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In-vitro PHYSIOCHEMICAL RESPONSES OF *Viola odorata* PLANT TO COMBINED SALT AND DROUGHT STRESS

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ABSTRACT

Nowadays sweet violet (*Viola odorata*) is an ornamental-medical plant that considered as endangered and threatened species. On the other hand, biotic and abiotic stresses impose a major threat to agriculture. Here, we investigated the effects of salinity and drought stresses, based on polyethylene glycol (PEG; 1, 1.5, 2, 2.5, 3 and 4%) and NaCl (0, 50, 100 and 150 mM), on growth characteristics, physiological parameters and antioxidant defense system of sweet violet under *in-vitro* conditions. The influences of NaCl and PEG gradients in the culture media on plant height, green leaf percentage, root dry weight (DW), and electrolyte leakage (EL) was described by a linear or quadratic model. All measured parameters (except EL) decreased when NaCl or PEG concentration increased. In contrast, EL increased other traits. Moreover, with increasing in salinity and drought severity, shoot DW decreased, while antioxidant enzymes activity such as catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) and proline content increased. However, total soluble carbohydrates (TSC), at all drought levels, increased with increasing NaCl concentration up to 50 or 100 mM, and then decreased. Most variations in the shoot DW, CAT activity, proline and TSC contents due to salt stress occurred at low concentration of PEG. Overall, our findings highlight that the effect of combined drought and salt stress was more severe. However, the sensitivity of the plant to drought or salinity stress was higher in the absence of other stress.

Key words: biotic stress, antioxidant enzymes activity, NaCl, osmotic stress, PEG

INTRODUCTION

External stresses such as temperature, drought, and salinity influence plant yield and plant react to abiotic stresses through a range of variations at the molecular level (gene expression) which result in physiological changes [Mantri et al. 2012]. In many areas of the world environmental streses such as salinity and drought severely affect plant yield and quality which threaten the security of food and become more critical with the existence of worries about global climate change. Hence, today lots of researches are going on abiotic stress resistance globally [Niu et al. 2012]. As we know, all of these stresses induced oxidative stress as a result of excessive production of reactive oxygen species (ROS), as well as their own specific effects [Rao et al. 2006] which damaged cellular macromolecules, such as proteins, lipids, carbohydrates and DNA [Gill and Tuteja 2010]. Plants utilize both enzymatic and nonenzymatic defense systems to scavenge and detoxify ROS [Mittler 2002]. The balance between ROS production and activities of antioxidative enzyme determines whether oxidative signaling and/or damage will occur [Bian and Jiang 2009].

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For the adaptation to stress conditions, plant reacts with accumulation of compatible solute as a biochemical strategies to limit the absorption of salt and adjust their osmotic pressure. Proline is one of the most investigated compatible solutes which protect plants from osmotic stress [Rai et al. 2011]. This amino acid is accumulated by plants during different abiotic stresses and plays adaptive roles in stress tolerance mechanisms [Verbruggen and Hermans 2008]. It is a compatible solute (an osmotically active agent) which able to retain water within the cells and protects membranes and proteins under severe osmotic stress [Janska et al. 2010]. Level of accumulated proline could be a basis for selection of water stress-tolerant plants. For this reason in many studies, increasing the proline content in plants under stress condition were identified as tolerant to drought or salinity [Hussain et al. 2011, Piwowarczyk et al. 2014].

Because of difficulties in controlling environmental conditions in field, plant tissue culture techniques are used for stress investigations which provide numerous opportunities for researcher to investigate the unique and complicated responses shown by plants in confronting with environmental stresses [El-Missiry 2012]. To induce stress conditions in plant under *in vitro* cultures, in many researches, NaCl and PEG has been used in many plants as well as ornamentals [Hossain et al. 2007].

