

Treatment with 24-epibrassinolide mitigates NaCl-induced toxicity by enhancing carbohydrate metabolism, osmolyte accumulation, and antioxidant activity in *Pisum sativum*

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Abstract: A pot culture study was performed to evaluate the differences between salt-stressed pea (*Pisum sativum* L.) plants of tolerant (Climax and Samarina Zard) and sensitive (Ambassidar and PF-400) genotypes, and to determine whether treatment with 24-epibrassinolide (EBL) could enhance the accumulation of osmolytes and antioxidant activity and thereby induce tolerance in salt-stressed plants. Three-week-old seedlings were subjected to +NaCl (5 dS m⁻¹) and -NaCl conditions (0 dS m⁻¹). After 4 days of salt stress, the plants were sprayed with Milli-Q water or EBL (0.125 mg L⁻¹). The plants were sampled at 32 days after sowing and at the final maturity stage. Half-strength Hoagland solution was used as the nutrient medium. Both the tolerant and sensitive plants subjected to saline condition showed decreases in germination percentage, embryo axis length, plant fresh/dry biomass, leaf area, chlorophyll content, relative water content (RWC), photosynthesis, rubisco content, rubisco activity, stomatal conductance, number of stomata, stomatal size, number of epidermal cells, and yield attributes and increases in lipid peroxidation, total phenolics content, proline content, glycine betaine content, total amino acids content, total soluble sugars content, sucrose content, sucrose phosphate synthase activity, sucrose synthase activity, and activities of antioxidant enzymes (superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and guaiacol peroxidase). Salt stress had a less deleterious effect on Climax and Samarina Zard than on Ambassidar and PF-400. The foliar application of EBL mitigated the deleterious effects of NaCl by improving growth, productivity, and gas exchange attributes and by further enhancing the concentrations of the above osmolytes and antioxidants, particularly in the tolerant genotypes. The enhanced concentrations of osmolytes and antioxidants may have induced salt tolerance in the plants subjected to saline conditions, resulting in improved growth, gas exchange attributes, and yield.

Key words: 24-Epibrassinolide, antioxidants, lipid peroxidation, NaCl toxicity, osmolytes, photosynthesis, rubisco

1. Introduction

Abiotic stresses drastically affect crop production by reducing yield. Plants face many distinctive abiotic stresses at different stages of their growth and development. Water, light, heat, and salt stress are the major abiotic stresses that ultimately affect plant growth. Among these, salt stress (salinity) also has a limiting effect on plant growth and productivity (Parida and Das, 2005) and this effect varies from place to place. Plants cope with these abiotic stresses through a variety of mechanisms including changes in morphological, physiological, and biochemical processes (Shahid et al., 2011a; Balal et al., 2012).

High concentrations of salts affect physio-biochemical aspects such as photosynthesis rate, transpiration rate,

stomatal conductance, water use efficiency, and sugar, protein, and water metabolisms. Nutritional imbalance can also result from the antagonistic effect between sodium (Na⁺) and potassium (K⁺) for crucial binding sites in various physiological and cellular metabolic processes (Tester and Davenport, 2003).

Various biochemical and mechanical techniques have been adopted to mitigate the harmful effects of salinity. One approach, the reclamation of soils to make them suitable for plant growth, is highly labor-intensive and expensive. As an alternative, many growth hormone- or chemical-related studies have attempted to induce or increase salt tolerance in plants. The salt tolerance potential can be augmented by the exogenous application of growth promoters such

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as gibberellins, auxins, silicates, oxalic acid, ascorbic acid, proline, polyamines, glycine betaine, and brassinosteroids (BRs) (Karlidag et al., 2011). In some successful studies, growth hormones were found to be involved in inducing tolerance against abiotic stresses (Roychoudhury et al., 2011). BRs, 1 of the 6 classes of plant hormones, have high growth promoting activity and comprise approximately 60 types of phytosteroids (Bajguz and Tretyn, 2003). They are found in various vegetative structures (buds, leaves, roots, and shoots), reproductive structures (flowers, pollens, and seeds), and vascular bundle tissues (cambium) (Bajguz and Hayat, 2009). Approximately 70 types of BRs have been identified from various plant parts in either free form or combined with fatty acids or sugars (Bajguz and Tretyn, 2003). BRs have been shown to accelerate many growth and developmental processes, including the formation of roots, shoots, flowers, and fruits (Hayat and Ahmad, 2003). BRs have also been identified as inducers of tolerance against abiotic stresses in many plant genotypes. BRs in combination with gibberellins and auxins regulate stem elongation. BRs at very low concentrations are involved in cell differentiation, cell elongation, root development, hypocotyl development, and the promotion of adventitious roots (Clouse, 1996). BRs, when applied in combination with other growth hormones, accelerate cell division (Pereira-Netto et al., 2003). While salt stress enhances the generation of radical oxygen species (ROS), BRs induce salt tolerance by enhancing the enzymatic activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) and the concentrations of ascorbic acid, tocopherols, carotenoids, and glutathione in plants growing under environmental stresses, particularly salt stress (Bajguz and Hayat, 2009). BRs (particularly 24-epibrassinolide (EBL)) have been reported to increase seed germination, seedling growth, chlorophyll activity, and nitrate reductase activity (NRA) in salinized plants (Bajguz and Hayat, 2009). BRs also regulate various morphological, physiological, and biochemical processes or attributes including photosynthesis, stomatal conductance, transpiration, and water use efficiency in many plant species under stressful conditions (Yuan et al., 2010).

In our previous study (Shahid et al., 2011b), we investigated the effect of EBL to mitigate the drastic effect of salt stress, when applied as seed treatments before sowing. The results of that study indicated that seed treatment with solution of EBL is highly effective in alleviating the salinity-induced drastic effects in *Pisum sativum* L. However, there was a need to check the mitigating role of EBL against salt stress, when applied as foliar spray instead of seed treatment. Therefore, the present study was planned to examine the changes in antioxidant

systems and osmolyte accumulation in response to foliar application of EBL in salt-tolerant and nontolerant *Pisum sativum* plants grown under high NaCl conditions and to evaluate possible correlations among antioxidant enzymes, organic osmolytes, and salt-tolerance potential in terms of enhanced growth, productivity, and photosynthetic apparatus. The hypothesis tested is that foliar application of EBL enhances osmolyte concentrations and the enzymatic activities of antioxidants and thereby protects plants from NaCl-induced stress.

2. Materials and methods

2.1. In pot experiments

Seeds of the *Pisum sativum* genotypes Climax (CMX) and Samarina Zard (SMZ) (salt tolerant) and Ambassador (AMB) and PF-400 (PF) (salt sensitive) (screened in a separate study; unpublished data) were sown in plastic pots (9-L capacity; perforated bottom) filled with Astatula fine sand (hyperthermic, uncoated typic quartzipsamments) as a growth medium. Before, sowing seeds were disinfected with 10% sodium hypochlorite solution. The temperature at the time of germination was kept in the range of 22–24 °C. The tested *Pisum sativum* genotypes were selected on the basis of percent increase/decrease in growth, physiological, biochemical, enzymatic, and ionic attributes. The sand properties were pH 6.0–6.5, field capacity 7.2%, and incipient wilting at 1.2% (volume basis). Ten seeds per pot were sown; following the emergence of the first true leaves (15 days after germination), the number of plants per pot was adjusted to 6 by thinning out the weak and less vigorous plants. The plants were irrigated according to their need, which was estimated by observing the wetness of the sand. Overall, approximately 300 mL of Milli-Q water having Hoagland solution (30 mL per liter) was applied to each pot to fulfill their irrigational requirements. The pots were placed in a growth chamber adjusted to 26/16 °C day/night, RH 85%, and light intensity 2200 lx from fluorescent tubes. Twenty-one days after sowing (DAS), the plants were subjected to +NaCl (5 dS m⁻¹) or -NaCl (0 dS m⁻¹) stress. The salinity was applied by dissolving a specific quantity of pure NaCl in irrigational water (Milli-Q + Hoagland solution). The desired salinity concentration (5 dS m⁻¹ NaCl) was achieved by steps to avoid osmotic shock. For this purpose, plants were exposed to a low salinity level (2.5 dS m⁻¹) and after 2 days the desired salinity level (5 dS m⁻¹) was created. The saline solution of electrical conductivity (EC) 2.5 and 5.0 dS m⁻¹ was made by dissolving 1.46 g and 2.93 g of NaCl, respectively, per liter of Milli-Q water. The EC of the saline solution and the sand within the pots was confirmed by EC meter (Model HI-9813-0, Hanna, New York, USA) every 3–4 days. This NaCl concentration was chosen because preliminary studies showed that concentrations above 5 dS m⁻¹ were highly damaging to

the plant. After 96 h of salt stress, the plants were sprayed with -EBL (Milli-Q water only) or +EBL (0.125 mg L⁻¹ EBL in Milli-Q water) solution. The EBL was sprayed using a locally made, small, hand-operated sprayer for 3 consecutive days (once a day) after 96 h of salinity application. The desired EBL concentration was attained by diluting the stock solution (made by dissolving EBL in 3 mL of ethanol) to the final volume by addition of Milli-Q water. The EBL concentration (0.125 mg L⁻¹) used in this study was selected based on a preliminary optimization experiment using plastic trays (data not shown). This EBL concentration gave excellent results in terms of various morphological and growth attributes (data not shown). Plants were harvested at 32 DAS for the estimation of leaf area, RWC, photosynthetic activity, stomatal conductance, number of stomata and epidermal cells, stomatal size, amino acids, proline, glycine betaine, total soluble sugars, chlorophyll content, lipid peroxidation, total phenolics, SOD, peroxidase (POD), CAT, APX, and GPX. For the estimation of the above-mentioned attributes except leaf area, RWC, photosynthetic activity, and stomatal conductance, 2 plants per replicate (10 plants for each treatment) were used. However, for the measurement of leaf area, RWC, photosynthetic activity, and stomatal conductance, 3 plants for each replicate per treatment were used. For the estimation of yield attributes, the remaining 4 plants for each replication per treatment were used.

