RESEARCH ARTICLE **(Open Access)**

Effects of 24-epibrassinolide on growth, lipid peroxidation, protein and antioxidative enzyme activities in seedlings of loquat under salinity stress

FATEMEH SADEGHI[1], AKHTAR SHEKAFANDEH[1]*

[1]Department of Horticulture, Faculty of Agriculture, Shiraz University, Shiraz Iran.

Abstract

In this research, the changes in plant growth, lipid peroxidation, protein content and activities of antioxidant enzymes in loquat seedlings subjected to NaCl stress and 24-epibrassinolide (24-EBL) application were investigated. Plants were treated with 5 levels of salt (0.5, 2, 4, 6 and 8 dS m^{-1}) and 4 levels of 24-epibrassinolide solution (0, 0.25, 0.5 and 0.75 mg L^{-1}). Salinity decreased growth, catalase activity and total protein concentrations and increased superoxide dismutase and peroxidase activities, electrolyte leakage and malondialdehyde synthesis. The plant growth, activities of antioxidative enzymes and protein content increased in 24-epibrassinolide-treated plants in comparison with non-treated plants. Leaf cell electrolyte leakage and malondialdehyde synthesis reduced by exogenous application of 24-epibrassinolide under saline conditions. Our results suggest that 24-epibrassinolide application under salt stress conditions alters the equilibrium between free radical production and enzymatic defence reactions in loquat by enhancing the protein content and free radical scavenging capacity.

Keywords: Antioxidative enzyme, Loquat, 24-epibrassinolide, Salt, Lipid peroxidation.

1. Introduction

Loquat (*Eriobotrya japonica* Lindl.) is an important sub-tropical fruit tree belonging to the Rosaceae family that blooms in fall and early winter (32) hence is a good source of nectar. Although, the tree is traditionally considered to be confined in subtropical regions, loquat is actually very well adapted to virtually environments and mid-temperate climates and has been widely distributed throughout the world (11).

Loquat is commonly propagated by seed; as a result, the plants possess variable performance and different fruit characteristics owing to heterozygosis and cross-pollination. Therefore, in some parts of the world, the seedlings employ as a rootstock for genotypes with high fruit quality (25).

Salinity stress is the major environmental factors limiting plant growth and productivity which leads to ion toxicity, nutrient imbalance and osmotic stress. In addition, during salinity induced oxidative stress, several cytotoxic reactive oxygen species (ROS) are continuously generated in the mitochondria, peroxisomes and cytoplasm, which can destroy the normal metabolism through oxidative damage of lipids, proteins and nucleic acids (4, 35).The active oxygen species such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$) are produced during normal aerobic metabolism when electrons from the electron transport chains in mitochondria and chloroplasts are leaked and react with O_2 in the absence of other acceptors (6). However, plants have the ability to counteract oxidative stress, by detoxifying ROS by up-regulating antioxidative enzymes, like superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

Recently, rapid and economically viable approaches have been proposed to alleviate the adverse effects of salt stress (5). In a number of studies it has been emphasized that exogenous application of osmoprotectant, antioxidants, or plant growth regulators is a meaningful approach in inducing salt tolerance in crops (5). Of plant growth regulators, brassinosteroids (BRs) have been used to provide plant protection against abiotic stresses such temperature stress (15), water stress (41), pathogen infection (30) and salinity (1). BRs possess biochemical effects on plants, such as detoxification of active oxygen and increased levels of antioxidants (13).

The present study was aimed to test the hypothesis that application of 24-EBL, an analogue of brassinosteroids, will induce tolerance to salt stress in loquat and the induced tolerance is associated with

*Corresponding author: Akhtar Shekafandeh; E-mail: shekafan@shirazu.ac.ir
(Accepted for publication 20 June 2014)
***ISSN**: 2218-2020, © Agricultural University of Tirana*

increase in the growth and the protection of the plants from oxidative stress.