Viola odorata (sweet violet) belonging to family Violaceae is an evergreen perennial herb and native to Europe, North Africa and Western Asia (Iran) [Lim 2014]. Sweet violet is a medicinal plant which leaves are known as good traditional remedy in bronchitis, mucus, coughs, asthma and cancer of the breast, stomach, lungs and digestive system [Bown 1995]. The ruinous harvesting of medicinal plants for the production of medicines causes severe decrease in plant resources and get endangered all over the world [Naeem et al. 2013]. There is a general feeling that the populations of sweet violet are decreasing at an alarming rate [Kaloo et al. 2013]. So, conservation of the plant diversity heritage, especially the rare and native medicinal plants for future generations is very important [Mokhtari 2015]. Due to medicinal applications, landscape using and not being cultivated commercially, the germplasm of sweet violet should be preserved in natural habitats. So, with consideration of the increasing human population and global climate changes, water deficit is getting more and more serious. Hence, finding and cultivation of some species which are able to tolerate abiotic stresses while maintaining high productivity could be a solution to this problem.

With respect to important of sweet violet as a medicinal and valuable plant and because of low information regarding its physiological reactions under environmental stress conditions, the present study will be helpful in our knowledge of this plant resistance mechanisms to drought and salinity stress. Therefore, this study aims to evaluate the effect of salinity and drought and their combination, based on PEG and NaCl treatments, on some growth, physiological and biochemical parameters as well as the evaluation of the resistance level of sweet violet plant to stress.

MATERIALS AND METHODS

Plant material and growth conditions. Wild plants of sweet violet were collected as source of explants from plants grown in the forest areas in the north part of Iran. Plantlets regenerated from petiole callus of sweet violet placed under stress treatments for 30 days.

Murashige and Skoog [1962] medium (MS) was used as a basal medium supplemented with 3% (w/v) sucrose and 0.8% agar. The experiment was performed in culture glass (90 × 60 mm) which contained 25–30 ml of culture medium. The growth room temperature was kept at 25 ±1°C under a 16 h photoperiod with a light intensity of 3000 lux supplemented with white fluorescent tubes and relative humidity of 65–70%.

NaCl and PEG treatment of regenerated plants. Regenerated plantlets (1–1.5 cm in length) were treated in MS medium supplemented with various concentrations of NaCl (0, 50, 100 and 150 mM) and polyethylene glycol (PEG; 1, 1.5, 2, 2.5, 3 and 4%). experiment was done with three replicates (culture containers) and each replication (container) including three seedlings. It means that each treatment was consist of 9 explants. The treated plant samples were analyzed after 30 days.

Measurement of Growth Parameters. Plants were removed from culture media and plant height was measured by a ruler. The percentage of green leaves was also calculated by ratio of green leaves to total leaves and expressed as percent. Plants were separated

into shoot and root samples and dried in an oven at 72°C for 48 h and then their dry weights (DW) were recorded.

Electrolyte leakage (EL). Electrolyte leakage measurement was carried out as presented in Shi et al. [2012].

Antioxidant enzyme assays

Fresh leaf/petiole tissue (250 mg) was homogenized using chilled mortar and pestle in 1 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, then kept in ice bath. The homogenate was centrifuged at 15,000 × *g* at 4°C for 15 min. Clear supernatant was used for enzyme assays.

Catalase (CAT). To measure CAT, the method described by Chandlee and Scandalios [1984] was used. The enzyme activity was presented in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Peroxidase (POX). The guaiacol oxidation method [Kochba et al. 1977] was used to POX activity evaluation. A unit of peroxidase activity was presented as the alteration in absorbance per minute and specific activity as enzyme units per mg soluble protein.

Superoxide dismutase (SOD). According to Beyer and Fridovich [1987] protocol, SOD activity was determined by nitro blue tetrazolium (NBT) photochemical assay. The absorbance of solution was recorded at 560 nm. SOD activity was presented as U (unit) mg^{-1} protein.

Hydrogen peroxide content (H_2O_2). The H_2O_2 content was specified according to Velikova et al. [2000]. The absorbance of the supernatant was determined at 390 nm in a spectrophotometer (T92+, United Kingdom).

Free proline content. Proline content was determined spectrophotometrically with the use of the method of Bates et al. [1973]. Absorbance of chromophore which contained toluene was measured at 520 nm. Proline content was shown as μmol g^{-1} DW.

Total Soluble Carbohydrates (TSC). A phenol-sulfuric acid procedure [Dubois et al. 1956] was used in order to measure TSC. The absorption was then determined by spectrophotometry at 490 nm.