2.2. Petri dish experiments

Germination percentage and embryo axis length were determined in petri dishes. Seeds were disinfected with 10% sodium hypochlorite and grown in petri dishes on Whatman filter paper (20 seeds per dish) wetted with Milli-Q water (without NaCl) or a defined saline solution (5.0 dS m⁻¹). The saline solution of electrical conductivity (EC) 5.0 dS m⁻¹ was made by dissolving 2.93 g of NaCl per liter of Milli-Q water. The EC of the saline solution was confirmed with an EC meter (Model HI-9813-0, Hanna, New York, USA). The EBL was applied as spray on seeds (3 consecutive sprays at 24-h interval), with a small handheld sprayer. During spraying all seeds were turned one by one with the help of forceps, to ensure the full saturation of seed from all sides by EBL spray. The treatments were replicated 5 times and placed in a growth chamber at 20 to 22 °C. Germination was calculated for a 5-day period. The germination percentage was calculated as follows:

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

2.3. Determination of morphological attributes

At the end of the experiment (90 DAS), 3 plants from each replicate were gently uprooted and washed with distilled water to remove sand particles. The plants were then blotted with filter paper to remove any water present on

the leaves and shoots and placed on a digital balance for the measurement of fresh weight. The plants were then oven dried (Mettler-110, Schawabach, Germany) at 72 °C for 1 week, and the average dry weight of each replicate was recorded. The leaf area was calculated (from 9 leaves per replicate) using a leaf area meter (LI-3100; LI-COR Inc., Lincoln, NE, USA), and the average leaf area per replicate was recorded. These 9 leaves were selected from upper, middle, and lower portions of the plants.

2.4. Photosynthesis and stomatal conductance

For the measurement of physiological attributes such as net photosynthesis rate (P_n) and stomatal conductance (g_s), 3 young, fully developed and healthy leaves per plant (3 plants from each replicate per treatment) were selected and placed individually in the chamber of a portable infrared gas analyzer (IRGA) (Analytical Development Company, Hoddesdon, UK). All recordings of photosynthesis rate and stomatal conductance were taken between 1000 and 1200 hours with the following conditions: leaf chamber volume gas flow rate (v) 296 mL min⁻¹, molar air flow per unit leaf area 389.4 mmol m⁻² s⁻¹, atmospheric pressure 97.5 kPa, water vapor pressure in the chamber 6.3–8.4 mbar, photosynthetic active radiation (PAR) at the leaf surface maximum 1633 μmol m⁻² s⁻¹, leaf temperature 27.2 °C, ambient temperature 21.8 °C, and ambient CO₂ concentration 337 μmol mol⁻¹.

2.5. Stomatal size and the numbers of stomata and epidermal cells

The numbers of stomata and epidermal cells were counted by separating a very thin abaxial layer (2 × 3 mm) from the lower epidermis of the leaf. This dry film was carefully separated, placed on a microscope slide under a coverslip, adjusted on the stage of a Nikon EFD-3 microscope (F-601, Type-104, Tokyo, Japan), and observed at various magnifications. The numbers of stomata and epidermal cells were counted under a magnification of 40 × 10 on the full screen. The lengths and widths of stomata and epidermal cells were measured in micrometers (Moya et al., 2003).

2.6. Relative water content (RWC)

Three mature leaves were detached from each of 10 randomly chosen plants (2 plants from each replicate per treatment). The leaves were tagged, washed under tap water for at least 5 min, blotted with tissue paper, weighed, kept in tap water for 24 h, and weighed again (turgid weight: TW). The leaves were then oven dried at 72 °C and their dry weight was measured on a digital electrical balance (Bosch AE-160, Germany). The average RWC per replicate was calculated by the method of Barrs and Weatherley (1962):

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TD} - \text{DW}} \times 100$$

2.7. Determination of osmolytes and biochemical attributes

All osmolytes and biochemical attributes were determined by using fresh leaf samples. The chlorophyll concentration (Chl) was estimated by the method of Arnon (1949). The free proline content of fresh leaves was estimated by the method of Bates et al. (1973). The glycine betaine content of fresh leaves was determined by the method of Grieve and Grattan (1983). Total free amino acids were estimated by the method of Hamilton and Van Slyke (1973). Total soluble sugars were estimated by the method of Riazi et al. (1985). Leaf sucrose content was estimated by the phenol-sulfuric acid method (Buysse and Merckx, 1993). Total soluble proteins were estimated by the method of Lowry et al. (1951). Lipid peroxidation (LPO) was estimated by measuring the concentrations of malondialdehyde (MDA) and thiobarbituric acid (TBA) as described by Heath and Packer (1968) in fresh leaf samples. Total phenolics in fresh leaves were determined by the method of Julkunen-Tiitto (1985). The rubisco content was assessed by the spectrophotometric method of Makino et al. (1994). The upper fully expanded young leaves were cut into small segments and then homogenized in 50 mM Na-phosphate buffer (pH 7.0) containing 120 mM 2-mercaptoethanol, 2 mM iodoacetic acid, and 5% (v/v) glycerol at a leaf buffer ratio of 1:9 (g mL⁻¹) in a chilled mortar and pestle. To estimate the rubisco activity, a Triton X-100 solution to a final concentration of 0.1% (v/v) was added to a portion of the leaf homogenate. After centrifugation of 1000 × g for 3 min, the supernatant fluid was treated with a lithium dodecylsulfate solution (1.0% (w/v), final concentration) at 100 °C for 1 min. This preparation was stored at -35 °C until analyzed by SDS-PAGE. The amount of rubisco was determined spectrophotometrically after formamide extraction of Coomassie brilliant blue R-250-stained subunit bands separated by SDS-PAGE. A calibration curve was made with rubisco purified from *Pisum sativum* leaves. However, rubisco activity was estimated by the spectrophotometric method of Lilley and Walker (1974).

2.8. Determination of antioxidant enzyme activities

For estimating antioxidant enzyme activities, fresh leaves (0.5 g) were ground in an ice-cooled tissue grinder in 5 mL of 50 mM cooled phosphate buffer (pH 7.8). The homogeneous mixture was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used to determine the activities of the following enzymes. SOD activity was analyzed by calculating its potential to hinder the photoreduction of nitrobluetetrazolium (NBT), as described by Giannopolitis and Ries (1977). CAT and peroxidase (POD) activities were measured by the method of Maehly (1954) with some modification as follows. The CAT reaction solution (3 mL) comprised 50 mM phosphate buffer (pH 7.0), 5.9 mM H₂O₂, and 0.1 mL of

enzyme extract. Changes in the absorbance of the reaction solution at 240 nm were recorded every 20 s. One unit of CAT activity was defined as an absorbance change of 0.01 units min⁻¹. The POD reaction solution (3 mL) comprised 50 mM phosphate buffer (pH 5.0), 20 mM guaiacol, 40 mM H₂O₂, and 0.1 mL of enzyme extract. Changes in the absorbance of the reaction solution at 470 nm were recorded every 20 s. One unit of POD activity was defined as an absorbance change of 0.01 units min⁻¹. The activity of each enzyme was expressed on the basis of protein content. APX activity was estimated by the method of Nakano and Asada (1981). GPX activity was estimated by the method of Urbanek et al. (1991).

2.9. Determination of Na⁺ and Cl⁻

Before the estimation of Na⁺, the plant material was digested. For this purpose the dried ground plant material (0.5 g) (roots and leaves) was digested individually with concentrated sulfuric acid (5 mL) in digestion tubes as described by Wolf (1990). The digested samples of roots and shoots were analyzed for Na⁺ by flame photometer (Jenway PFP-7, UK). A standard curve (SC) was drawn on the basis of graded series of standards (ranging from 10 to 100 mg L⁻¹) of Na⁺. The values of Na⁺ from the flame photometer were compared with SC and actual ratios were calculated.