2. Material and Methods

2.1.Plant material and treatments

The seeds were extracted from mature loquat fruits and immediately washed with tap water and located at 4˚C for 2 weeks. Germinated seeds were allowed to grow in perforated polyethylene pots contained a mixture of peat-moss, sand and clay (1:1:1, v/v/v). After the seedlings were attained the height of about 30 to 40 cm, the same vigour seedlings were transferred to 7 litre plastic pots filled with 6 kg soil mixture as mentioned above. The field capacity of the soil used for potting was determined according to the protocol described by Richards (33). Potted seedlings were irrigated to field capacity level for 8 months. The seedlings were grown in the greenhouse at day/night temperature: 30/25±4°C and relative humidity of 40-45% under natural sun light. In order to achieve optimum seedling vegetative growth, Fasemko complete fertilizer (pH 6.7) was applied to each pot with irrigation water each fortnight. After this period, 5 levels of NaCl salt, were applied which created an electrical conductivities (EC) of 0.5 (control), 2, 4, 6 and 8 dS m^{-1}, in pots. The salts were added to pots by irrigation water step-wise until the appropriate electrical conductivity was attained. After that, plant were treated with exogenous application of 24-epibrassinolide (24-EBL) at run-off. Treatments were 4 levels of 24-EBL (0.0, 0.25, 0.5 and 0.75 mg L^{-1} of distilled water). This action was repeated 3 times with one week interval, then six weeks after spraying, data were recorded.

2.2. Growth measurements

Growth of the plants was measured in terms of stem dry weight (DW) and leaf dry weight per treatment.

2.3. Electrolyte leakage

The percentage electrolyte leakage (EL) from fresh leaf tissues was determined using an electrical conductivity meter, based on the method of Lutts et al. (26) to assess changes in cell membrane permeability. The leaf samples (fifth fully developed leaf from the top) were cut into 1 cm segments; after rinsing 3 times with distilled water to remove surface contamination they were then placed in individual stoppered vials containing 10 mL of distilled water. The vials were kept at 40°C for 30 min. Electrical conductivity of the bathing solution (EC1) was read after this time. Samples were then placed in a boiling water bath for 20 min and after the bathing solution was cooled to room temperature a second reading (EC2) was taken. EL % was calculated as:

EC1/EC2×100

2.3. Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) formed using the thiobarbituric acid (TBA) reactive substances method described by Heath and Parker (22). Frozen leaf samples (0.5 g) were homogenized in 10 mL of 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 15,000 rpm for 15 min. To a 1mL aliquot of the supernatant, 4 mL of 0.5% thiobarbituric acid was added in 20% TCA. The mixture was then heated at 95°C for 30 min in an oven, and then cooled in an ice bath. After centrifugation at 10,000 × g for 10 min, the absorbance of the supernatant was recorded at 532 and 600 nm. The MDA content (nmol g^{-1} FW) was calculated using an extinction coefficient of 155 mM cm^{-1} after subtracting the non-specific absorbance at 600 nm.

2.4. Protein

Total soluble protein content was measured according to Bradford (10) using bovine serum albumin (BSA) as a protein standard. Fresh leaf samples (1 g) were homogenized with 4 mL Na-Phosphate buffer (pH 7.2) and then centrifuged at 4°C. Supernatants and dye were pipetting in spectrophotometer cuvettes and absorbance was measured using a spectrophotometer (Model UV-120-20.Japan)) at 595 nm.

2.5.Enzyme assay

For extracting antioxidant enzymes, fresh leaves (0.5 g) were ground using a tissue grinder in 5 mL of 50 mM cooled phosphate buffer (pH 7.8) placed in an ice bath. The homogenate was centrifuged at 15000 x g for 20 min at 4ºC. The supernatant was used for determining the activities of enzymes.

Superoxide dismutases (SOD, EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (8). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 µM EDTA, 4 µM riboflavin and enzyme extract. The reaction was based on the measurement of inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) using spectrophotometer at 560 nm. The reaction was started by adding riboflavin and placing the tubes

under two 15 W fluorescent lamps for 15 min. The reaction mixture with no enzyme developed maximum colour due to maximum reduction of NBT. A non-radiated reaction mixture did not develop colour and served as the control. The reduction of NBT was inversely proportional to the SOD activity. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

Activities of catalase (CAT, EC 1.11.1.6) and Peroxidase (POD, EC 1.11.1.7) were measured by the methods of Chance and Maehly (14). CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient of 39.4 $mM^{-1}.cm^{-1}$) at 240 nm over a 2 min intervals. Reaction mixture of 3 mL contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 µl of the enzyme extract. The reaction was initiated by adding the enzyme extract.

