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Research Paper

Morphological and Physiological Responses of Euonymus (*Euonymus japonicus*) to Exogenous Paclobutrazol under Salinity Stress

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Abstract

Paclobutrazol (PBZ) is one of the most important triazole compounds. This compound increases plant tolerance to environmental stresses. The present study aimed to investigate the effects of PBZ on morphological and biochemical traits of euonymus (*Euonymus japonicas*) exposed to NaCl stress. Plants were treated with three levels of PBZ (0, 500 and 1000 mg L^{-1}) and four levels of NaCl (0, 50, 100, 150 mM). Morphological characteristics, electrolyte leakage (EL), chlorophyll content, and antioxidant enzyme activity were changed by the treatment with PBZ and NaCl stress. As compared to the control plants, PBZ at the rates of 1000 and 500 mg L^{-1} decreased height by 22.8% and 10.44%, respectively. The highest number of lateral shoots was recorded at 1000 mg L^{-1} PBZ (no salt treatment). The interaction of salinity and PBZ was significant for shoot fresh weight. At 150 mM NaCl + 1000 mg L^{-1} PBZ, shoot fresh weight was increased by 30% compared to 150 mM NaCl. The PBZ-treated plants had higher superoxide dismutase (SOD) activity than those not treated with PBZ. PBZ at the rate of 1000 mg L^{-1} was the best treatment for increasing the activity of this enzyme. PBZ mitigated the salinity stress and reduced EL of euonymus. Compared with 150 mM NaCl alone, EL was decreased by 24.4% in the plants treated with 1000 mg L^{-1} PBZ + 150 mM NaCl. According to the results, it can be concluded that PBZ can be effective in improving the tolerance of euonymus to 150 mM NaCl.

Keywords: Electrolyte leakage, Enzyme activity, Height, Shoot fresh weight.

NTRODUCTION

Plants may endure numerous biotic and abiotic stresses throughout their lifecycle. Among these stresses, soil salinity is one of the most important stresses that impedes the growth and development of plants and causes significant yield losses (Ashraf and Harris, 2004). Salinity impairs growth by reducing leaf water potential and altering various metabolic activities, such as ion imbalances, enzymatic inhibition, and changes in solute accumulation (Porcel *et al*., 2012). In plants, strategies to protect against salt stress include controlling salt ion uptake by roots, controlling the movement of ions from roots to shoots, altering photosynthetic pathways, altering the activity of antioxidant enzymes, and modifying plant hormone levels (Zhao *et al*., 2010; Liu *et al*., 2018).

Studies have shown that triazoles play a role in resistance to environmental stresses, e.g., drought, salinity, temperature stress, sulfur dioxide gas, and fungal infections (Nguyen *et al*., 2018). Commercial triazole derivatives (such as paclobutrazol [(2RS, 3RS) -1- (4-chlorophenyl) -4, 4-dimethyl-2- (1, 2, 4-triazolyl)-pentan-3-ol]) have been recommended for use as fungicides or plant growth regulators. The morphological and anatomical characteristics of triazole can be attributed to the reduction of plant growth and trichome length, larger chloroplasts, and expanded root system (Berova *et al*., 2002). Triazoles have an effect on the isoprenoid pathway and hormonal changes, such as increasing cytokinin and ethylene depletion and inducing plant resistance to stressful conditions. It has been reported that PBZ protects stress-exposed plants by reducing oxidative damage through enhancing antioxidants or reducing the activity of oxidative enzymes. In one study, wheat (*Triticum aestivum*) seeds were exposed to different concentrations of PBZ (0, 25 and 50 mg L^{-1}) for 24 hours. Seven-day-old plants were exposed to temperature stress in a cold room (2 ± 1ºC) for 10 days. After stress, the seedlings were returned to a controlled climatic chamber. The low temperature stress caused lipid peroxidation, increased peroxidase activity, and decreased chlorophyll content. The increase in growth rate, such as shoot length, fresh and dry weight, after temperature stress was higher in the PBZ-treated seedlings than in the control (Berova *et al*., 2002). There is a report that PBZ application reduced the height of peony (*Paeonia lactiflora* Pall.) plants. Furthermore, PBZ application significantly increased photosynthetic rate and water use efficiency (Xia *et al*., 2018). The application of 1-2 mg PBZ produced shorter plants, and darker green foliage color in potted sunflowers (*Helianthus annuus*) than in non-treated plants (Ahmad *et al*., 2015). In a study on mango (*Mangifera indica*), 1500 mg L^{-1} PBZ reduced salinity stress and ion leakage in seedlings compared to non-PBZ-treated salinized plants (Srivastava *et al*., 2010). The application of PBZ to tomato (*Solanum lycopersicum* L.) seedlings decreased plant height, increased leaf number, and improved stem diameter under drought stress (Pal *et al*., 2016). Sumit *et al*. (2012) reported that PBZ significantly decreased plant height of *Camelina sativa* when compared to the control. In one study, water deficit stress reduced plant height and fresh weight in *Curcuma alismatifolia*. Water stress decreased RWC and increased protein and electrolyte leakage content in leaves. However, by adding PBZ to the treatments, conflicting results were obtained. Some antioxidants, including vitamin C, vitamin E, and CAT and SOD activities, were induced by PBZ (Jungklang *et al*., 2017). In the winters of 2009 to 2010, heavy snowfall in Beijing led to the use of 30,000 tons of a chloride de-icing salt in downtown area roads. Reportedly, *E. japonicus*, as a hedge plant, is severely affected (Zhang, 2010).