The chloride was determined by grinding the dried roots and leaves in a grinder. Then this fine plant material (1 g) of both roots and leaves was heated overnight in distilled water (20 mL) in test tubes at 65 °C in an oven. The extract obtained after overnight heating was filtered with Whatman-40 filter paper and used for the determination of chloride contents with the help of an analyzer (Corning-920, Germany).

2.10. Determination of yield attributes

The yield attributes (1000-seed weight, number of pods plant⁻¹, and number of seeds pod⁻¹) were determined at the end of the experiments (90 DAS). After the use of 2 plants per replicate (for the estimation of plant biomass at 32 DAS), the remaining 4 plants per replicate of each treatment were used for the determination of yield attributes. The number of pods plant⁻¹ was counted, and the average pod number for the replicate was computed. After the pod numbers were counted, seeds were separated from the same pods, the seeds were counted, and the average number of seeds pod⁻¹ was calculated. Uniformly sized seeds (300 seeds per replication of each treatment) from the pods of every plant of each replication per treatment were taken and weighed using a digital balance, and then recalculated into 1000-seed weight.

2.11. Statistical analysis

The experiment was laid out in 3-factor (salinity, EBL, and genotypes) factorial arrangement under a completely randomized design with 5 replications. There were 5 pots

for each treatment and 1 pot with 6 plants was considered as 1 replicate. The data were analyzed by standard statistical procedures as described by Gomez and Gomez (1984). Tukey's test was used to evaluate the significance of differences between the treatments at $P \leq 0.05$ ($n = 5$).

3. Results

3.1. The effect of EBL on growth attributes

The plants of salt-sensitive genotypes (AMB and PF-400) subjected to +NaCl conditions showed significant reductions in growth attributes, i.e. germination percentage, embryo axis length, plant biomass, and leaf area, in comparison with the plants grown under -NaCl conditions (Figure 1). However, the degree of reduction of germination in the salt-tolerant genotypes (CMX and SMZ) was nonsignificant. On the other hand, salt stress significantly reduced embryo axis length in SMZ, whereas a nonsignificant effect was observed in CMX. The foliar application of EBL enhanced these growth attributes under +NaCl conditions compared with -NaCl conditions (controls). The EBL treatment improved the germination in salt-sensitive genotypes by 24% to

30%, while it was ineffective for salt-tolerant genotypes. Similarly, EBL was also ineffective for embryo axis length in genotypes AMB, CMX, and PF-400 both under saline and nonsaline conditions, but foliar application of EBL significantly improved embryo axis length in SMZ. Considerable increases in plant fresh and dry weights were recorded for salt-stressed plants of SMZ and CMX, but no considerable effect of EBL was recorded in AMB and PF-400 (salt-sensitive genotypes). Overall, EBL had a greater growth promoting effect in stressed plants of the tolerant genotypes (CMX and SMZ) than in those of the sensitive genotypes (AMB and PF-400).

3.2. The effect of EBL on photosynthesis rate and chlorophyll content

Salt stress reduced the photosynthesis rate (P_n) and chlorophyll content in the tested *Pisum sativum* genotypes, but the largest percentage reduction was observed in the salt-sensitive genotypes (AMB and PF-400). This deleterious effect of NaCl was significantly mitigated by EBL treatment in terms of the augmentation of P_n and chlorophyll content (Figure 2). EBL application also increased P_n and chlorophyll content in the plants grown

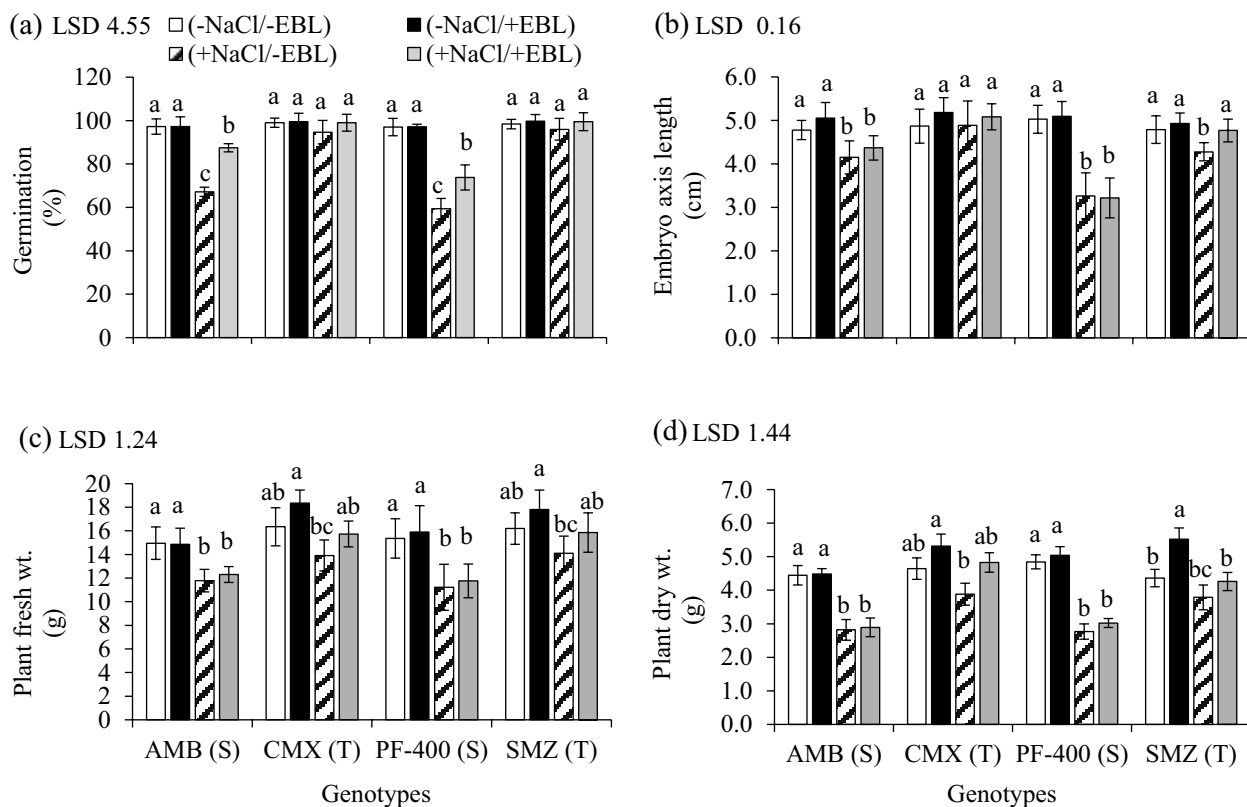


Figure 1. Effect of EBL on germination (a), embryo axis length (b), plant fresh weight (c), and plant dry weight (d) on salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and the vertical bars give the standard error (SE) of the mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassador), CMX (Climax), SMZ (Samarina Zard).

