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Original Article

Induced Mutations through EMS Treatment and *In Vitro* Screening for Salt Tolerance Plant of *Petunia* × *atkinsiana* D. Don

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Abstract

In vitro culture is a practical plant breeding tool in developing plant resistant to different abiotic stresses such as salinity. In this study, the focus is on inducing mutations for salt tolerance using ethyl methane sulphonate (EMS) in calli of petunia (*Petunia* × *atkinsiana* D. Don 'Prism Red'), followed by cell line selection and subsequent plant regeneration. Callus cultures were initiated from leaf fragments soaked in mutagen solution in two concentrations 0.5 and 5.0 mM for 60, 120 and 180 min, respectively. Then, the callus was rinsed three times in deionized, sterile water and was transferred onto embryo formation medium Murashige and Skoog (MS) supplemented with 2.0 mg l^{-1} 1-napthaleneaceticacid (NAA) and 5.0 mg l^{-1} (6-benzyloaminopurine) BAP. After 28 days, somatic embryos were transferred onto MS medium supplemented with 4.0 mg l^{-1} BAP for regeneration. Results of our studies showed that the callus immersion in the 5.0 mM EMS solution caused damage to the cells, and indicated by the change in callus colour into brown. The plants regenerated from the somatic embryos induced from callus treated with 0.5 mM solution of mutagen for 60 and 120 min were determined as M1 and M2 mutant variants, respectively. After the multiplication of the obtained plants, their tolerance to salinity stress was determined. The obtained results have showed that the M1 and M2 mutant lines were characterized by elevated tolerance to the stress factor in comparison to non-mutated plants.

Keywords: chemical mutagen, micro propagation, salinity stress

Introduction

The genus *Petunia* is a plant of high economic importance in world-wide horticulture and provides a model plant for studying plant development, due to various favourable biological features and the availability of technical tools for genomic, biochemical, cytogenetic and functional analyses (Berenschot *et al*., 2008).

Salt solution is one of the abiotic factors that influences growth and vigor of many crops. However, a negative influence of salinity not only reduces horticultural production with the concomitant increase of its costs, but it also causes soil erosion and disturbs the ecological balance (Manchanda and Garg, 2008; Piwowarczyk *et al*., 2016). High concentration of salt in the environment may cause stress in plants in two ways: by the increase in osmotic potential of the rooting medium as a result of high solute content, and by the toxic effect of high concentration of ions (Demir and Kocaçalişkan, 2002). The first way causes inhibition of the capability to absorb water from the substrate, contributing to the inhibition of growth of the plant subject to salinity stress. The second way is a long-lasting process, stemming from the disturbance of the water-mineral balance of plants, via accumulation of high concentrations of Na^+ and Cl^- ions in the cells (Khalid *et al*., 2015).

Plants show a wide spectrum of response to salinity but all have their growth or/and field reduced by salt. Thus, the effect of salinity constitutes a result of many reactions that take place in the processes of morphological, physiological and biochemical changes within the plant (Sabir *et al*., 2012). Plants subject to salt stress are characterized by a clear decrease of plant tissue dry weight, increase of the root length to stem length ratio, a decrease of the area of leaves, eventually leading to yield reduction (Manchanda and Garg, 2008; Khalid *et al*., 2015; Piwowarczyk *et al*., 2016). Moreover, a high concentration of salts in the substrate influences the basic metabolic processes of

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the plant, such as photosynthesis or lipid metabolism (Agami, 2014). Large amount of salt can be accumulated in chloroplasts and cause damage of photosynthetic pigments, lowering leaf area or by decreasing the activity of photosynthetic enzymes (Nandy *et al*., 2007; Khalid *et al*., 2015; Piwowarczyk *et al*., 2016). In addition, salinity has a negative effect on cell membranes, causing lipid peroxidation, which, as a consequence, leads to malondialdehyde (MDA) accumulation (Khalid *et al*., 2015).