Euonymus japonicus from the family of Celastraceae is a popular ornamental plant for urban space. There is little research on the effect of PBZ on salinity resistance in important urban space species. Therefore, the present study aimed to investigate the effect of PBZ on morphological and biochemical changes in euonymus under salinity stress.

MATERIALS AND METHODS

Plant materials and treatments

Two-years-old healthy *E. japonicus* plants with uniform sizes were grown in plastic pots (20 cm length, 15 cm diameter) containing sand and garden soil (3:1 V/V) in a greenhouse. Some properties of media are presented in Table 1. To investigate the effect of exogenous PBZ on salt tolerance of euonymus, a factorial experiment was conducted in a completely randomized design with three replications. The first factor was paclobutrazol (PBZ at three rates of 0, 500, and 1000 mg L^{-1}) and the second factor was salinity stress (at four levels of 0, 50, 100, and 150 mM).

Table 1. Properties of the culture medium used in this study.

Mg^{2+} (mg kg^{-1})	Ca^{2+} (mg kg^{-1})	K^+ (mg kg^{-1})	Na^+ (mg kg^{-1})	Cl^- (mg kg^{-1})	Field capacity (%)	C (%)	EC (dS m^{-1})
1240	480	890	1280	2440	18.02	2.04	1.01

The plants were maintained in a greenhouse under natural light conditions with day/night temperatures of 23/15°C and relative humidity of 75%. First, all plants (except the control treatment) were sprayed with different concentrations of PBZ. A second spraying was applied after one week. The soil surface was covered in each spraying. One week after the second foliar application, salinity was applied by using 99.9% pure NaCl. To prevent osmotic shock, salt concentration was gradually increased until the desired concentration was reached. Tap water was used as the control. After three months, traits such as height, injury rating value, number of lateral shoots, shoot and root fresh and dry weight, chlorophyll content, enzyme activity, and electrolyte leakage were recorded

Plant growth

Plants were removed from the pot after 90 days of salinity stress. Plant height, number of new growth lateral shoots, and shoot and root fresh and dry weight were recorded. Dry weight was determined after drying the samples at 80°C for 48 hours.

Injury rating value

Injury rating value was calculated according to the percentage of plant damage. At the end of the experiment, the appearance of plants, such as necrosis in leaves, weak branches, withered plants, leaf fall, etc., was evaluated by assigning a number from 1 to 5 in which 1 meant the least damage and 5 meant complete damage to the plant.

Chlorophyll content

Chlorophyll was extracted from fresh leaves with 80% acetone, and its contents were determined by spectrophotometers according to the method of Arnon (1949).