For POD, the oxidation of guaiacol was measured by the increase in absorbance at 470 nm. The assay mixture of 3 mL contained 30 µl of enzyme extract, and 2970 µl of guaiacol (45 mM) and H_2O_2 (100 mM) which was prepared in 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA. POD activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2 (extinction coefficient of 26.6 $mM^{-1}.cm^{-1}$) at 470 nm over a 2 min intervals.

2.6. Statistical design

A factorial experiment was conducted in completely randomized design with 4 replications. Data analyses were performed by PROC GLM of SAS (SAS.9.1) and means were compared by LSD test at $P \leq 0.05$.

3. Results and Discussion

3.1. Stem dry weight

The results showed that there was a highly significant interaction between salinity and 24- EBR on seedling growth (Table 1). In 24-EBL non-treated plants, seedlings growth in term of stem dry weight increased in EC 2 dS m^{-1} then, with higher concentrations of salt in the pots decreased. The highest stem dry weight was observed in EC of 2dS m^{-1} when seedlings were sprayed with 24-EBL. In EC of 4dS m^{-1}, when plants were treated with 0.25 or 0.5 mg L^{-1} 24-EBL, stem dry weight increased by 43 and 24% respectively with comparison to the non treated seedlings in the same condition of EC.

Salt stress affects many physiological aspects of plant growth. Stem growth was reduced by salinity due to inhibitory effect of salt on cell division and enlargement in growing point (27).The increased seedling growth in stress condition after treatment by 24-EBL could be attributed to the positive effect of 24- epibrassinolide, which stimulate cell elongation and division via enhancing microtubules and cellulose biosynthesis and thus changing mechanical characteristics of the cell wall (18). Also, it has been reported that this growth promoting effect of BRs under normal and stress conditions is probably through their auxin like hormonal effect on cell division and cell enlargement (29). Catunda et al. (12) also reported that seedlings of 'Imperial' pineapple sprayed with a brassinoesteroid analogue (BIOBRAS-16) showed higher growth of shoots with greater numbers of leaves, fresh and dry matter production.

3.2. Leaf dry weight

Leaf dry weight decreased in salt-stressed plants. However 24-EBL application increased the leaf dry weight, especially in plants treated with 24-EBL at 0.5 mg L^{-1} and grown under 2 dS m^{-1} NaCl (Table1).

Leaf growth promoting effect of EBL on loquat as observed in the present study could be due to the role of EBL in growth promotion by activating cell elongation, vascular differentiation and/or proton pump. The greater fresh mass of leaves of loquat plants may be explained by the greater water contents observed in these organs after application of brassinosteroids (16).

3.3. Ion leakage (%)

Salinity affected cell membrane stability. With increasing the concentration of salts in pots ion leakage significantly increased (Table 2). The results showed that, irrespective of salt treatment, the application of 24-EBL decreased ion leakage. Minimum ion leakage (25.3%) occurred in seedlings sprayed with 0.5 mg L^{-1} 24-EBL which was significantly lower than non- treated plants (29%). Although, the interaction of salinity and 24-EBL on leaf cell ion leakage was not significant but in EC of 6 dS m^{-1} plants treated with 0.5 mg L^{-1} 24-EBL showed a decrease of 14% in ion leakage.

Table 1. The effect of 24-epibrassinolide (EBL) on growth of loquat plant under NaCl stress.

24-EBL mg L^{-1}	NaCl EC dS m^{-1}	Stem DW g	Mean	Leaf DW g	Mean
0	Cont(0.5)	46efg		59.1c	
	2	66.5bc		62.4bc	
	4	44.7efg		47.4de	
	6	40.5fg		32.2g-j	
	8	37.4g	47.1B	20.7k	44.4B
0.25	Cont(0.5)	65.3bc		63.3abc	
	2	58.8cd		59.9c	
	4	63.9c		57.1cd	
	6	45.2efg		37.2fgh	
	8	42.4fg	55.1A	26.3ijk	48.7AB
0.5	Cont(0.5)	77.8ab		70.4ab	
	2	82.8a		72.4a	
	4	55.6cde		48.5de	
	6	42.5fg		39.4fgh	
	8	41.4fg	60.1A	29.4h-k	52A
0.75	Cont(0.5)	55.9cde		59.3c	
	2	57.3cde		59.6c	
	4	50.4def		45.7ef	
	6	41.4fg		35.4ghi	
	8	40.2fg	49.1B	23.6jk	44.7B
Analysis of variance (F values)					
Salinity		25.84***		76.68***	
24-EBL		8.70***		4.41**	
Salinity × 24-EBL		2.75**		0.79ns	