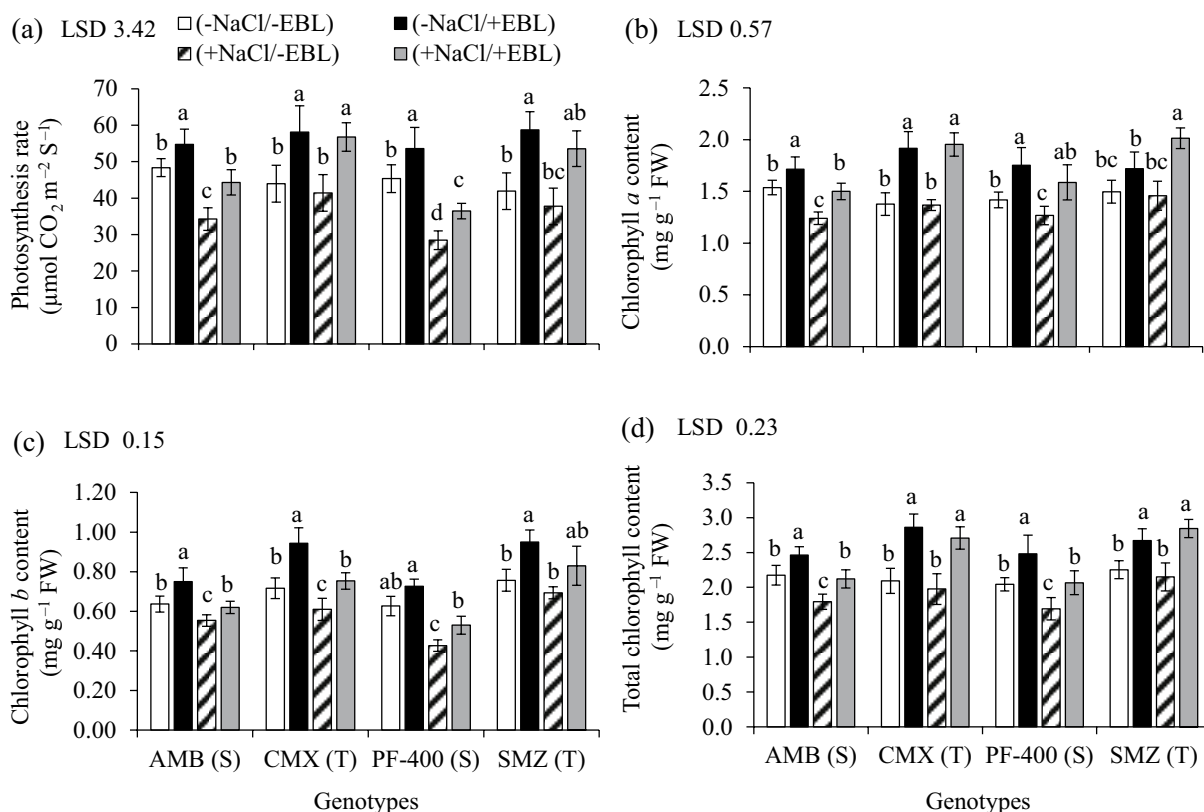


Figure 2. Effect of EBL on photosynthesis (a), chlorophyll a (b), chlorophyll b (c), and total chlorophyll contents (d) on salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassidar), CMX (Climax), SMZ (Samarina Zard).

under unstressed conditions. The plants treated with +NaCl/+EBL showed increases in P_n of 29.85%, 33.32%, 22.74%, and 20.76% for CMX, SMZ, AMB, and PF-400, respectively, in comparison with the plants treated with +NaCl/-EBL. NaCl stress reduced the chlorophyll content (total chlorophyll, chlorophyll a, chlorophyll b) in both the tolerant and sensitive genotypes, but to a greater degree in the sensitive genotypes. The exogenous application of EBL markedly increased the chlorophyll content in salt-stressed plants of the studied genotypes. The plants treated with +NaCl/+EBL showed higher rates (28%) of chlorophyll content than those treated with +NaCl/-EBL. Under stressed conditions, EBL enhanced total chlorophyll by 36.93%, 32.24%, 18.21%, and 22.04%; chlorophyll a by 42.92%, 38.21%, 20.96%, and 25.26%; and chlorophyll b by 23.49%, 19.71%, 12.04%, and 12.5% for CMX, SMZ, AMB, and PF-400, respectively, compared with the control (+NaCl/-EBL).

3.3. The effect of EBL on leaf area, stomatal conductance, stomatal size, and the numbers of stomata, epidermal cells, and RWC

All the plants of tested genotypes subjected to -NaCl/+EBL conditions exhibited significant increases in leaf area, but

under +NaCl/+EBL conditions only salt-tolerant genotypes (SMZ and CMX) had increases in leaf area, by 10% and 15%, respectively, with respect to the control (Figure 3). However, a nonsignificant effect of EBL was recorded for the plants of salt-sensitive genotypes (AMB and PF-400) grown under saline conditions. Regarding stomatal conductance (g_s), EBL treatment did not have a significant effect on stressed AMB and PF-400 plants but in CMX and SMZ plants caused a 12.94% and 10.24% increase, respectively, relative to the controls (+NaCl/-EBL) (Figure 3). The plants of all tested genotypes, except PF-400, grown under stressed (+NaCl/-EBL) conditions had leaves with smaller stomatal size and a lower number of epidermal cells compared with those grown under unstressed (-NaCl/-EBL) conditions (Figure 3). There was no significant effect of salt stress or EBL treatment on the number of stomata in either the tolerant or sensitive genotypes (Figure 3). EBL treatment significantly facilitated gaseous exchange by increasing stomatal size under both stressed (+NaCl) and unstressed (-NaCl) conditions. CMX, SMZ, AMB, and PF-400 showed increases in stomatal size of 37.28%, 41.74%, 29.31%, and 28.0%, respectively, when exposed to +NaCl/+EBL treatment. However, EBL

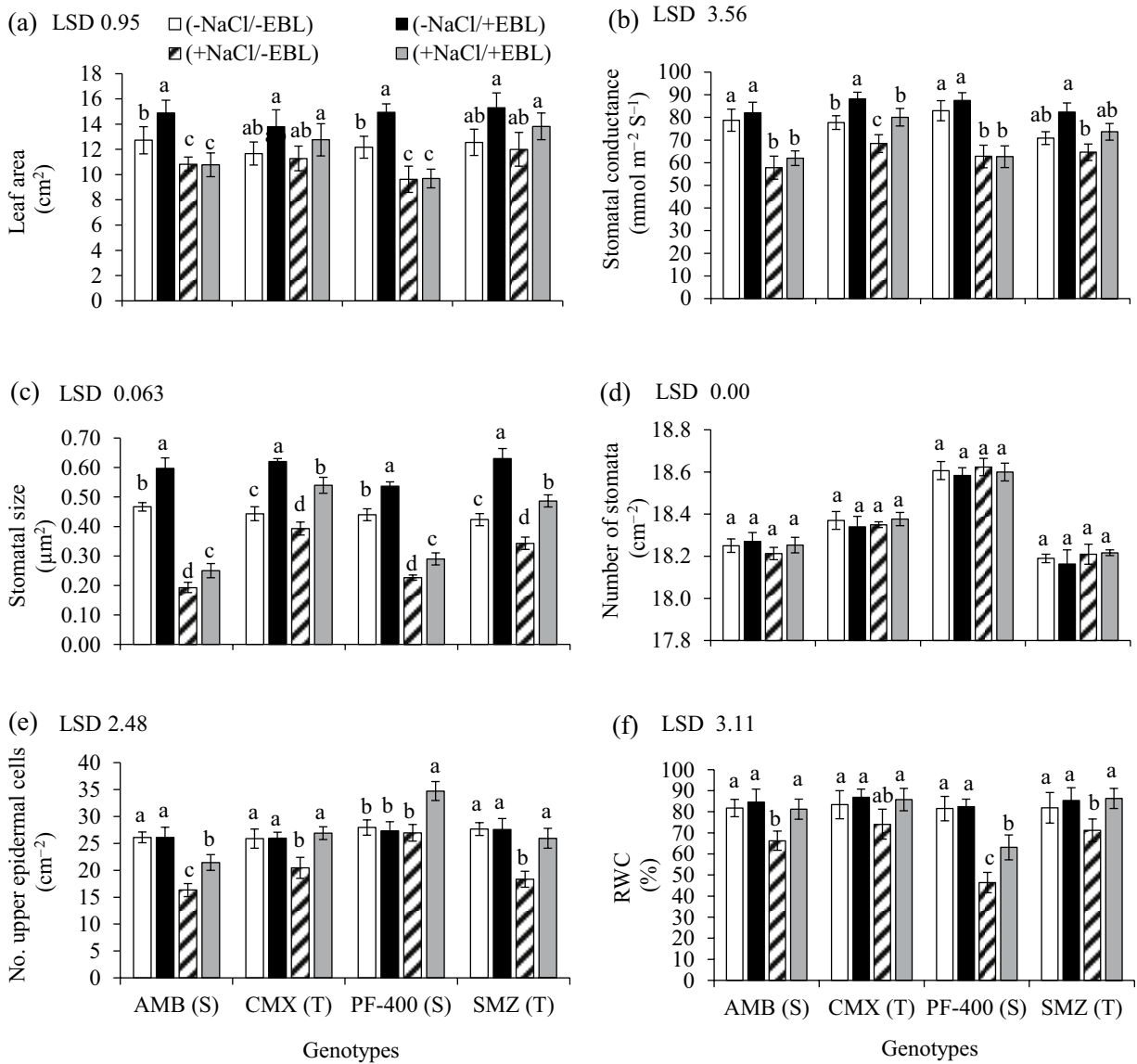


Figure 3. Effect of EBL on leaf area (a) stomatal conductance (b), stomatal size (c), number of stomata (d), number of upper epidermal cells (e), and relative water content (RWC) (f) of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments excluding number of stomata were significant at $P \leq 0.05$. AMB (Ambassidar), CMX (Climax), SMZ (Samarina Zard).

treatment increased the number of upper epidermal cells under stressed conditions by 19.77% (Climax), 36.79% (Samarina Zard), 43.68% (Ambassidar), and 28.78% (PF-400), but had no marked effect on unstressed plants (Figure 3). Salt stress significantly reduced the leaf relative water content (RWC) in both salt tolerant and nontolerant *Pisum sativum* genotypes, but the greatest reduction was observed in the salt-sensitive genotypes AMB and PF-400 (Figure 3). This reducing effect of NaCl on RWC was significantly mitigated by the exogenous application of EBL. The plants treated with EBL had high RWC when

grown under saline conditions with respect to their controls. Stressed plants of the sensitive genotypes (AMB and PF-400) showed the greatest increase in RWC (15.42% and 24.42%, respectively) with respect to the controls.