Statistical analyses. The present experiment was carried out based on a completely randomized design (CRD). The data for all parameters were examined with the analysis of variance procedure of the Statistical Analysis System (SAS) software, version 6.12. Mean comparison of three replications was carried out by Duncan's multiple range test at 0.05 probability level.

RESULTS

Growth parameters. After 30 days of plant's exposure to NaCl and PEG stress treatments, results indicated a significant reduction in plant height, green leaf percentage, root and shoot DW (Figs. 1a-g, 2a, b). These growth parameters declined linearly or quadratic, when NaCl and PEG concentration increased (Fig. 1a-f). The DW of shoots also declined under PEG and NaCl stressed plants. It showed a maximum reduction in the presence of both stresses. The highest DW of shoots was recorded in control plants (Fig. 1g).

Electrolyte leakage (EL). The response of EL to NaCl and PEG concentration gradients in the MS medium was described using linear ($R^2 = 0.998$) and quadratic ($R^2 = 0.787$) models, respectively (Fig. 3a, b). The addition of NaCl to the culture medium induced a significant increase in electrolyte leakage ($P < 0.001$) and reached the maximum values at 150 mM NaCl (Fig. 3a). The EL significantly increased with increasing PEG concentration from 2.0 to 4.0% in the culture medium. However, increase in PEG concentration from 0 to 2.0% had no significant effect on the EL (Fig. 3b).

Antioxidant enzymes activity. Under saline and drought conditions, a significant increase in CAT and POX activity was observed (Fig. 4a,b). An increase in CAT and POX activity under NaCl stress (78–385% and 54–78%, respectively) was more than PEG stress (139–226% and 27–75%, respectively). However, the highest CAT and POX activities were found under combined stress levels (more than 6 and 5-fold, respectively) compared to control. SOD activity under different NaCl and PEG concentrations is illustrated in Fig. 4c. It's indicated that SOD activities in leaves of sweet violet increased due to the increase in salt and PEG concentrations in the culture media. The increase rate in SOD activity was the highest (more than 3.5-fold as compared to control) in 150 mM NaCl and 2.5% PEG concentrations. NaCl at a rate of 150 mM in combination with various concentrations of PEG resulted in a sudden increase in SOD activity. However,