In each column, means with different letters are significantly different (P<0.05)
*,**, * * *; significant at 0.05, 0.01, and 0.001 levels, respectively.
ns ; non-significant

3.4. Malondialdehyde (MDA)

The interaction of salinity and 24-EBL treatments on leaf MDA content was highly significant. Application of 24-BER diminished the destructive effect of salt. For example in EC of 6 dS m^{-1} when seedlings were treated with 0.25 or 0.5 mg L^{-1} 24-EBL, leaf MDA content was 1.6 and 2 nmol g^{-1} FW respectively which were significantly lower than non-treated plant (2.7nmol g^{-1} FW). However, in EC of 6 and 8 dS m^{-1} spray with 0.75 mg L^{-1} 24-EBL could not prevent destructive effect of salinity and leaf MDA content increased significantly in comparison with the control.

Increased electrolyte leakage (EL) under salt stress, as an indirect measure of membrane stability, was observed in various crops and was probably due to a reduced ability of the cell to regulate movement through channels (17, 19, 26).

In our experiment, it appears that 24-EBL has protective effect on membrane damage in loquat which is concordant with the results obtained by Ali et al. (2) on mustard.

It has been reported that up regulation of stress protective bio-molecules in 24-EBL-treated plants have enhanced the capacity to limit the damage caused by species of reactive oxygen. Arora et al. (7) had also reported that BRs may help membrane integrity by enhancing the level of the antioxidant system that protects the plant from the oxidative damage. On the other hand, MDA a decomposition product of polyunsaturated fatty acids produced during the peroxidation of membrane lipids is used as an indicator of oxidative damage (28). The higher

levels of MDA found in our stressed plants also implies that they had higher bulk oxidative lipid metabolism in their leaves compared with the control. Salinity can cause oxidative damage to cell membrane and lipids (lipid peroxidation), leading to an increase in electrolyte leakage (3). Supporting this idea, the electrolyte leakage was positively correlated with the MDA content (r =0.66), indicating that the membrane injury induced by salt stress is a result of oxidative damage.

Table 2. The effect of 24-epibrassinolide (EBL) on ion leakage and MDA content of loquat plants under NaCl stress.

24-EBL mg L^{-1}	NaCl EC dS m^{-1}	Ion leakage (%)	Mean	MDA nmol g^{-1} FW	Mean
0	Cont(0.5)	14.2hi		1.7d-h	
	2	18.3h		2.1de	
	4	27.5g		2.5bc	
	6	37.5cd		2.7ab	
	8	47.1a	29A	3a	2.4A
0.25	Cont(0.5)	13.4i		0.8i	
	2	15.3hi		1.3ijk	
	4	26.9g		1.7e-i	
	6	33.2de		1.6g-j	
	8	45.4ab	26.9B	2.1def	1.5C
0.5	Cont(0.5)	13.3i		0.9kl	
	2	13.2i		1.5hji	
	4	24.4g		2.1de	
	6	32.2ef		2d-g	
	8	43.4ab	25.3B	1.9d-h	1.6C
0.75	Cont(0.5)	14hi		1.2jkl	
	2	17.3hi		1.6f-j	
	4	28.6fg		2.2cd	
	6	34.2de		2.9ab	
	8	41.2bc	27.1AB	3.1a	2.2B
Analysis of variance (F values)					
Salinity		249.3***		48.3***	
24-EBL		4.3**		39.9***	
Salinity × 24-EBL		0.5 ns		2.5**	

In each column, means with different letters are significantly different (P<0.05)
*, **, * * *; significant at 0.05, 0.01, and 0.001 levels, respectively.
ns ; non-significant

The plants treated with EBL, both in presence and absence of stress had higher membrane stability index and decreased peroxidation of membrane lipids. It is assumed that BRs act as secondary messengers for the induction of antioxidant defenses in stressed plants (23). Furthermore, BRs also modify the membrane structure/stability under stress conditions with scavenging ROS by increasing the activity of antioxidant enzyme systems (2).