3.4. The effect of EBL on total phenolics and lipid peroxidation

The leaf total phenolic contents of +NaCl-treated plants were lower than those of -NaCl-treated plants. This inhibiting effect of NaCl on phenolic contents was counteracted by the foliar application of EBL (Figure 4). The +NaCl/+EBL treated plants of CMX, SMZ, AMB, and

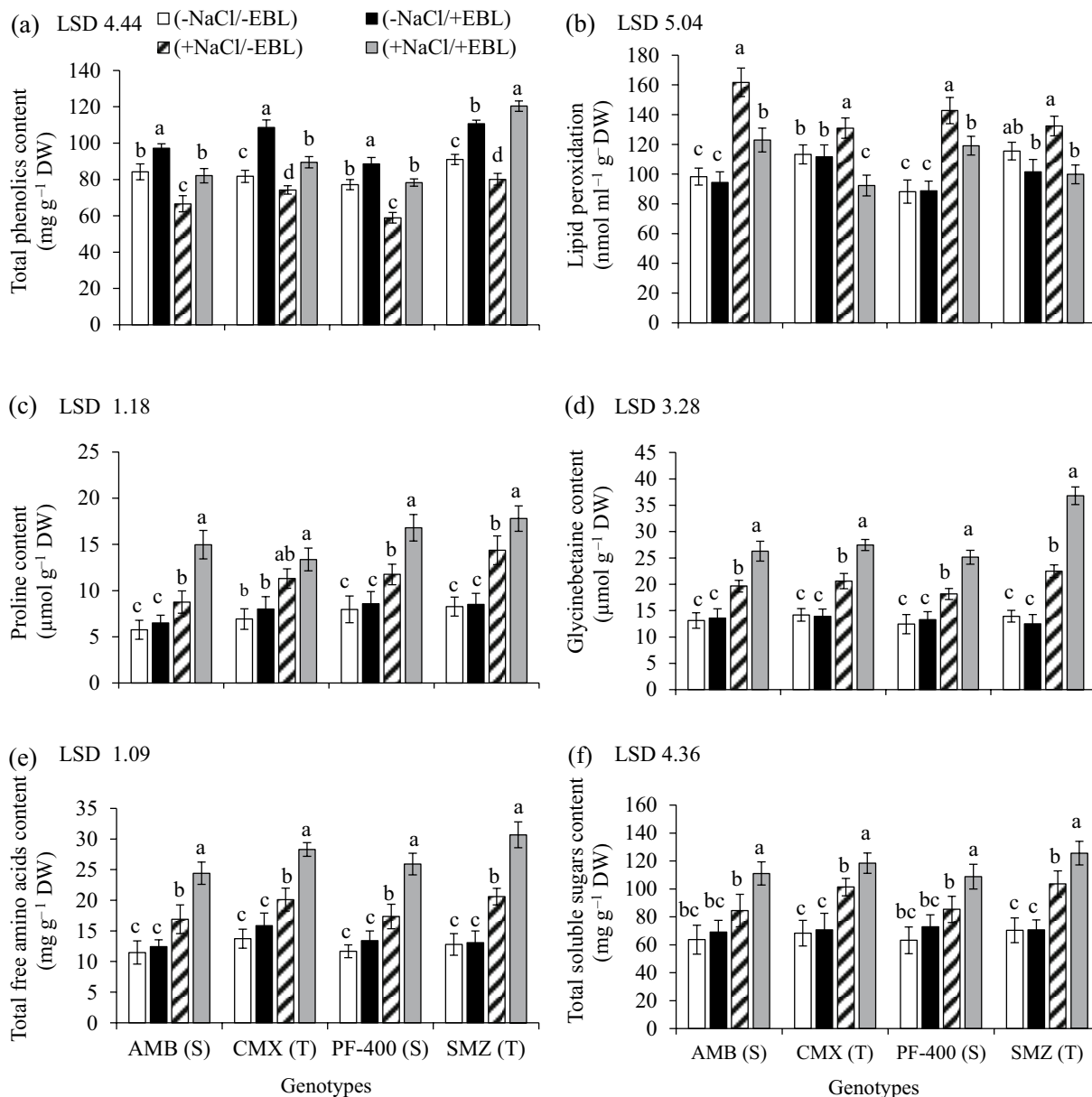


Figure 4. Effect of EBL on total phenolics content (a), lipid peroxidation (b), leaf proline content (c), leaf glycinebetaine content (d), leaf amino acids content (e), and leaf total soluble sugars content (f) of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassidar), CMX (Climax), SMZ (Samarina Zard).

PF-400 showed increases in total leaf phenolic contents of 20.52%, 50.26%, 23.26%, and 32.92%, respectively, in comparison with +NaCl/-EBL treated plants (controls).

A significant increase in lipid peroxidation was observed in all of the plants grown under +NaCl conditions (Figure 4). The degree of this increase was greater for AMB and PF-400 (sensitive) than for CMX and SMZ (tolerant). The follow-up application of EBL markedly reduced the process of lipid peroxidation in the plants subjected to NaCl stress

(Figure 4). The stressed plants (+NaCl/+EBL) of the CMX, SMZ, AMB, and PF-400 genotypes showed reductions in lipid peroxidation of 29.48%, 24.55%, 23.96%, and 16.60%, respectively, with respect to the controls (+NaCl/-EBL).

3.5. The effect of EBL on proline, glycine betaine, amino acids, and total soluble sugars

The concentrations of proline, glycine betaine, total free amino acids, and total soluble sugars showed increases in response to NaCl stress for all the studied genotypes

(Figure 4). The degree of such increase was higher for the tolerant (CMX and SMZ) than for the sensitive genotypes (AMB and PF-400). This high concentration of osmolytes differentiated the salt-tolerant genotypes from the sensitive genotypes in terms of salt tolerance potential. The exogenous application of EBL to the +NaCl-treated plants significantly elevated the concentration of the above-mentioned osmolytes, while no significant effect of EBL was observed in -NaCl-treated plants (Figure 4). EBL treatment increased the content of proline by 42%–71% (salt-tolerant) and 18%–24% (salt-sensitive), glycine betaine by 33%–64% (salt-tolerant) and 33%–34% (salt-sensitive), total free amino acids by 49%–57% (salt-tolerant) and 45%–49% (salt-sensitive), and total soluble sugars by 17%–21% (salt-tolerant) and 27%–31% (salt-sensitive) in the stressed plants. Regarding changes in concentrations of the above osmolytes and sugars (total soluble sugars and sucrose), EBL was ineffective under unstressed conditions (-NaCl) but was highly effective for plants subjected to a +NaCl environment.

3.6. The effect of EBL on rubisco content, rubisco activity, sucrose content, sucrose phosphate synthase, and sucrose synthase enzymes

Salt stress caused a significant reduction in the rubisco content and rubisco activity of both the tolerant and sensitive genotypes (Figure 5). EBL treatment strengthened the photosynthetic apparatus by accelerating rubisco activity and increasing rubisco content in the tested *Pisum sativum* genotypes. The stressed plants sprayed with EBL showed significant increases in rubisco content (20.45%, 23.07%, 18.35%, and 15.33% for CMX, SMZ, AMB, and PF-400, respectively) compared with the controls (+NaCl/-EBL). The foliar application of EBL promoted rubisco activity by 38.54% (CMX), 45.95% (SMZ), 47.71% (AMB), and 57.15% (PF-400). The exogenous application of EBL improved carbohydrate metabolism under NaCl stress by enhancing the sucrose content, activities of sucrose phosphate synthase (SPS), and sucrose synthase (SS) in both the tolerant and nontolerant genotypes (Figure 5). The plants of tested *Pisum sativum* genotypes submitted to salinity had an elevation in their SPS and SS activities, but foliar application of EBL further strengthened the carbohydrate metabolism and osmotic adjustment potential by increasing the enzymatic activities of SPS and SS under saline conditions (+NaCl). However, salinity had a nonsignificant effect on sucrose contents of AMB. The salt-tolerant (CMX and SMZ) and salt-sensitive (AMB and PF-400) genotypes grown under +NaCl/+EBL conditions had increases in sucrose content by 17%–43% and 23%–38%, respectively, as compared to the controls (+NaCl/-EBL). Salt stress caused a reduction in SPS and SS activities, but this reduction was significantly mitigated by EBL treatment (Figure 5). The EBL-treated stressed plants

(+NaCl/+EBL) showed increases in SPS and SS activities by 39% and 27% (Climax), 56% and 37% (Samarina Zard), 33% and 50% (Ambassidar), and 55% and 50% (PF-400), respectively, with respect to the controls (+NaCl/-EBL).