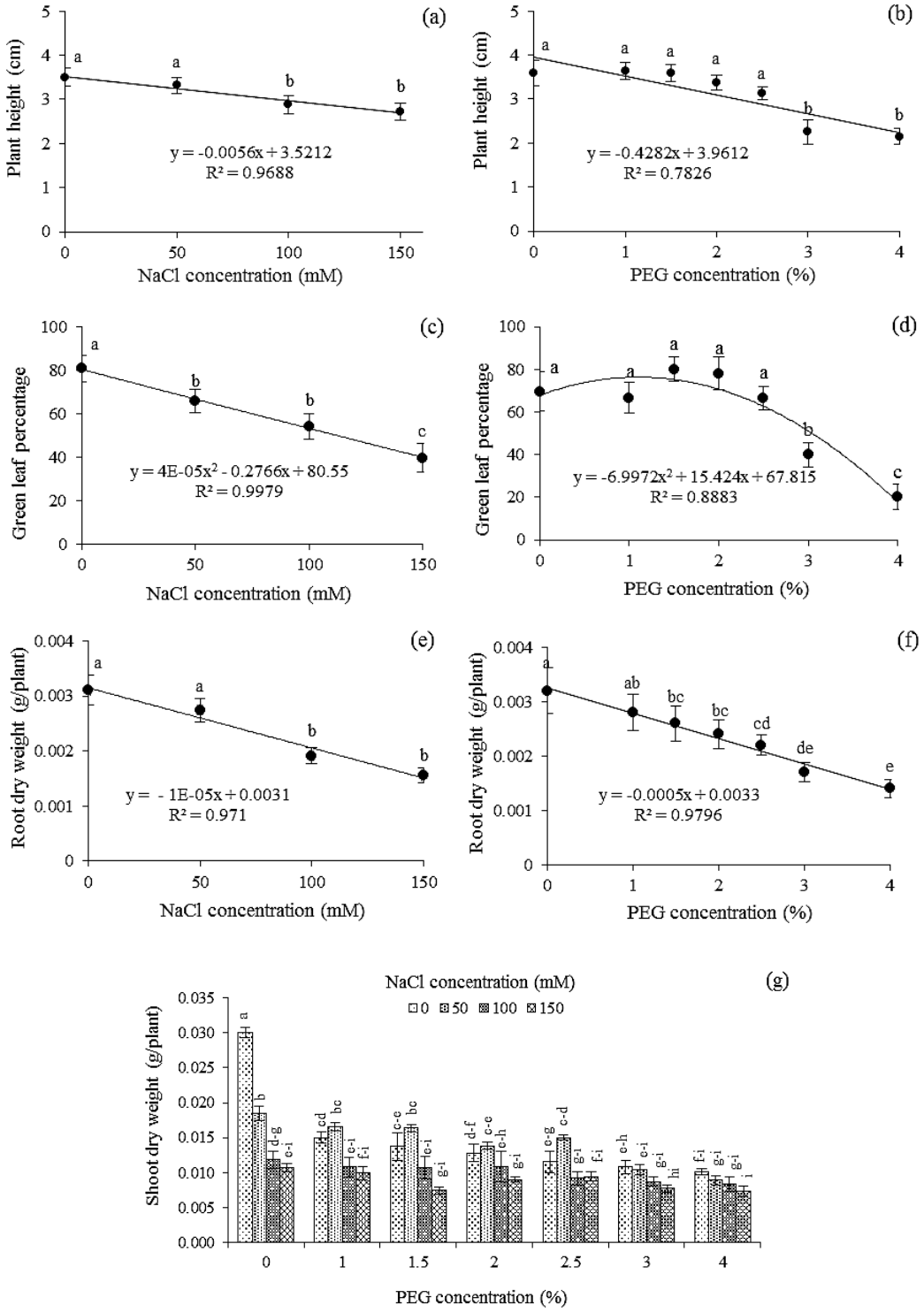

Fig. 1. The response of plant height (a, b), green leaf percent (c, d), root (e, f) and shoot DW (g) of *Viola odorata* to NaCl and PEG concentrations in the MS medium. Values are mean of three replicates and vertical bars indicate ±SE (n = 3). Values with different letters are significantly different at 5% level according to Duncan's multiple range test (DMRT)

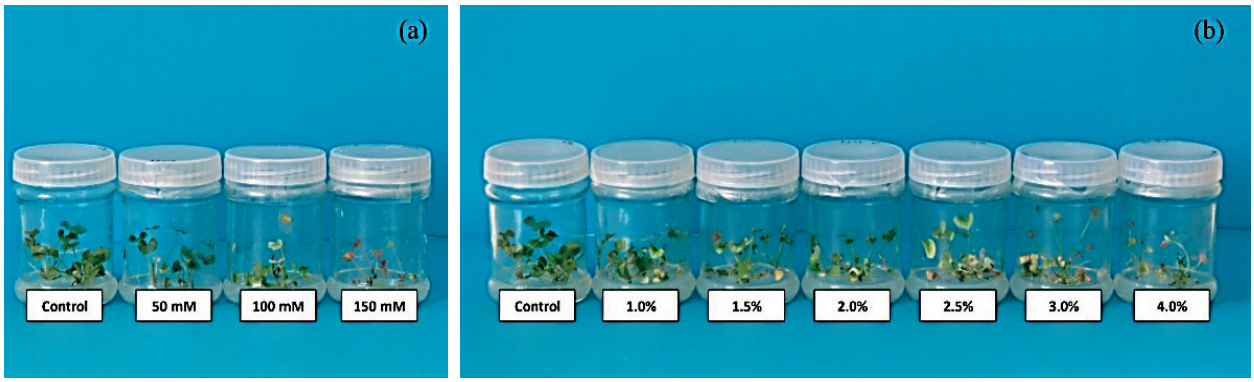

Fig. 2. The response of *Viola odorata* growth to NaCl (a) and PEG (b) concentrations in the MS medium