3.5.Protein content

Increased EC by addition of NaCl caused a significant reduction in leaf total soluble proteins content. This reduction was more pronounced in EC of 6 and 8 dS m^{-1}. In the lower salt concentrations (control, EC of 2 and 4 dS m^{-1}), when plants were sprayed with 0.5 mg L^{-1} 24-EBL, they showed higher amount of protein in comparison with their controls. For example, in EC of 4 dS m^{-1}, an increase of 60 % protein was found in the leaves of seedlings sprayed with 0.5 mg L^{-1} 24-EBL. In EC of 6 dS m^{-1}, 0.25 mg L^{-1} 24-EBl was found to be most effective (Figure 1).

Protein synthesis is an early activated process during seedling growth, which is suppressed by NaCl treatment (9). The protein reduction in a saline environment might be due to the decrease in protein

synthesis, accelerated proteolysis, decrease in the availability of amino acid and denaturation of enzymes involved in protein synthesis (38).

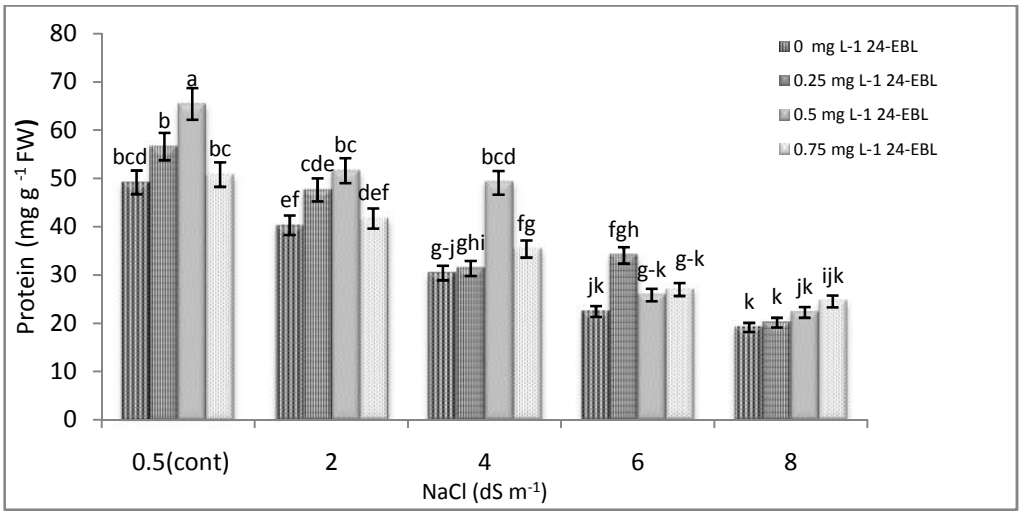

Figure 1. The effect of 24-EBL on potein of loquat plants grown under NaCl stress. Means with different letters are significantly different (P<0.05)

In our experiment, the inhibition of total protein content by salt stress was partially alleviated by 24-EBL application which may be due to synthesis of stress protective protein as reported by Arora et al. (7) in maize seedlings. Increased protein concentrations following the application of BRs have been also reported in the case of mung bean epicotyls (39) and groundnut (36).

3.6. Antioxidant enzymes activities

There was a high significant interaction between salinity and 24-EBL treatments on SOD activity in seedlings leaf. In non-24 EBL treated plants, with increasing EC in the pots, SOD activities increased until 4 dS m^{-1} and then decreased (Figure 2). In EC of 4 dS m^{-1}, the highest activity of SOD occurred in seedlings sprayed with 0.75 mg L^{-1} 24-EBL which was not significantly different from those sprayed with 0.25 mg L^{-1} 24-EBL. In EC of 6 and 8 dS m^{-1}, it was 0.25 mg L^{-1} that caused higher activity of SOD (Figure 2).

In all levels of salinity (except EC8 dS m^{-1}), the activity of POD in seedlings treated with different concentrations of 24-EBL significantly increased. The highest POD activity was found in EC 6 dS m^{-1} when plants were treated with 0.25 mg L^{-1} 24-EBL (Figure 3).

In non-24 EBL treated plants, CAT activity decreased with increasing salinity in the pots. In all levels of salinity (EC 2, 4, 6 and 8 dS m^{-1}), 0.5 mg L^{-1} 24-EBL amplified the leaf CAT activity. The highest activity of CAT was recorded in seedlings treated with 0.5 mg L^{-1} 24-EBL solution in EC 2 dS m^{-1} (Figure 4).