Assay of enzyme activity

Superoxide dismutase (SOD) (EC 1.11.1.7)

Superoxide dismutase activity was assayed according to the method of Stewart and Bewley (1980). The reaction mixture was prepared by mixing 0.1 mM nitroblue tetrazolium, 0.1 mM EDTA, and 50 µM xanthine and xanthine oxidase in 50 mM potassium phosphate buffer, pH 7.8. One unit of SOD is defined as the amount of enzyme that inhibits the control rate by 50% (0.025 units of absorbance at 550 nm min^{-1}).

Peroxidase (POD) activity

For POD activity, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and the enzyme extract (Cakmak and Marschner, 1992). Peroxidase activity was determined following the oxidation of *o*-Dianisidine in the presence of H_2O_2 at 470 nm.

Catalase (CAT) (EC 1. 11.1.6)

For CAT assay, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2, and the enzyme extract (Cakmak and Marschner, 1992). Protein contents were determined following the method of Bradford and Williams (1976).

Determination of electrolyte leakage (EL)

First, 0.2 g of fresh leaf was washed with deionized water. The leaves were placed in closed tubes containing 5 ml of deionized water and incubated at 10°C for 24 h. Subsequently, the initial electrical conductivity of the solution (EC_1) was determined using a conductor. The samples were then immersed in a water bath at 95°C for 20 min, cooled to 25°C, and their EC_2 was measured. Electrolyte leakage (EL) was calculated by the following equation (Deshmukh *et al*., 1991):

$$\mathrm{EL}(\%) = \frac{\mathrm{EC}_1}{\mathrm{EC}_2} \times 100$$

Statistical analysis

Data were statistically analyzed using SAS software (version 9.1). Means were compared by using the least significant difference (LSD) at the 5% level of probability.

RESULTS

Plant height and number of lateral shoots

The effect of salinity and interaction of salinity and PBZ were significant ($P < 0.01$) on plant height and number of lateral shoots (Table 2). Decreases were observed in the height of the plants treated with PBZ. As compared to the control (no salt and no PBZ), a decrease in height of 22.8 and 10.44% was observed in the plants treated with 1000 and 500 mg L^{-1} PBZ, respectively (Fig. 1A). The highest number of lateral shoots was recorded in the plants treated with 1000 mg L^{-1} PBZ (no salt) and the lowest in 150 mM NaCl without PBZ (Fig. 1C).

Table 2. Variance analysis of measured traits in euonymus under different levels of PBZ and salinity.

S.o.V	df	Plant height	Number of lateral shoots	Injury rating value	Chlorophyll content	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Salt stress (A)	3	14.02^{**}	13.18^{ns}	3.78^{*}	6.80^{*}	37.16^{**}	1.40^{ns}	39.99^{**}	0.85^{ns}
PBZ (B)	2	0.44^{ns}	0.88^{ns}	18.44^{ns}	22.55^{**}	27.53^{**}	1.87^{*}	7.81^{ns}	0.46^{ns}
A × B	6	33.33^{**}	26.00^{**}	4.76^{**}	5.08^{*}	8.88^{*}	1.90^{*}	25.03^{**}	1.52^{*}
Error	24	1.56	8.69	1.06	1.50	2.75	0.73	5.78	0.61
CV (%)		4.21	25.02	27.28	25.16	10.36	16.51	15.95	22.30

*, ** and ^{ns}: Significantly different at the $P < 0.05$ and $P < 0.01$ levels, and non-significant, respectively.

Injury rating value

The effect of salinity and the interaction between salinity and PBZ were significant ($P<0.05$ and $P<0.01$, respectively) on injury rating value (Table 2). The results showed that salinity increased the apparent damage of the plants. The highest damage was in 150 mM NaCl without PBZ treatment. The application of PBZ reduced the injury rating value of the plants under salinity stress. Compared to 150 mM NaCl without PBZ treatment, 1000 and 500 mgL^{-1} PBZ reduced the damage by 33.3% and 22%, respectively (Fig. 1B).