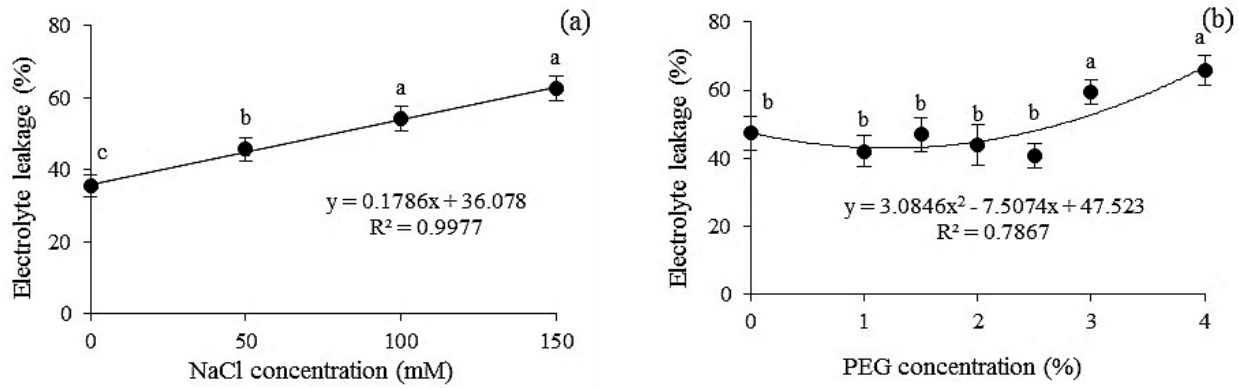

Fig. 3. The response of Electrolyte leakage in *Viola odorata* leaves to NaCl (a) and PEG (b) concentrations in the MS medium. Values are mean of three replicates and vertical bars indicate ±SE (n = 3). Values with different letters are significantly different at 5% level according to Duncan's multiple range test (DMRT)

plant under 50 and 100 mM NaCl displayed gradual increase in SOD activity.

H_2O_2 content. In leaves of treated plants, H_2O_2 content was increased under salinity and drought stresses and returned to the control level in high concentrations of NaCl (150 mM) and (PEG 2.0 and 2.5%) (Fig. 4d). The maximum amount of H_2O_2 contents was recorded under 100 mM NaCl treatment in combination with 0, 1 and 1.5% PEG. H_2O_2 content decreased with increasing NaCl level from 100 to 150 mM.

Proline content. The accumulation of proline was significantly greater in salt and drought stressed plants in comparison to control (Fig. 4e). A positive correlation was shown between proline accumulation and increasing salinity/drought level. In maximum salinity/drought levels, the highest accumulation of proline was recorded.

Total Soluble Carbohydrates (TSC). As salt and drought stress levels increased to 100 mM NaCl and 1.5%, respectively, TSC content significantly increased and then decreased gradually, throughout the experiment. At all concentrations of PEG, the highest amounts of TSC were obtained in moderate salt stress conditions (50 and 100 mM NaCl). Maximum value of TSC was recorded at 50 mM NaCl + 2.5% PEG, followed by 100 mM NaCl + 1 and 1.5% PEG levels (Fig. 4f).