3.7. The effect of EBL on antioxidant activities

The foliar application of EBL significantly ($P \leq 0.05$) alleviated the salinity-induced deleterious effects by strengthening the antioxidant system (Figure 6). Plants subjected to +NaCl/+EBL conditions showed increases in the activities of SOD by 51%–61% and 33%–34%, of POD by 46%–72% and 40%–42%, of CAT by 39%–45% and 20%–24%, of APX by 12%–21% and 15%–20%, and of GPX by 21%–44% and 40%–56% in salt-tolerant and salt-sensitive *Pisum sativum* genotypes, respectively, compared with the plants under +NaCl/-EBL conditions (Figure 6). The SOD, POD, and CAT activity increases were greater for CMX and SMZ (tolerant) than for AMB and PF-400 (sensitive) under +NaCl/-EBL conditions. However, EBL application elevated the APX and GPX activities more in salt-sensitive genotypes than in salt-tolerant ones. EBL treatment had no significant effect on SOD or APX activities in unstressed plants (-NaCl).

3.8. The effect of EBL on root/shoot Na and Cl contents

NaCl stress significantly elevated the concentrations of Na and Cl in both the leaves and roots of salt-stressed *Pisum sativum* plants of AMB, CMX, and SMZ (Figure 7), but there was no significant effect of root zone salinity on roots Cl contents of genotype PF-400. However, foliar applied EBL reduced the root Na contents by 12% and 10% in salt-tolerant genotypes (CMX and SMZ). The salt-stressed plants of CMX and SMZ sprayed with EBL had decreases in leaf Cl contents by 8% and 7%, but EBL did not affect leaf Cl contents of salt-sensitive genotypes (AMB and PF-400). In the case of plants exposed to salt stress and treated with EBL, a considerable reduction in root Cl contents was observed for AMB (6%), CMX (19%), and SMZ (24%). Overall, a nonsignificant effect of EBL on Na and Cl concentrations was observed in *Pisum sativum* plants subjected to -NaCl conditions.

3.9. The effect of EBL on yield attributes

Salt stress significantly ($P \leq 0.05$) reduced the number of seeds pod⁻¹ in both the tolerant (CMX and SMZ) and sensitive genotypes (AMB and PF-400), but the degree of such reduction was greater in sensitive ones (Figure 8). Likewise, salt stress also reduced the 1000-seed weight in AMB and PF-400, but did not affect CMX and SMZ in this regard. This reducing effect of NaCl on number of seeds pod⁻¹ and 1000-seed weight was significantly counteracted by EBL treatment. The plants grown under +NaCl/+EBL conditions displayed increases in seeds pod⁻¹ by 21%–30% and 28%–44% and in 1000-seed weight by 18%–22% and 29%–34% for salt-tolerant and salt-sensitive genotypes, respectively, with respect to the controls (+NaCl/-EBL).

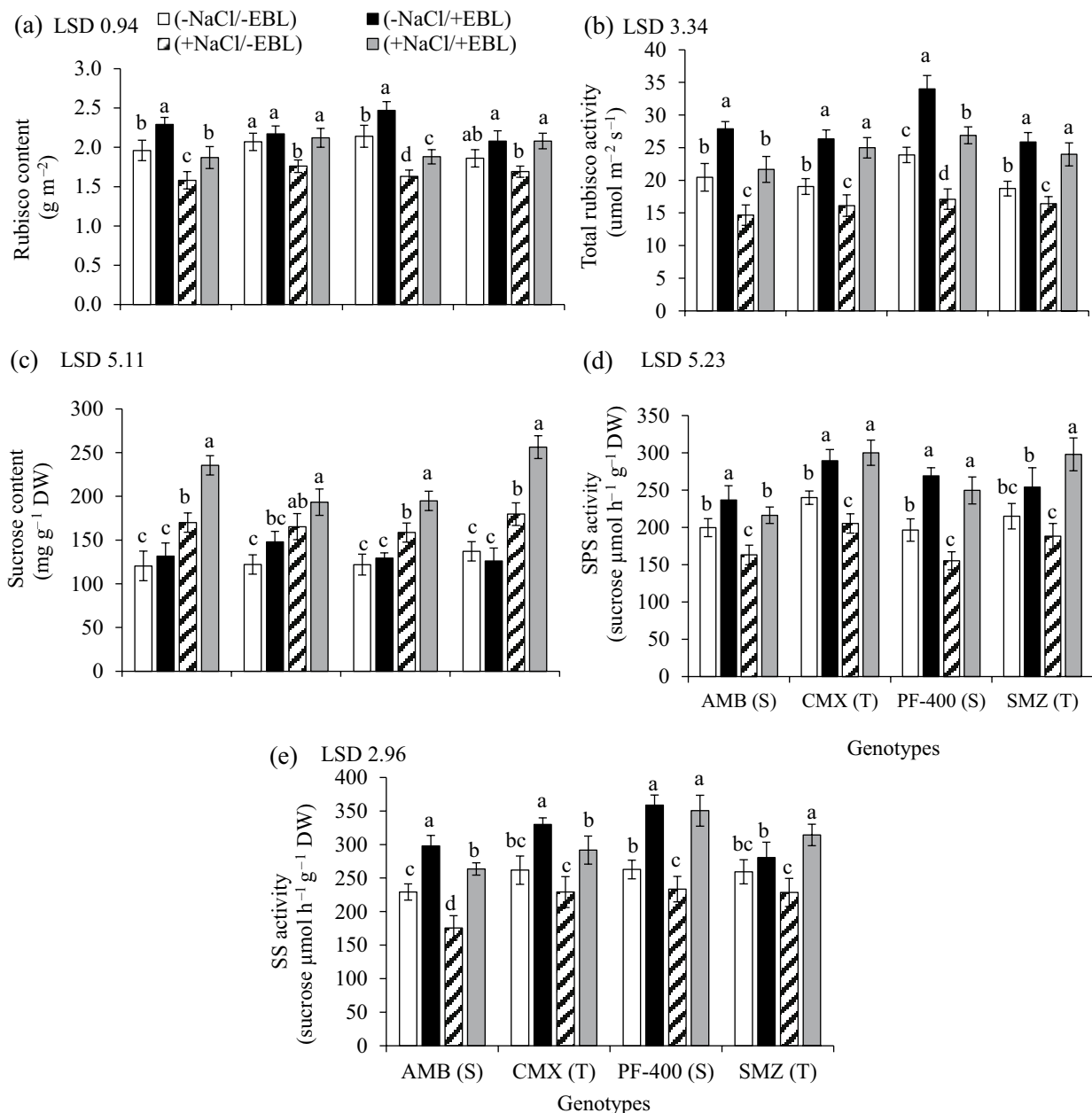


Figure 5. Effect of EBL on leaf rubisco content (a), leaf total rubisco activity (b), leaf sucrose content (c), sucrose phosphate synthase activity (SPS) (d), and sucrose synthase activity (SS) (e) of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey’s test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassidar), CMX (Climax), SMZ (Samarina Zard).

EBL treatment had no significant effect on the number of pods plant⁻¹ under saline (+NaCl) or nonsaline conditions (-NaCl).

4. Discussion

The generation of ROS such as hydroxyl radical (OH⁻), superoxide radical (O₂⁻), and hydrogen peroxide (H₂O₂) is a common phenomenon under stressed environments.