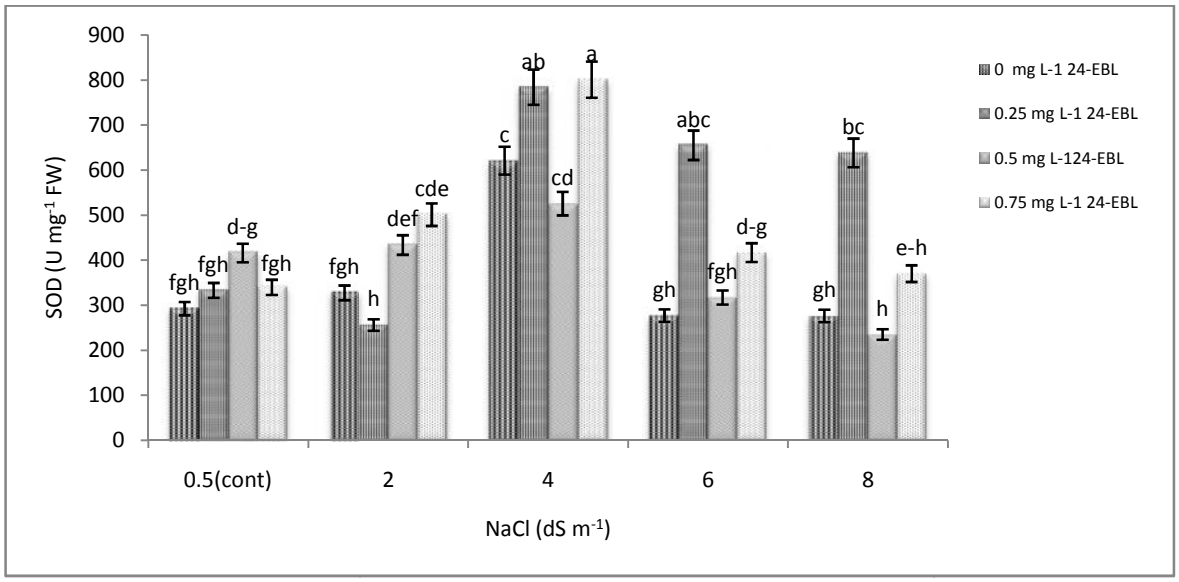

Figure 2. The effect of 24-EBL on the activity of SOD of loquat plants grown under NaCl stress. Means with different letters are significantly different (P<0.05)

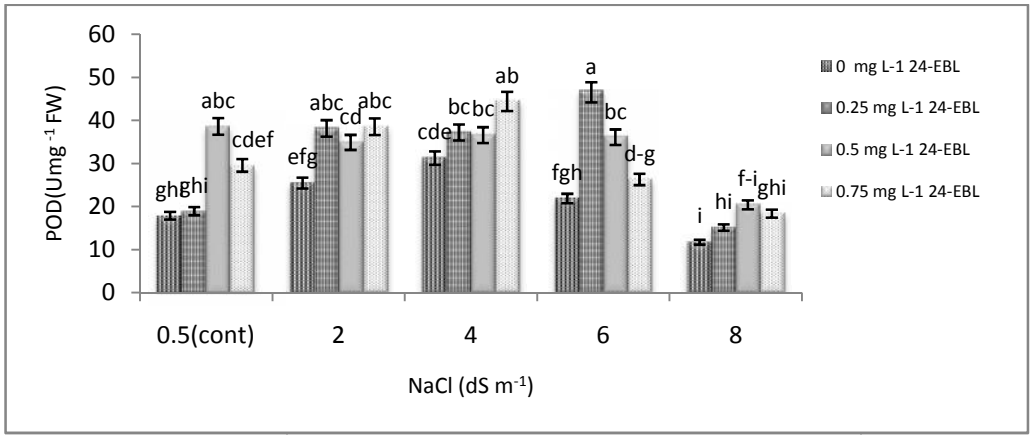

Figure 3. The effect of 24-EBL on the activity of POD of loquat plants grown under NaCl stress. Means with different letters are significantly different (P<0.05)