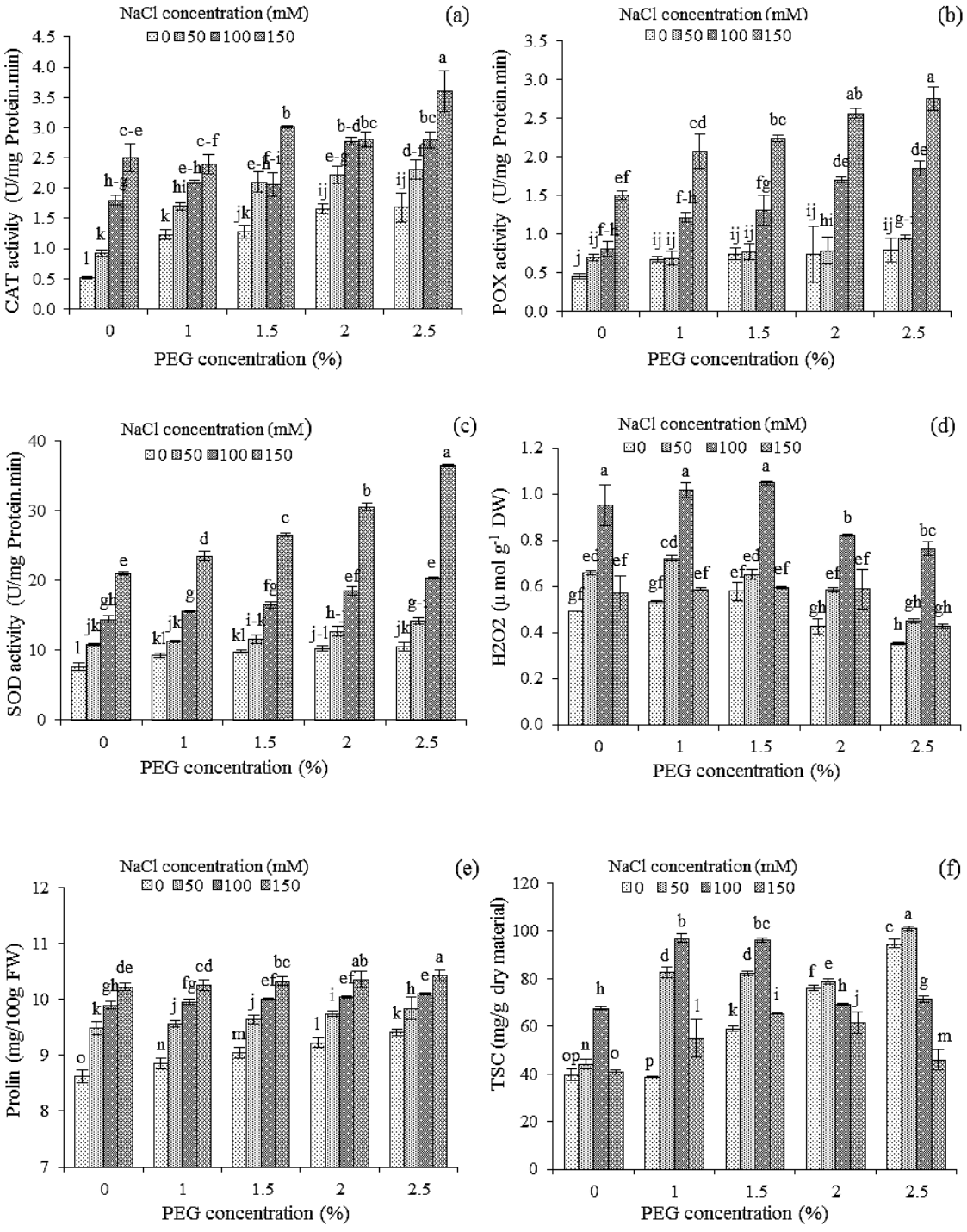

Fig. 4. The response of CAT (a), POX (b) and SOD (c) enzymes activity and H_2O_2 (d), proline (e) and TSC content (f) in *Viola odorata* leaves to NaCl (a) and PEG (b) concentrations in the MS medium.Values are mean of three replicates and vertical bars indicate ±SE (n = 3). Values with different letters are significantly different at 5% level according to Duncan's multiple range test (DMRT)

DISCUSSION

One of the most important strategies that plants have adopted in front of abiotic stress including salinity and drought is to slow down their growth. As a matter of fact, slower growth is an adaptive trait for plant survival under stress conditions [Queiros et al. 2007]. This reduction in growth not only helps the plant to save the energy for defense purpose but also limits the risk of heritable damage [Hossain et al. 2007]. In our study, the presence of NaCl in the medium decreased all of the growth parameters. Plant height, green leaf percentage, and shoot and root DW were considerably reduced at high levels of salinity (100 and 150 mM NaCl) and drought (3 and 4% PEG) stress. Growth inhibition is considered as a common phenomenon of salt/drought-stressed plants, also seen in cultured cells, tissues or organs on medium supplemented with NaCl and the degree of salt tolerance often appears to be inversely related to growth rate [Queiros et al. 2007]. These results are in line with previous study in *Atriplex prostrata*, in which the leaf area, dry mass of leaves and roots showed a significant reduction with the increasing of salinity [Wang et al. 1997]. Kulkarni and Deshpande [2007] reported that the reduction in shoot growth was severe with the increasing PEG concentration in tomato (*Solanum lycopersicum* L.). Pirdashti et al. [2012] also obtained similar results in rice plants under salinity stress. In our experiment, the leas shoot DW was obtained in combined stress conditions (150 mM NaCl and 4% PEG). Salinity and drought affects cell development, leaf area, biomass and yield as a response of plant to stress. Ahmed et al. [2013] reported a significant inhibition in plant growth parameters of barley (*Hordeum vulgare* L.) under single or combined salt and drought stress, with the largest reduction in the combined stress.