The understanding of the mechanisms enabling the tolerance of salt stress at the level of the entire organism and at molecular level is the key element, which would enable the improvement of tolerance to salinity of many crops (Piwowarczyk *et al*., 2016). Proper selection of tolerant genotypes from cultivated plants depends on the availability of verified assessment methods. Conventional breeding by hybridization or cross-pollination usually takes longer, yet sometimes also carries unexpected traits. This may result in an output that is not good as expected as a commercial cultivar (Rai *et al*., 2010; Gaswanto *et al*., 2016). Mutation is one of possible alternatives to conventional breeding for crop improvement program (López Colomba *et al*., 2013; Gaswanto *et al*., 2016; Oladosu *et al*., 2016; Purnamaningsih and Hutami, 2016). Genetic variability as a result of induced mutations by various mutagens has contributed to plant breeding efforts. It has played a major role, along with recombinant and transgenetic breeding, in the development of superior plant varieties demonstrating economic value of the mutation breeding technology. Mutagenesis is the process that causes changes in the genetic material via the use of chemical, physical or biological agents (Oladosu *et al*., 2016). The most commonly used chemical mutagens include ethyl methane sulfonate (EMS), which is characterized by causing the highest frequency of genetic changes and lowest frequency of chromosome aberrations in plant (van Harten, 1998). The chances of a mutation depend on the number, age and the growth stages of plant which are used as plant material (explant). The mutation frequency can be increased via the use of callus tissue as the starting material (Shalaby and El-Banna, 2013; Oladosu *et al*., 2016; Purnamaningsih and Hutami, 2016). Callus consists of a shapeless mass of non-differentiated and rapidly dividing cells (Purnamaningsih and Hutami, 2016). Moreover, according to Patade and Suprasanna (2008), genetic instability and somaclonal variation of callus tissue might considerably increases the chances of a mutation.

The evaluation of salt tolerance in tissue culture may be more useful for breeding programs, because selection is earlier and faster in tissue culture than in the field (Sabir *et al*., 2012). However, the determination of the proper dose of EMS mutagen is necessary to obtain useful mutants, without the excessive cell destruction and/or inhibition of embryo germination, with concomitant maintaining of regeneration. The objective of the present study was to obtain salt tolerant genotypes via application of EMS on calli of petunia followed by *in vitro* plant regeneration.

Materials and Methods

Biological material

The research was conducted in a Department of Plant Genetics, Breeding and Biotechnology, West Pomeranian University of Technology in Szczecin (Poland). The plant of petunia (*Petunia* × *atkinsiana* D. Don) cultivar 'Prism Red' was used in the present study. Auxiliary buds from stabilized sterile *in vitro* culture were collected. Explants were cultured on plant growth regulators free MS (Murashige and Skoog, 1962) medium. After 4 weeks of culture, leaf segments were collected and cut into small pieces of about 10 × 10 mm.

Experimental procedures

For callus induction MS medium were supplemented with 5.0 mg l^{-1} BAP (6-benzyloaminopurine) and 3.0 mg l^{-1} IAA (indole–3 acetic acid). Callus culture was incubated in flask containing 20 ml medium. After 30 days, calli were cut into 2-3 mm^3 clumps and soaked in solution of EMS in concentration 0.5 and 5.0 mM for 1, 2 and 3 h. There were 40 clumps for each dose. After EMS treatment, the clumps were rinse tree times in sterile distillate water and cultured on MS medium without the addition of plant growth regulators. In preliminary experiment showed that long time of subculture reduced the regeneration rate, so calli culture were subcultured 4 times (21 days each). Percentage of live callus and callus colour were observed. The survived callus were regarded as mutant cell lines and transferred onto somatic embryo formation MS medium supplemented with 2.0 mg l^{-1} NAA (1-napthaleneaceticacid) and 5.0 mg l^{-1} BAP. The selection of plant growth regulators was used on the basis of preliminary studies. After 4 weeks, the formed somatic embryos were transferred to regenerative MS medium supplied with 4.0 mg l^{-1} BAP. Thus regenerated plants were propagated 4 times on the MS medium without the addition of plant growth regulators.