Chlorophyll content

Salinity stress, PBZ treatment, and their interaction were significantly effective on chlorophyll contents (Table 2). The results showed that NaCl reduced chlorophyll contents in the euonymus leaves. However, PBZ prevented the reduction of chlorophyll content under salt stress. The PBZ concentration of 1000 mgL^{-1} was the most effective in reducing salt stress. Compared to 50, 100, and 150 mM NaCl alone, the chlorophyll contents were increased by 33.3%, 63.7 %, and 60.7% in the 1000 mg L^{-1} treatment, respectively (Fig. 1D).

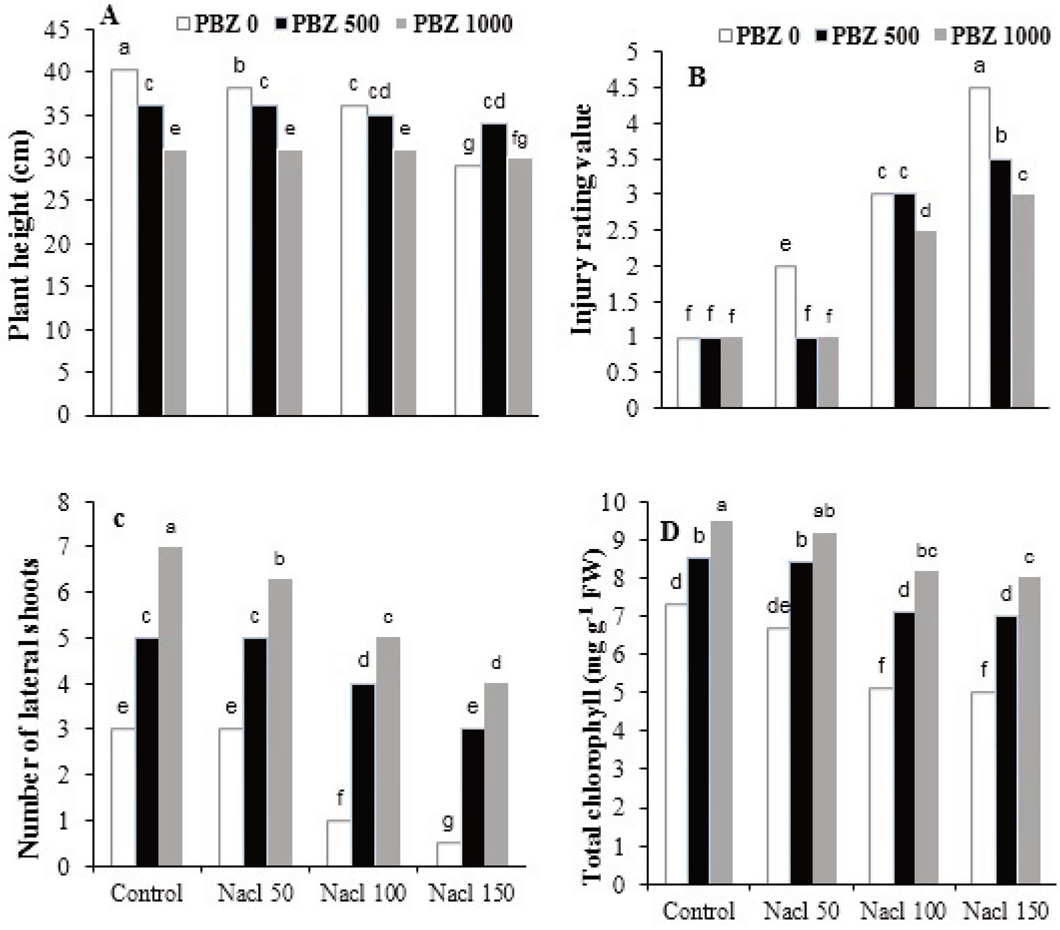

Fig. 1. The effects of exogenous PBZ (0, 500 and 1000 mg L^{-1}) on plant height, injury rating value, number of lateral shoots, and total chlorophyll in the euonymus plants exposed to NaCl (0, 50, 100 and 150 mM) stress. The columns with the same letters are not significantly different at the 5% probability level.