ROS cause damage to molecules (lipid, proteins, nucleic acids, pigments, photosynthesis) and disturb cellular structures. Therefore, the scavenging of ROS is a common mechanism to counteract abiotic stresses. To overcome oxidative damage under stressed conditions, particularly salinity stress, plants have developed antioxidant defense systems composed of various antioxidant enzymes such as APX, GPX, CAT, SOD, and POD (Shahid et al., 2011b;

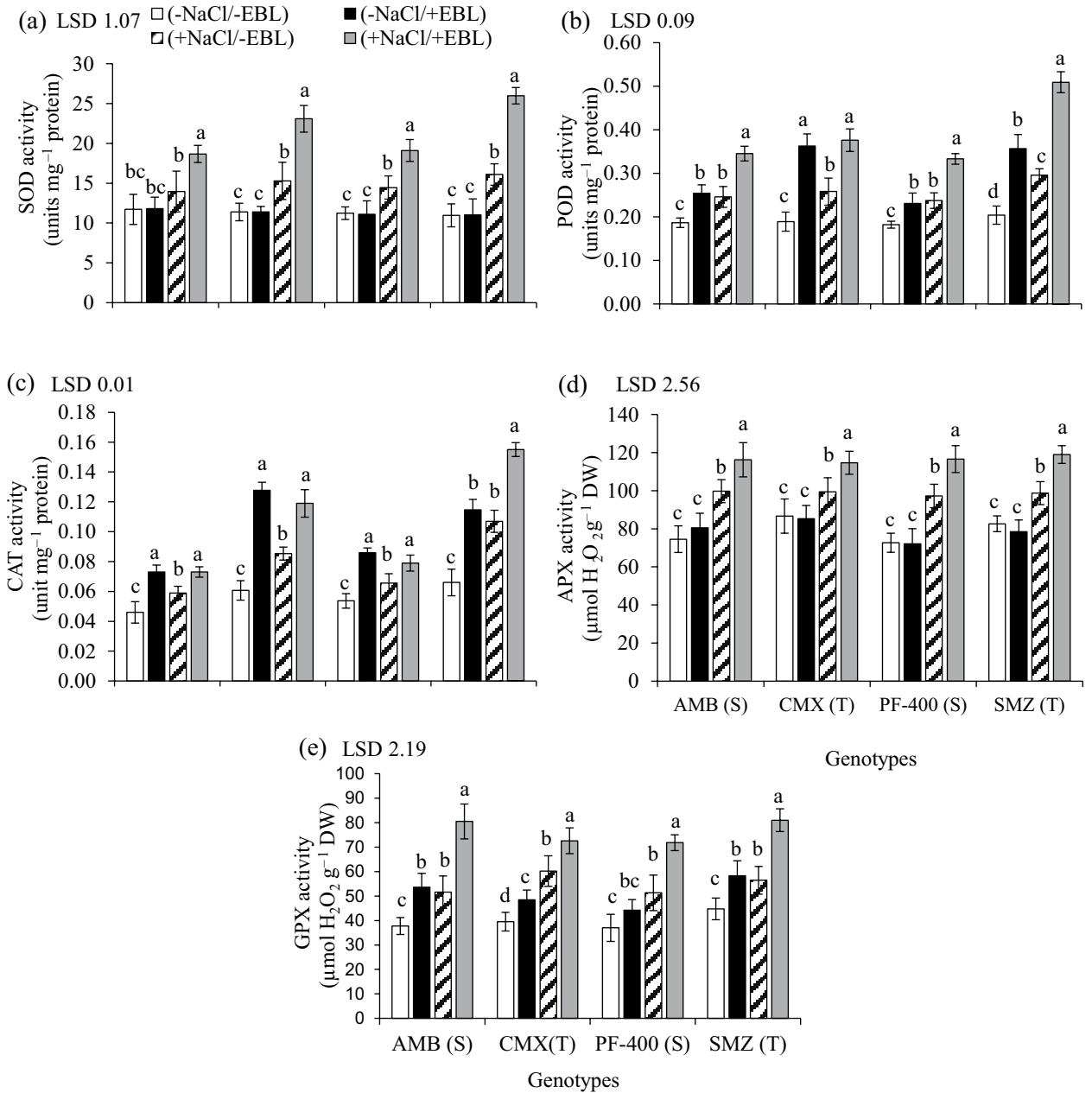


Figure 6. Effect of EBL on superoxide dismutase (SOD) (a), peroxidase (POD) (b), catalase (CAT) (c), ascorbate peroxidase (APX) (d), and guaiacol peroxidase (GPX) (e) in the leaves of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassador), CMX (Climax), SMZ (Samarina Zard).

Sekmen Esen et al., 2012) and low molecular weight compatible osmolytes such as proline, glycine betaine, total amino acids, and total soluble sugars (Meloni et al., 2004; Zhang et al., 2013). We therefore hypothesized that NaCl might enhance the antioxidant activities and concentrations of compatible solutes in both salt tolerant and nontolerant *Pisum sativum* genotypes.

However, the findings of the present study show that EBL treatment enhanced the antioxidant activities as well as the osmolyte concentrations of plants subjected to both +NaCl and -NaCl conditions. EBL treatment enhanced the antioxidant activities and accumulation of organic osmolytes in both the tolerant and nontolerant genotypes. The augmentation of antioxidant activities and

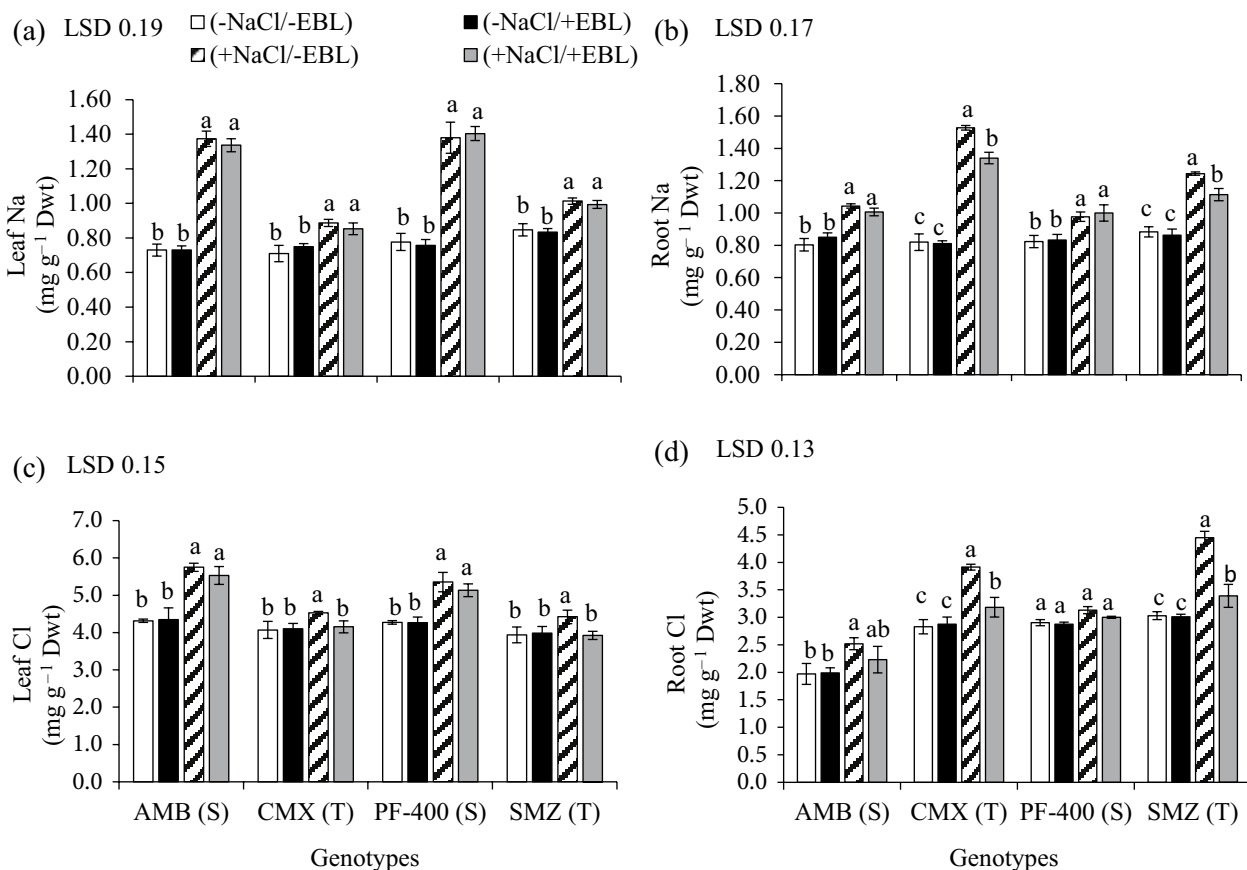


Figure 7. Effect of EBL on sodium (Na) and chloride (Cl) contents in the leaves and roots of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$. AMB (Ambassidar), CMX (Climax), SMZ (Samarina Zard).

osmolyte concentrations is controlled by specific genes (Banu et al., 2009). Various reports (Mussig et al., 2002; Nam and Li, 2002) stated that BRs regulate the expression of different genes within plants; therefore EBL treatment may have enhanced the expression of genes regulating the antioxidant activities and osmolyte concentrations in the studied *Pisum sativum* genotypes, resulting in increased antioxidant activities and high accumulation of compatible solutes. BRs have a significant potential to regulate antioxidant activities under stressed conditions (Roychoudhury et al., 2011). We therefore cannot rule out the possibility that EBL scavenged the ROS by enhancing the antioxidant activities and consequently induced salt tolerance in the +NaCl treated plants. Studies by Bajguz and Hayat (2009) and Shahid et al. (2011b) similarly showed that BRs enhanced osmolyte accumulation under stressed conditions. Osmotic stress resulting from salinity, due to excessive amounts of toxic ions, i.e. Na and Cl in plant tissues, stimulates abscisic acid (ABA) production, which causes leaf damage along with leaf drop, lowers the

stomatal conductance, intercellular CO₂ concentration, green pigments, and rubisco activity and disturbs electron transport, thereby reducing photosynthetic activity. Plants with better osmotic adjustment potential under stressed conditions are therefore considered to display lower deleterious effects of ABA compared with those having less capacity for osmotic adjustment. In the present study, the excellent performance of +NaCl/+EBL-treated plants may be linked with reduced osmotic stress and higher osmotic adjustment potential resulting from minimal production of ABA coupled with the high accumulation of osmolytes, which are necessary for osmotic adjustment to mitigate ion toxicity (Demetriou et al., 2007) under osmotic stress. EBL treatment may accelerate the high accumulation of osmoprotectants in the stressed *Pisum sativum* plants, thus inducing an efficient osmotic adjustment potential that is an important adaptation to withstand the stressed conditions. This phenomenon may also explain the improved germination percentage under saline conditions. Finally, the elevated activities of antioxidants and the