In order to evaluate the level of tolerance of the obtained mutants to salinity stress, 3 combinations of media with different NaCl content (50, 100 and 150 mM) were used. Petunia plants regenerated from callus and not treat of the EMS constituted the control. Two cm fragments of stems of the obtained mutants and control plants were placed on the proper media.

All cultures were incubated in growth room at the temperature of 25 °C under 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1}. All the media were contained with 8 g l^{-1} agar (Biocorp, Poland), 30 g l^{-1} sucrose and 100 mg l^{-1} myo-inositol, pH was adjusted to 5.7. The media were heated and 30 ml were poured into 450 ml flask and next they were autoclaved at 121 °C (0.1 MPa) during the time required according to the volume of medium in the vessel.

After 35 days explants were removed and washed with deionized distilled water, and the length of the shoots and roots (cm), and the number of roots were measured. The plants were weighed for estimation of plant fresh mass (g) and were dried at 70 °C for 48 h. The dried plants were weighed to establish a plant dry mass (g).

Estimation of electrical conductivity

Leaf discs of 1 cm^2 were washed with distilled deionized water and placing in a test tube containing 10 ml distilled water and incubated at 25 °C on shaker (100 rpm) for 24 h. At the end of incubation the electrolytic conductivity I (EC I) of bathing solution was recorded (Electrical Conductivity Meter). The samples were autoclaved at 121 °C for 19 min to completely decompose the tissues and release electrolytes. EC II was recorded after cooling the solution to room temperature. Electrical conductivity was measured using the formula:

$$EC(\%) = (EC\ I/EC\ II) \times 100$$

Determination of proline and MDA content

The concentration of free proline in petunia plants has been measured in three replications. The proline accumulation was determined according to Bates (1973). Content of the malondialdehyde (MDA) in plant tissue was determined by the method described by Sudhakar *et al*. (2001).

Determination of pigments

The levels of chlorophyll *a*, *b* and carotenoid (Car) were measured in 80 % (v/v) acetone extracts according to Arnon *et al*. (1956) in modification to Lichtenthaler and Wellburn (1983). The concentration of Chl *a* and Chl *b* were calculated from equations derived by Hendry and Grime (1993).

Chlorophyll *a* (mg g^{-1} fw) = [(12.7 A_{663} – 2.69 A_{645})/ 1,000 × fw] ×V

Chlorophyll *b* (mg g^{-1} fw) = [(22.9 A_{645} – 4.68 A_{663})/ 1,000 × fw] ×V

Carotenoid content was determined by the equation of Price and Henry (1991):

Carotenoid (mg g^{-1} fw)=[(A_{480} + 0.114 A_{663}) – (0.638 A_{663}) ×V/112.5 × fw]

where V is volume of the sample (ml), A is absorbance and fw is fresh weight (g).

Leaf pigmentation

The leaf pigmentation measurement was carried out using spectrophotometer CM-700d (Konica Minolta, Japan), in glass cuvettes of 1 mm optical length. Measurements were made in CIE L*a*b* system, in which L* stands for white (100) and black colour (0), a* - green (–100) and red colour (+100), b* - blue (–100) and yellow colour (+100) (Hunterlab 2012). The 10° observer type and D65 illuminant was applied. Colour was measured in triplicates for each experimental combination.

Statistical analysis

Results obtained for plants in *in vitro* cultures were statistically analysed. The significance of differences was determined by means of variance analysis (ANOVA) and Tukey's test, at the level of significance of $\alpha = 0.05$. Homogenous groups between analysed combinations were labelled with successive letters of alphabet.

Results and Discussion

Effect of EMS on callus tissue

Callus, which was soaked in EMS solution, showed different growth responses depending on the time of immersion and concentration used. Moreover, the treated explants showed considerable differences in callus coloration (Fig. 1). In general, it was determined, that the callus tissue viability decreased with the increase of treatment time and mutagen concentration (Table 1).