Fresh and dry weight of shoots and roots

The interaction of salinity and PBZ was significant for the fresh and dry weight of shoots and roots (Table 2). The results revealed that salinity decreased shoot and root fresh and dry weight. The minimum fresh and dry weight of the shoots was recorded in 100 and 150 mM NaCl (no PBZ) with 10.44% and 25.88% decrease compared to the control, respectively (Fig. 2A). At 150 mM NaCl + 1000 mg L^{-1} PBZ, shoot fresh weight increased was by 30% compared to 150 mM NaCl without PBZ (Fig. 2A). With increasing the NaCl concentration, shoot dry weight was decreased. Compared with 150 mM NaCl alone, the shoot dry weight was increased by 27.5 % in the 150 mM NaCl + 1000 mg L^{-1} PBZ treatment (Fig. 2B). Salinity stress decreased root growth. The highest growth reduction was at 150 mM NaCl (non-PBZ). PBZ reduced the negative effect of salinity. The lowest fresh and dry root weights were recorded in 150 mM NaCl (Figs. 2C, D).

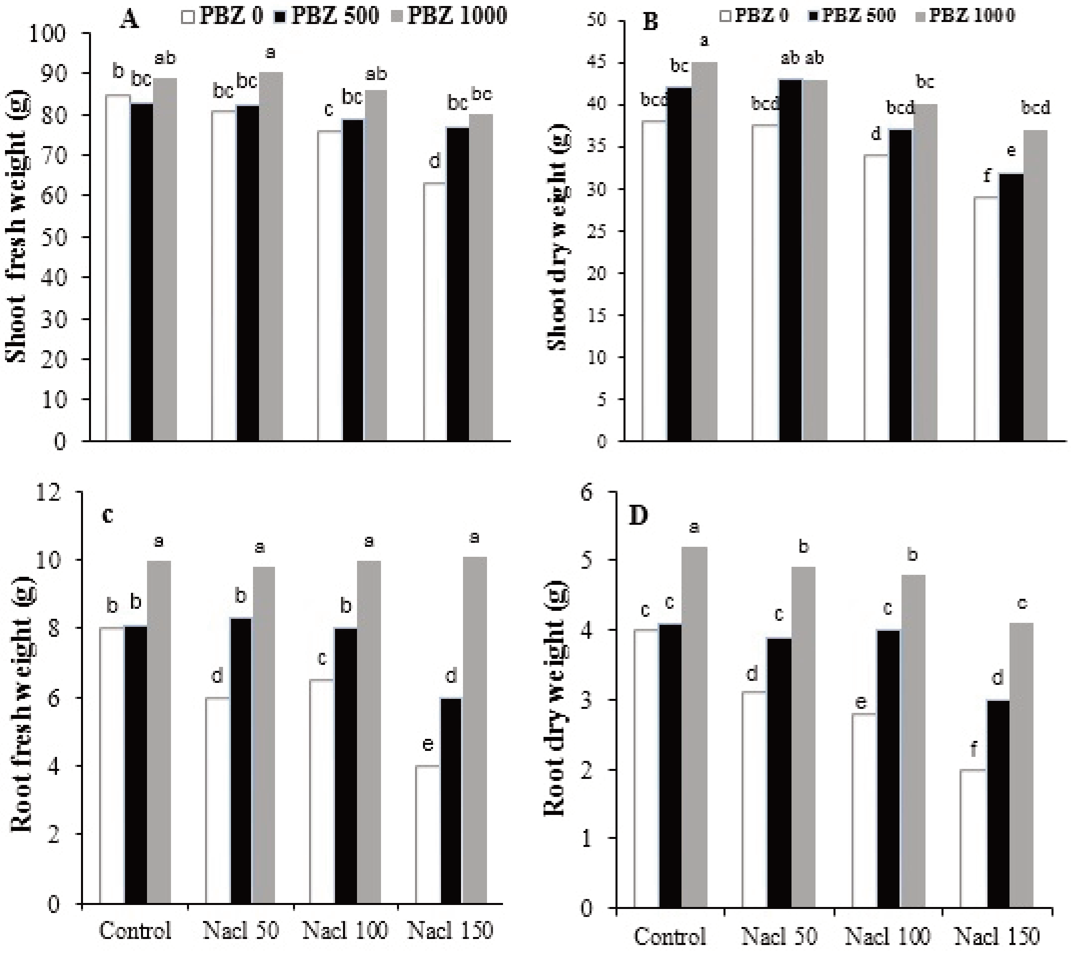

Fig. 2. The effects of exogenous PBZ (0, 500 and 1000 mg L^{-1}) on the fresh and dry weight of shoots and roots in the euonymus plants exposed to NaCl (0, 50, 100 and 150 mM) stress. The columns with the same letters are not significantly different at the 5% probability level.