Different concentrations of NaCl as well as higher concentrations of PEG (3 and 4%) led to significant increase in the EL rate in sweet violet; this observation is consistent with previous study [Farghaly et al. 2016].

Antioxidant enzymes play important roles in the defense mechanism against oxidative stress. Antioxidant system may provide a strategy to enhance salt tolerance in plants, though the detailed mechanisms are not yet clear [Jaleel et al. 2007]. To endure oxidative damage under increased oxidative stress, tolerant plants possess efficient antioxidant systems. In plants, there are antioxidant systems in the form of enzymes such as SOD, POD, and CAT; they also have an efficient system for scavenging ROS, using the enzyme SOD in chloroplasts [Asada 1999]. Protective enzymes activity increase to a high level to remove ROS and keep them at a low level. Consequently, the function and structure of undamaged membranes are maintained [Zhang et al. 2013]. Environmental stress induces the production of O_2^- and the other ROS [Gill and Tuteja 2010].

The CAT, POX, and SOD activities increased in all of the stressed plants compared to control. CAT activities, together with SOD are the most effective antioxidant enzymes in preventing cellular damage [Asadi-Sanam et al. 2015] and both of them were significantly increased in response to the salt or drought stresses [Patade et al. 2011]. In mulberry [Sudhakar et al. 2001], legumes and other crops [Talukdar 2012], cooperative increase in antioxidant enzyme capacity particularly peroxidase-catalase system in fine regulation of salt/drought-generated ROS has also been reported. POX is among the enzymes that scavenges H_2O_2 in chloroplasts which is produced through dismutation of O_2^- catalyzed by SOD [Bor et al. 2003].

POX activity increased significantly under salt and drought stress conditions, especially in 150 mM NaCl and 2.5% PEG. Greater POX activity has also been reported in salt-tolerant and sensitive species of tomato [Koca et al. 2006] and rice cultivars [Dionisio-Sese and Tobita 1998].

Results showed that H_2O_2 content increased when NaCl and PEG concentration in the culture media was increased from 0 to 100 mM and from 0 to 1.5%, respectively. H_2O_2 is produced as a result of dismutation reaction of O_2 mostly catalyzed by SOD [El-Missiry 2012]. H_2O_2 is formed in the peroxisomes as part of photorespiratory, and also produced from β-oxidation of fatty acids as a by-product. H_2O_2 is not a free radical, but participates as an oxidant or a reductant in several cellular metabolic pathways [Reddy and Raghavendra 2006].

Our results revealed a significant increase in level of proline in shoots under highest concentration of NaCl and PEG. However, proline accumulation does not main role against stress at the plant. In the present study, there was no correlation between proline accu-

mulation and the ability of plant to endure stress condition, so that, the growth rate and height of treated plants declined at highest concentration of NaCl and PEG. These results are in accordance with Talukdar [2012].

TSC concentrations increased significantly under moderate salt and drought stress and the maximum TSC was obtained at 50 mM NaCl in combination with 2.5% PEG followed by 100 mM NaCl + 1 and 1.5% PEG levels. Another one of the self-defense responses during water stress period in seeds and plants is the increase in osmolyte content which protects the enzyme system [Muscolo et al. 2014]. Similar results for TSC under salt and drought stress were shown by Hussain et al. [2010] in sweet violet plants.

CONCLUSION

In conclusion, our findings highlight that the effect of combined drought and salt stress is more severe; however, the sensitivity of the plant to drought or salinity stress is higher in the absence of other stress. Moreover, it has been observed that the sweet violet plant has been relatively resistant to low levels of salinity and drought stress. Therefore, this plant can be considered as a relatively stress resistant plant and can be introduced to the areas other than its main habitat.

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