The highest percentage (100%) and the highest weight (2.47 g) of regenerated callus were observed on the control medium (without EMS). The use of 5.0 mM EMS, independent of the treatment time, caused death of the entire callus tissue. 50 and 33.3% of calli treated by 0.5 mM EMS for 1 and 2 h respectively were viable, so these ones were selected for the subsequent experiment. However, 0.5 mM concentration for 3 h was highly toxic and gave adverse effect of calli proliferation. Callus of putative mutants of petunia which were able to withstand mutagenic treatment could develop shoot after being transferred onto the somatic embryo formation medium. The highest mean number of germinated embryos (2.1) was formed on the callus tissue on the control medium. However, it was observed, that with the increase of time of treatment of 0.5 mM EMS solution the number of germinated somatic embryos decreased (Table 2). From the germinated somatic embryos, a somaclonal plant population was obtained, originating from the callus tissue treated with 0.5 mM EMS for 1 h (M1) and 0.5 mM EMS for 2 h (M2). Svetleva and Crinó (2005) treating callus of common bean with EMS and NEU (N-nitrose-N′-ethyl urea) mutagens observed, that in both cases mutagen application of 90 min caused two relevant effect, either browning or callus growth inhibition. For the study conducted by Koch *et al*. (2012) a significant reduction in sugarcane plantlet production was observed at 32.2 to 96.6 mM EMS. However, Revathi and Arumugam Pillai (2015) noticed that the lowest rice callus regeneration frequency was observed less than 120 min of exposure to 0.5% EMS. Purnamaningsih and Hutami (2016) using the EMS mutagen onto the sugarcane callus tissue determined, that the variability of the callus decreased with the increase of the mutagen concentration (0.1 to 0.5%) and time of its effect (30 to 120 min). According to many authors (Bhagwat and Duncan 1998; Koh and Davies 2001; Latado *et al*., 2004; Svetleva and Crinó, 2005; Gadakh *et al*., 2015; Revathi and Arumugam Pillai, 2015) the use of 0.5% EMS solution is sufficient to obtain visible changes resulting from mutation. Koch *et al*. (2012) suggest that in order to increase the frequency of desired mutations, the mutagen concentration, in the range from 8 to 40 mM, should be used, and its exposure time should be 3 h. However, response of the callus tissue to the effect of EMS is variable and it depends on plant genotype, and the concentration of mutagen. Induced chemical mutation can be successfully employed not only for early maturity (Gadakh *et al*., 2015; Oladosu *et al*., 2016), but also for induction of salt tolerance in sweet potato (Luan *et al*., 2007), rice (Revathi and Arumugam Pillai, 2015); low pH media and Al tolerance in sugarcane (Purnamaningsih and Hutami, 2016), and herbicide tolerance in sugarcane (Koch *et al*., 2012).

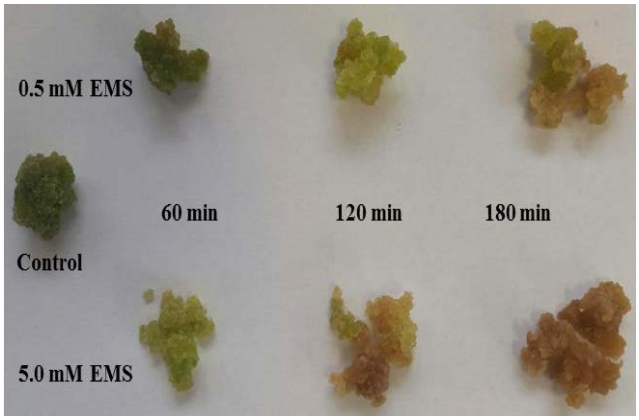

Fig. 1. Callus culture of *Petunia* × *atkinsiana* D. Don on MS medium after 0.5 and 5.0 mM EMS treatment in different time (60, 120 and 180 min)

Table 1. The percent (%), weight (g) and colour of petunia's callus after immersion in 0.5 and 5.0 mM EMS solution