Antioxidant enzymes activities

Salinity stress, PBZ treatment, and their interaction were significantly effective on antioxidant enzymes activities (Table 3). The plants treated with PBZ had higher SOD activity than the PBZ-untreated plants. PBZ at the rate of 1000 mg L^{-1} was the best treatment for increasing the activity of this enzyme. The SOD activity was 33.3% (PBZ 1000 mg L^{-1} + NaCl 150 mM) to 25% (PBZ 1000 mg L^{-1} + NaCl 150 mM) higher than non-treated plants at the same NaCl concentration (Fig. 3A). Salinity, PBZ, and their interactions significantly affected the CAT activity in euonymus. Compared with the control, the CAT activity was increased by 16% in the treatment of 1000 mg L^{-1} PBZ (Fig. 3B). The POD activity in euonymus followed a similar trend to SOD. Non-salt-treated plants with PBZ showed more POD activity than non-salt-treated plants without PBZ. The highest activity of this enzyme was recorded in 1000 mg L^{-1} PBZ under 50 and 100 mM salinity stress (Fig. 3C).

Electrolyte leakage (EL)

The interaction of salinity and PBZ was significant for electrolyte leakage (Table 3). Both NaCl and PBZ had an effect on leaf membrane damage, and significant changes were observed in electrolyte leakage. PBZ mitigated the salinity stress and reduced EL of euonymus. Compared with 150 mM NaCl alone, the EL was decreased by 24.4% in 1000 mg L^{-1} PBZ + 150 mM NaCl (Fig. 3D).