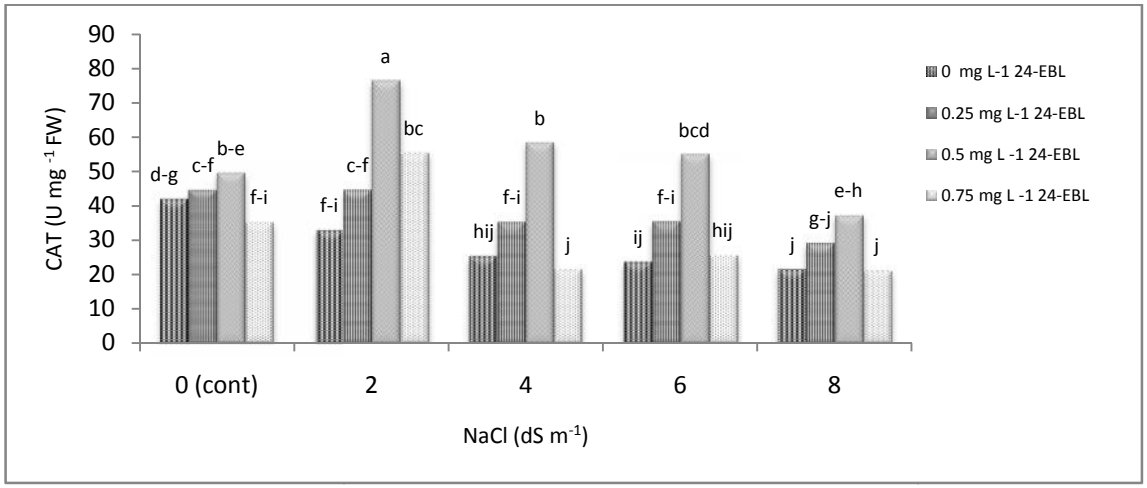

Figure 4. The effect of 24-EBL on the activity of CATof loquat plants grown under NaCl stress. Means with different letters are significantly different (P<0.05)

Super oxide dismutase (SOD) functions as the first line of defense against oxidation at the membrane boundaries (28). SOD catalyses the dismutation of superoxide anion radicals (O_2^-) with great efficiency resulting in the production of H_2O_2 and O_2 (34) which improves the scavenging systems of cell and reduces the accumulation of free radicals.

Catalase is a heme-containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen. (34).

POD is also one of the major systems for the enzymatic removal of H_2O_2 in plants. The increased activities of POD in plants suggest the protective role of the enzyme in salinity stress.

Salt-treated plants having 24-EBL supplements showed higher SOD, CAT and POD activities than the same plants without 24-EBL treatments. Results clearly suggest the positive role of 24-EBL in up regulating the SOD, CAT and POD activities in loquat under salinity. Similar effects of BRs in increasing the antioxidant enzymes activities have also been observed by other studies (1,20, 24). Zhang et al. (41) and Ozdemir et al. (31) also reported that BR-treated plants showed very efficient antioxidative defense mechanism for detoxifying and scavenging of toxic oxygen species through an adoptive means involving up regulation of antioxidative enzymes such as SOD, CAT and POD. The reason for the increase in the activity of these enzymes may be the effects of BRs on expression of biosynthetic genes of these enzymes that resulted in increased oxidation of harmful substrates. Hayat et al. (21) also indicated that the elevation in the activities of antioxidative enzymes by BRs is a gene regulated phenomenon. The results of present study are in agreement with findings of Yu-xian et al. (40) who reported that under different concentrations of natural brassinolide, MDA content was decreased dramatically, while SOD and POD activities were increased significantly in Fuji apple.

Verma et al. (37) found that application of homobrassinolide increased catalase and peroxidase activities and decreased membrane lipid peroxidation in groundnut.

These results, accompanied with the decrease in lipid peroxidation contents, probably represent a decline in ROS and an indicator of removal of stressful conditions by antioxidant enzymes activated by BR.

4. Conclusions

In conclusion, the present study demonstrated that 24-EBL can ameliorate the detrimental effect of salinity stress on loquat growth. It seems that 0.25-0.5 mg L^{-1} 24-EBL treatments in EC 4 dS m^{-1} was the best. Our results suggest that 24-EBL application under salt stress conditions alters the equilibrium between free radical production and enzymatic defence reactions in loquat by enhancing the protein content and free radical scavenging capacity.

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