Time of immersion in EMS solution	%	Callus weight (g)		Colour of callus
Without immersion	100	2.47	a	green
60 min 0.5mM	50	1.75	b	green
120 min 0.5 mM	33.3	1.36	b	light green
180 min 0.5 mM	10	0.75	c	light brown
60 min 5.0 mM	0	0	d	brown
120 min 5.0 mM	0	0	d	brown
180 min 5.0 mM	0	0	d	brown
$LSD_{\alpha 0.05}$=0.48				

Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

Table 2. Number of germinated somatic embryo on MS medium after callus immersion in 0.5 and 5.0 mM EMS solution

Time of immersion in EMS solution	No. of germinated somatic embryo		% control
Without immersion	2.1	a*	100
60 min 0.5mM	0.42	b	20
120 min 0.5 mM	0.21	c	10
180 min 0.5 mM	0.1	cd	5
60 min 5.0 mM	0	d	0
120 min 5.0 mM	0	d	0
180 min 5.0 mM	0	d	0
$LSD_{\alpha 0.05}$ = 0.20			

Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

Table 3. Effect of NaCl stress on plant height (cm), root length (cm) and root number in *Petunia* × *atkinsiana* D. Don control and variants (M1, M2)

NaCl concentration (mM)	Plantlet	Shoot length (cm)		Root length (cm)		No of roots	
	Control	2.12	d*	5.93	a	4.0	b
50	M1	5.50	a	5.62	a	4.0	b
	M2	4.40	b	5.34	a	5.75	a
Mean		4.02	a	5.64	a	4.58	a
	Control	2.0	d	0	a	0	c
100	M1	4.38	b	2.56	c	3.75	b
	M2	3.50	c	1.63	b	0.75	c
Mean		3.29	ab	1.39	b	1.5	b
	Control	1.81	d	0	c	0	c
150	M1	2.12	d	0	c	0	c
	M2	2.06	d	0	c	0	c
Mean		2.0	b	0	c	0	b
		LSD_{I}=0.66		LSD_{I}=1.21		LSD_{I}=1.38	
		LSD_{II}=1.07		LSD_{II}=1.98		LSD_{II}=2.25	

Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

Effect of salt stress on plant growth

The population of petunia somaclones obtained in our study was tested on selective media with different NaCl content (50, 100 and 150 mM, respectively). The influence of salinity stress on plant growth is often determined from stem and root length measurements (Piwowarczyk *et al*., 2016). According to Queirós *et al*. (2007) low growth coefficient may suggest the occurrence of adaptive processes for stress conditions within the plant. Another assessment point of the salinity stress effect on the plants should be evaluation of plants dry weight (Munns, 2005; Khalid *et al*., 2015). Manchanda and Garg (2008) found, that the increase of salinity tolerance is strictly related to the growth or unchanged dry weight content in plant under salinity stress. From the results of own study it was determined, that the addition of 150 mM NaCl to the medium showed highly inhibitory influence on morphological parameters of all petunia plants (Table 3). However, variants M1 and M2 line at each stress level had higher shoot and root length, fresh weight and plant water content then control (Tables 3, 4). Phenotypic observations have shown that control plants were more exposed to the negative effect of salinity stress than mutants, which is expressed by the lack of roots formed after the treatment with 100 mM solution of NaCl. Moreover, with the increase of salt concentration on the MS medium, the value of fresh and dry weight content in plants decreased (Table 4). In this case, the percentage of water content in the plant on the media with 100 and 150 mM NaCl was at similar level (Table 4). The addition of NaCl to the medium also had also a clear effect on electric conductivity (EC) in the plants (Table 4). It was observed, that with the increase of ion concentration the EC index was higher. Hence, control petunia plants were characterized by a higher EC index (44.4-60.0%) in comparison to the introduced variants M1 (58.5-70.1%) and M2 (59.4-74.06%). This was probably influenced by the higher growth rate of these explants in the initial phase of the culture in comparison to the remaining plants.