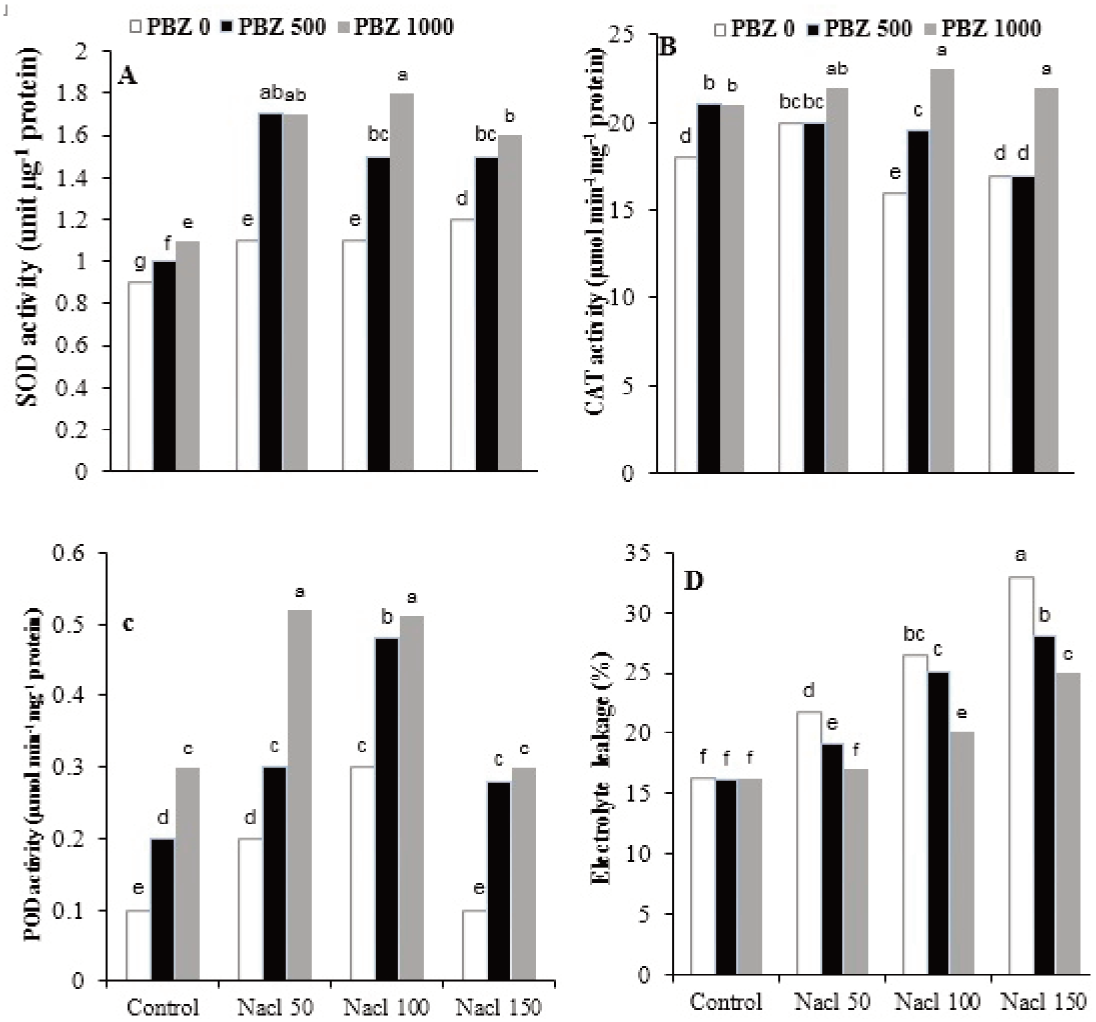

Fig. 3. The effects of exogenous PBZ (0, 500, and 1000 mg L^{-1}) on the activities of CAT, SOD, POD, and electrolyte leakage in euonymus plants exposed to NaCl (0, 50, 100, and 150 mM) stress. The columns with the same letters are not significantly different at the 5% probability level.

Table 3. The variance analysis of the measured traits in *Euonymus* under different levels of PBZ and salinity.

S.o.V	df	SOD	CAT	POD	Electrolyte leakage
Salt stress (A)	3	0.34**	60.91**	0.07**	45.27*
PBZ (B)	2	0.39**	36.67**	0.20**	34.65^{ns}
A × B	6	0.03*	5.89*	0.02**	81.12**
Error	24	0.01	1.13	0.001	16.79
CV (%)		6.92	5.29	11.04	22.19

*, ** and ^{ns}: Significantly different at the 5% and 1% of probability levels, and non-significant, respectively.

DISCUSSION

Plants are affected by various stresses, such as salinity, drought, freezing, cold, high temperature, waterlogging conditions, heavy metals, and UV radiation. Salinity is one of the most important abiotic stresses that restrict crop production, especially in arid and semi-arid climates (Ashraf and Harris, 2004; Hussain *et al*., 2009).

Environmental stresses can damage plants through the accumulation of toxic reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{.-}$) and hydroxyl (HO_2) (Noctor and Foyer, 1998). Excess ROS may damage cell membranes through the peroxidation of lipids, proteins, and nucleic acids. One of the defense mechanisms against oxidative stress is an increased activity of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) (Maestri *et al*., 2002). In the present study, the effect of salinity, PBZ treatment, and their interactions was significant on enzymatic activities. According to the results, the highest enzymatic activities were observed in the plants treated with 1000 mg L^{-1} PBZ. The minimum enzymatic activities were observed in the plants not treated with PBZ. POD is one of the important systems for H_2O_2 enzymatic degradation in plants. Increased POD activity in plants indicates the protective role of the enzyme in salinity stress (Fletcher *et al*., 2000). In plant cells, O_2^- is rapidly converted to H_2O_2 by the SOD enzyme while H_2O_2 is degradable by POD and CAT (Noctor and Foyer, 1998). Similar results have been observed for the effects of PBZ on increasing antioxidant enzyme activities in other studies (Fletcher *et al*., 2000; Srivastava *et al*., 2010). Sheikh Mohammadi *et al*. (2017) reported that the activity of APX, POD, SOD, and CAT in Iranian perennial ryegrass (*Lolium perenne* L.) was increased in response to PBZ under drought stress.