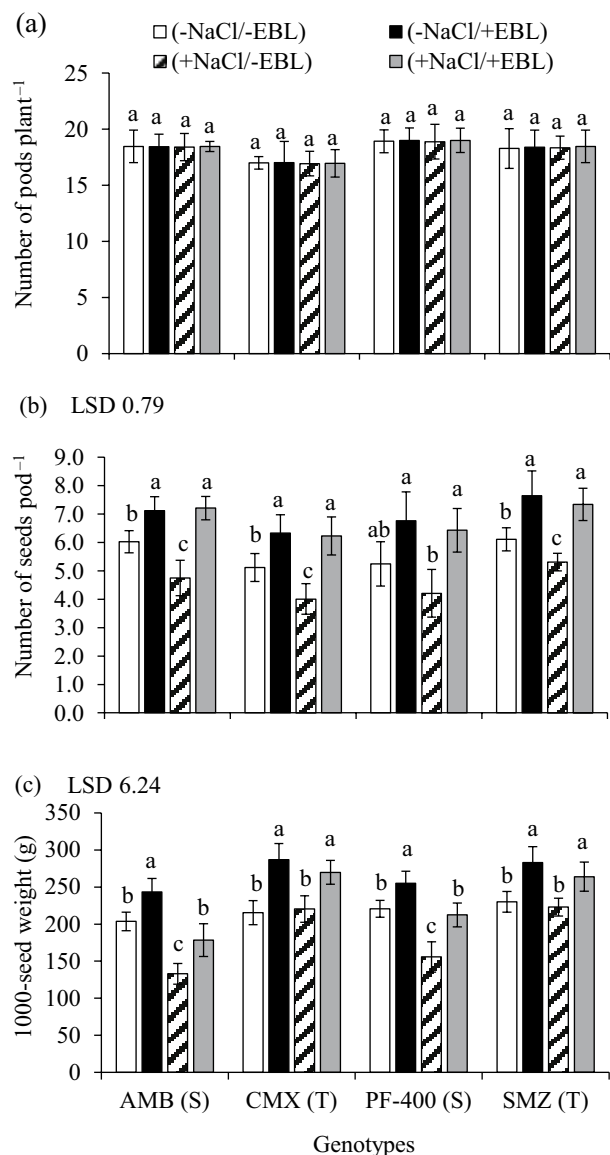


Figure 8. Effect of EBL on yield attributes, i.e. number of pods plant⁻¹ (a), number of seeds pod⁻¹ (b), and 1000-seed weight (c) of salt-tolerant (T) and salt-sensitive (S) *Pisum sativum* genotypes grown under saline (+NaCl) and nonsaline (-NaCl) conditions. Each value in the figure is the mean of 5 replicates and vertical bars give the standard error (SE) of mean. HSDs (Tukey's test) for genotypes and treatments were significant at $P \leq 0.05$.

high accumulation of osmoprotectants may result in an enhanced salt tolerance potential in the studied *Pisum sativum* genotypes as the high ratio of antioxidants and osmoprotectants scavenged high amounts of ROS by converting them into water and oxygen. The proposed augmentation of salt tolerance potential by EBL treatment is presented in the form of ameliorated growth, green pigments, stomatal size, number of epidermal cells, and

productivity. The results of the present study also indicate that plants grown under +NaCl conditions had a significant reduction in chlorophyll content. This reduction may be due to a decline in leaf area and to the high activity of a chlorophyll degrading enzyme (chlorophyllase), which was observed to be highly active under stressed conditions (Jiang et al., 2006). The increase in chlorophyll content of EBL-treated plants could have been due to inhibition of the chlorophyllase activity. Hassine and Lutts (2010) reported that the generation of ROS under saline conditions caused the peroxidation of chloroplast membranes and resulting reduction in chlorophyll content, which could be another reason for the reduced chlorophyll content observed in the present study. In the present study, EBL significantly scavenged ROS by increasing the activity of enzymatic and nonenzymatic antioxidants and thereby improved the chlorophyll content by inhibiting the peroxidation of chloroplast membranes caused by ROS. The improvement in chlorophyll contents in response to foliar spray of EBL in tolerant genotypes may also be due to its reducing action on root/leaf Na and Cl contents. Therefore, it can be assumed that EBL could have strengthened the endoderm of the mature basal cells and improved the extensibility of apical cells of the roots of salt-stressed *Pisum sativum* plants. Thus EBL application led to the strong and extensive root system that may also have inhibited the upward movement of toxic ions (Na and Cl).

The plants grown under +NaCl conditions showed significant reductions in leaf area, green pigments, stomatal conductance, stomatal size, rubisco activity, rubisco content, and relative water content, resulting in reduced photosynthetic activities. However, indirect interference by ABA of P_n as described above cannot be ruled out. The foliar application of EBL markedly strengthened the photosynthetic apparatus by improving the above attributes in both stressed and nonstressed plants. EBL also increased the concentration of total phenolics but decreased lipid peroxidation. According to the literature, brassinosteroids are highly involved in reducing the rate of lipid peroxidation in plants exposed to stressed conditions (Ahammed et al., 2013); therefore the inhibiting effect of EBL on lipid peroxidation in the present investigation may be associated with high scavenging of ROS resulting from the acceleration of antioxidant enzyme activities and increased osmolyte accumulation in plant tissues. Hamada (1986) reported that BRs are actively involved in the stabilization and maintenance of membrane structure under stressed conditions; thus, the improved growth of plants grown under +EBL conditions may have been due to lower lipid peroxidation. The improvement in photosynthetic activity may be associated with the above phenomenon or with efficient carbohydrate metabolism

resulting from increased activities of sucrose phosphate synthase and sucrose synthase enzyme in the leaves of EBL-treated plants. The salt stress disintegrates the green pigments (chlorophyll contents) by destroying their membranes and replacing the Mg, the vital component in chlorophyll structure, with Na and Cl (Vasilakoglou et al., 2011). This degradation of chlorophyll molecules due to the toxicity of Na and Cl leads to a reduction in P_n . Since EBL also lowered the root/leaf Cl and root Na in salt-tolerant genotypes, it may also be a significant reason for improved P_n under saline conditions. EBL treatment significantly enhanced the rubisco content as well as rubisco activity in the present study and may also be associated with high P_n in +EBL-treated plants in saline environments. Our findings are consistent with those of Yu et al. (2004), who reported that EBL enhanced photosynthesis by elevating the rubisco content, rubisco activity, SPS, and SS in *Cucumis sativus*. In addition to enhancing various physiological and biochemical processes, BRs help maintain plants in a healthy metabolic state by regulating the movement of photosynthates from source to sink and enhancing the nutritional absorption potential of stressed plants (Petzold et al., 1992). The high antioxidant activities, improved osmotic adjustment potential, elevation in carbohydrate metabolism, and increased rubisco activities of the plants subjected to +NaCl/+EBL conditions in the present study resulted in improvements in plant growth and productivity

in the form of increased plant fresh/dry weight, leaf area, number of seeds pod^{-1} , and 1000-seed weight.

In summary, it can be concluded that high antioxidant activities coupled with maximal accumulation of osmoprotectants was the major metabolic factor that differentiated the salt-tolerant *Pisum sativum* genotypes from the salt-sensitive genotypes. EBL treatment significantly mitigated the salinity-induced deleterious effects by improving the antioxidant enzyme activities (of SOD, POD, CAT, APX, and GPX), elevating the concentration of osmoprotectants (proline, glycine betaine, total amino acids, and total soluble sugars), and increasing the efficiency of carbohydrate metabolism. In addition, EBL improved the rubisco contents and rubisco activity, which leads to the strengthening of photosynthetic apparatus. All of the above factors promoted the photosynthetic apparatus, plant growth, and productivity under stressed conditions. Overall, it is concluded that spraying EBL on 24-day-old *Pisum sativum* plants will lead to better yield and more resistance to salt stress.

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