Effect of salt stress on biochemical parameters

The capability of the plants to grow under the salinity stress depends to a large extent on the proper functioning of the osmotic balance. In plants exposed to salinity stress such mechanism might be manifested by the accumulation of inorganic ions or organic compounds. In opinion of many authors (Demir and Kocaçalişkan, 2002; Bybordi, 2012; Smolik *et al*., 2013; Krupa-Małkiewicz *et al*., 2015) elevated proline and MDA level in plant tissues is quite good indicator of a negative effects of various stress factors to a plant. Excessive proline accumulation occurs at a strong action of environmental stress, which results from its uncontrolled biosynthesis, limited oxidation, inhibition of its incorporation into proteins, and even release from proteins due to proteolysis (Girija *et al*., 2002). Many studies demonstrate that plants tolerant to salinity accumulate more proline, than sensitive plants. According to Abdelhamid *et al*. (2013) MDA is one of the end products that are produced as a result of lipid peroxidation damage by free radical. These conclusions are confirmed with the results of this study, where in all studied plants grown on the medium supplied with 150 mM NaCl, a considerable increase of proline concentration and slight decrease of MDA concentration were observed (Table 5).

Table 4. Effect of NaCl stress on fresh (g) and dry weight (g) of plants, plant water content (%) and electric conductive (%) in *Petunia ×atkinsiana* D. Don control and variants (M1, M2)

NaCl concentration (mM)	Plantlet	Plant fresh weight (g)		Plant dry weight (g)		Plant water content (%)	EC (%)
	Control	2.18	a*	0.13	a	94.03	44.4
50	M1	1.20	b	0.12	ab	90.0	58.5
	M2	1.24	b	0.09	b	92.74	59.4
Mean		1.54	a	0.11	a	92.26	54.1
	Control	1.57	a	0.09	a	94.26	54.6
100	M1	0.34	b	0.04	b	88.23	61.48
	M2	0.58	b	0.06	ab	89.65	66.9
Mean		0.83	ab	0.06	a	90.71	57.66
	Control	0.19	a	0.07	a	63.16	60.0
150	M1	0.54	a	0.05	ab	90.74	70.1
	M2	0.28	a	0.03	b	89.28	74.06
Mean		0.34	b	0.05	a	81.06	61.38
		LSD_I=0.66 LSD_{II}=1.07		LSD_I=0.66 LSD_{II}=1.07			

*Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

Table 5. Effect of NaCl stress on proline and MDA concentration in *Petunia* × *atkinsiana* D. Don control and variants (M1, M2)

NaCl concentration (mM)	Plantlet	Proline (µmol g^{-1} fw)		MDA (nmol g^{-1} fw)	
	Control	5.88	b*	13.79	a
50	M1	10.95	a	13.22	a
	M2	10.77	a	12.89	a
Mean		9.20	c	11.79	a
	Control	10.57	b	13.62	a
100	M1	17.09	a	12.81	a
	M2	17.03	a	12.81	a
Mean		14.89	b	13.08	a
	Control	9.65	c	9.27	a
150	M1	43.22	a	10.64	a
	M2	38.50	b	10.88	a
Mean		30.46	a	11.77	a
		LSD_I=3.71 LSD_{II}=3.04		LSD_I=2.05 LSD_{II}=1.66	

*Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

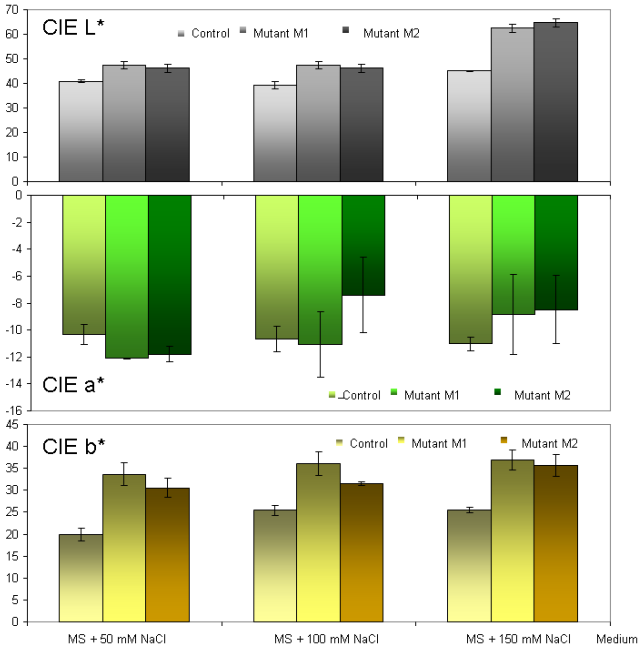

Fig. 2. Effect of NaCl stress on leaves colour space using CIE L*a*b* system, L* the lightness coefficient, a* (green colour) and b* (yellow colour) in *Petunia* × *atkinsiana* D. Don control (C) and mutants (M1, M2)

According to many authors (Demir and Kocaçalişkan, 2002; Bybordi, 2012; Krupa-Małkiewicz *et al*., 2015; Piwowarczyk *et al*., 2016) salinity stress has a negative effect on the photosynthetic pigment content, which constitutes an effect of photosynthetic apparatus degradation. In presented studies, salinity stress induced by 50 mM NaCl decrease the contents of chlorophylls in M1 and M2 variants (Chl *a* by 25 and 18%, respectively; Chl *b* by 18 and 22%, respectively) and increase carotenoid content (by 19 and 63% respectively), in comparison to the control plants (Table 6). In addition, it was observed that increase of NaCl concentration in MS medium caused decrease in Chl *a* and Car concentrations in all analyzed petunia genotypes (control, M1 and M2). However, the injurious effect of 100 and 150 mM salinity was more striking in control than M1 and M2 variants.

Content of photosynthetic pigments in petunia 'Prism Red' leaves is closely correlated to their colour (Fig. 2). Although leaves of petunia from the medium supplemented with 50 and 100 mM NaCl salt were at the same level of darkness and green colour. It is evidence by the value of L* and a* parameters (Fig. 2). It was observed that increasing in the medium concentration of NaCl to 150 mM, the value of L* parameter was higher in comparison to control by 13.5 and 17.6% (M1 and M2, respectively). Moreover, all petunia plants tested from the medium supplied with 150 mM NaCl were characterized by lighter leaf coloration in comparison to the remaining experimental combinations. This is showed by the value of the b* parameter, which has a clear increase tendency (Fig. 2).

Conclusions

The conducted review has shown that some man-made and disturbed habitats host many rare orchids and could provide a chance for survival of those rare and valuable plant species. The need for the protection of sites of valuable orchid species in habitats strongly transformed by man should be taken into consideration at the present time.

Table 6. Effect of NaCl stress on chlorophyll *a*, *b* (Chl *a*, Chl *b*) and carotenoid (Car) concentration in *Petunia* × *atkinsiana* D. Don control and variants (M1, M2)

NaCl concentration (mM)	Plantlet	Chl *a* (mg g^{-1} fw)		Chl *b* (mg g^{-1} fw)		Car (mg g^{-1} fw)	
50	Control	94.41	a*	14.93	a	3.57	b
	M1	70.91	c	12.24	a	4.25	b
	M2	77.56	b	11.69	a	5.81	a
Mean		80.96	a	12.95	a	4.54	a
100	Control	54.69	b	11.16	b	2.16	a
	M1	61.96	a	13.61	b	3.37	a
	M2	63.40	a	18.23	a	3.58	a
Mean		60.02	b	14.33	a	3.03	b
150	Control	37.32	a	7.39	b	0.51	a
	M1	36.92	a	12.11	a	0.86	a
	M2	38.97	a	15.39	a	0.76	a
Mean		37.74	c	11.63	a	0.71	c
		LSD_I=6.59 LSD_{II}=5.37		LSD_I =4.5 LSD_{II}=3.67		LSD_I=1.02 LSD_{II}=0.87	

*Means in the same column followed by the same letter are not significantly different (α<0.05; Least Significant Differences test LSD)

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