaCl to the media showed that concentrations above 80 mM of NaCl were lethal to *P. hybrida.*

As for proline content; results of table (2) showed that, proline content was in a direct proportional to the NaCl concentration and increased approximately 6 folds at the last concentration (80 mM) when compared to the control treatment. Figure (2) illustrates the relation between root length (cm) and proline content (µml). Root length was in inverse proportionate with the salt concentration, while the proline was directly proportional to the salt concentration, however, the obtained results are in agreement with those of Shin *et al.* (2000) and Kutis *et al.* (2009) who stated that proline has shown to be accumulated in a number of plant tissues in response to salt stress. Also, Harinasut *et al.* (2000) reported that when plants are subjected to high salt environment, they maintain their water content by accumulation of compatible organic solutes, such as proline, in their cytoplasm. These organic solutes act as osmoprotectants in response to abiotic stresses, such as increased salinity. Furthermore, proline accumulation under salt stress has been reported and suggested to be a biochemical marker for increased salt tolerance in plant species such as potato (Martinez *et al.,* 1996), mulberry (Harinasut *et al.,* 2000), acacia (Yokota, 2003), and sugarcane (Gandonou *et al.,* 2006).

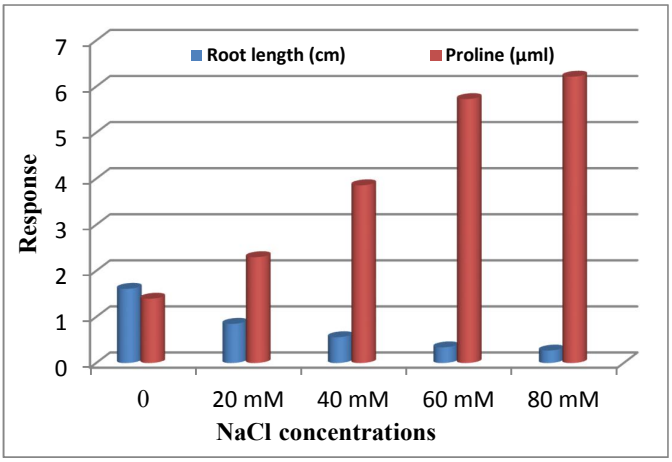

Fig. 2: Effect of NaCl concentration on root length and proline content of *Petunia hybrida*

In conclusion, the best *in vitro* propagation protocol of *Petunia hybrid* can could be done by culturing internodes (E_2) on MS + 0.5 mg/l BAP (M_2). Since it showed good tolerance towards salinity up to 80 mM via inducing 6 folds more of proline than the control treatment. It is recommended to plant *Petunia hybrid* as an ornamental plant in coastal areas using ground water, that has a high content of NaCl, as a source of irrigation and by that we are preserving more water that can be used elsewhere.

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