According to the results of the present study, salinity stress significantly increased electrolyte leakage (EL) in the leaves of the studied plants. However, the use of PBZ significantly reduced leaf EL during the experiment. The minimum EL was related to the concentration of 1000 mg L^{-1} PBZ. Srivastava *et al*. (2010) reported that this may be due to the fact that PBZ-treated salinized plants had less sodium and chloride in the tissues and suffered less damage to their cells. In addition, the rearrangement of stress-protective biomolecules in PBZ-treated plants has also increased the capacity to limit the damage caused by ROS.

One of the most important symptoms of environmental stress is leaf chlorosis that results in aging, which is accompanied by a simultaneous decrease in the concentration of photosynthetic pigments (Fletcher *et al*., 2000). In the present study, leaf chlorophyll content was significantly decreased after salinity stress. PBZ alleviated the severity of this injury. At a concentration of 1000 mg L^{-1} PBZ, maximum chlorophyll retention was observed. Berova *et al*. (2002) reported that one of the biochemical effects of PBZ is an increase in chlorophyll content. Increased content of chlorophyll in PBZ-treated plants may be associated with the minimized damage of reactive oxygen and changes in the levels of carotenoids, ascorbate, and ascorbate peroxidase (Nivedithadevi *et al*., 2015; Tesfahun, 2018). Plants treated with PBZ have also been reported to synthesize more cytokinins, which enhances chloroplast differentiation and biosynthesis of chlorophyll and prevents chlorophyll degradation. It has also been reported that PBZ delayed the onset of aging, indicating the extent of chlorophyll degradation in attached mung bean leaves, possibly due to the increased endogenous levels of cytokinins (Fletcher *et al*., 2000).

In this study, the PBZ treatment reduced the plant height and increased the number of lateral branches and leaf area (data not shown), thereby increasing shoot fresh weight. Salinity treatment also had a significant effect on decreasing height. PBZ are one of the most important compounds of sterol and gibberellin biosynthesis inhibitors (Khan *et al*., 2009). This compound is considered to be the most active inhibitor of growth that prevents stem elongation of the plants (Hwang *et al*., 2008; Megersa *et al*., 2018). Kim *et al*. (2012) reported that PBZ could significantly affect plant growth by altering photosynthetic rate and changing phytohormone levels. Growth inhibitors reduce the length of internodes and eliminate the end-dominance. Therefore, they increase the lateral branches (Chany, 2005). Growth inhibitors have also been shown to increase lateral shoots by increasing cytokinin hormone (Rossini Pinto *et al*., 2005). The application of 1-2 mg of PBZ per pot produced shorter plants, and darker green foliage color for potted sunflower (*Helianthus annuus*) than the non-treated plants (Ahmad *et al*., 2015).

In our study, salinity stress decreased root fresh and dry weight. But, the PBZ treatments mitigated the negative effects of stress. In saline conditions, PBZ improves stress-induced dehydration by establishing osmotic balance. It is inferred that paclobutrazol tries to increase the level of contact and access to water resources by increasing root growth under salinity conditions, thereby increasing plant tolerance to salinity stress (Tesfahun, 2018).

The results of Kamran *et al*. (2018) on maize showed that root activity and root sap flow were significantly higher in PBZ treatments compared to control. It has also been reported that uniconazole, a triazole, with a function such as PBZ, increased root bleeding sap, root activity, and root growth in soybeans (Yan *et al*., 2013). Also, higher root activities were found in wheat and rice plants treated with plant growth regulators (Zhao *et al*., 2006) Therefore, the use of PBZ by stimulating plant root activity can improve plant yield under stress conditions.

CONCLUSION

In summary, the present study showed that paclobutrazol increased salinity stress tolerance in euonymus plants. Under salt stress, PBZ improved the activity of antioxidant enzymes and, subsequently, reduced oxidative stress and ion leakage. PBZ contributed to maintaining chlorophyll content and increased production of lateral branches under NaCl stress.

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