



## Research article

# Epibrassinolide and proline alleviate the photosynthetic and yield inhibition under salt stress by acting on antioxidant system in mustard

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## ARTICLE INFO

## Keywords:

Photochemical efficiency  
*Brassica juncea*  
 Superoxide dismutase  
 Catalase  
 Transpiration rate

## ABSTRACT

Soil salinity is one of the major abiotic stress factors that hampers plant growth and productivity by limiting photosynthesis and other related metabolic processes. In this study we investigated whether treatment with proline and/or 24-epibrassinolide (EBL) to two contrasting cultivars of *Brassica juncea* (L.) Czern and Coss viz. Varuna and RH-30 could counteract with the adverse effects of salinity on photosynthesis and seed yield. Plants were treated with proline and/or 24-epibrassinolide (EBL) at 28 and 29d-stages of growth. Salt stress reduced plant growth, photosynthetic attributes, efficiency of PSII (Fv/Fm), leaf water potential and finally seed yield, at harvest but improved the activity of antioxidant enzymes in both the cultivars in a concentration dependent manner. Exogenous application of EBL with proline completely neutralised the adverse effects of salt at 78 mM or 117 mM stress levels whereas the treatment partially neutralised the impact of highest salt concentration of 156 mM, through the upregulation of the antioxidant system.

## 1. Introduction

Mustard [*Brassica juncea* (L.) Czern & Coss] is the member of the family Brassicaceae. It is an important crop known for its oil content, edible and medicinal uses. India ranks second in the world and produces nearly 7% of the world's edible oil (Wani et al., 2013). However, this production still remains insufficient to fulfil the daily requirement of the people. The insufficient economic yield can be attributed to various biotic and abiotic stresses among which salt stress is considered as a major constraint that limits the crop yield by reducing photosynthesis, protein synthesis and other metabolic processes (Yadav et al., 2011). The primary effect of salt stress is hyper-osmotic which in severe cases may cause oxidative stress in plants (Yadav et al., 2011; Astaneh et al., 2018) leading to the generation of reactive oxygen species (ROS), that cause deleterious impact on plants (Astaneh et al., 2018). To minimize the toxic effects of the oxidative stress, plants improve the activity of various antioxidant enzymes and accumulate various metabolites such as proline which besides acting as an osmolyte also acts as metal chelator, ROS scavenger and also acts as signal molecule during the stress (Liang et al., 2013). Moreover, the exogenous application of proline, at lower concentrations, is known to improve the rate of photosynthesis and the activity of various antioxidant enzymes during stress and stress-free conditions (Wani et al., 2016).

Brassinosteroids (BRs) is a class of poly-hydroxysteroids, recognised

as a sixth group of plant hormones. BRs promote cell elongation and division in stem, xylem differentiation, activate several enzymes, increase net photosynthetic rate and fruit set, confer tolerance against various stresses such as osmotic, salinity and heavy metals (Sharma et al., 2017). Although the individual effects of BRs or proline on stress physiology have received much attention but little information is available about their interactive effects on photosynthesis and seed yield under salt stress. In this study, we investigated the role of EBL and proline in combination to alleviate the oxidative damage, caused by salt stress in mustard plants, through the activation of antioxidant system.

## 2. Materials and methods

## 2.1. Experimental design

Present experiment was laid down to study the interactive effects of 24-epibrassinolide (EBL) and proline on sodium chloride (NaCl) induced changes in *B. juncea* cv. Varuna and RH-30. The experiment was conducted with 100 earthen pots (25 × 25 cm) in a way where each treatment had five replicates. Three plants were maintained in each pot, arranged under simple randomized block design. The seeds of Varuna and RH-30 were surface-sterilized with 0.01% mercuric chloride solution for 2 min followed by the repeated washing with double distilled water (DDW) in order to remove adhered mercuric chloride on the seed

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<https://doi.org/10.1016/j.plaphy.2019.01.002>

Received 25 September 2018; Received in revised form 1 January 2019; Accepted 1 January 2019

Available online 02 January 2019

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surface. These sterilized seeds were then sown in the earthen pots filled with an equal quantity of sandy loam soil mixed with farmyard manure (9:1) amended with different levels (78, 117, 156 mM) of NaCl. Sodium chloride is essential to maintain the electrolyte balance of fluids in plants. If levels of electrolytes become too low or too high, a plant can become dehydrated or over hydrated. The pots were stacked in the net house of the Botany department, Aligarh Muslim University, Aligarh, India under natural environmental conditions. The leaves of each plant were sprayed thrice at an interval of 10 min with 20 mM proline at 28 days after sowing (DAS) and/or  $10^{-8}$  M EBL at 29 DAS. The nozzle of the sprayer was adjusted in such a way that it pumped out about 1 mL of the solution in a single spray, therefore each plant received about 3 mL of the solution. Required number (10 plants from each treatment) of plants were sampled at 60 DAS to assess the following parameters. The remaining plants were allowed to grow up to maturity and harvested (about 120 DAS) to study the yield characteristics. The mean value for each treatment is given in this present study.

## 2.2. Growth attributes

The plants along with soil were removed at 60 DAS from each pot and dipped in water to dislodge the adhering soil particles without injuring the roots. The length of the root and shoot was measured on a meter scale. The roots were then separated from the shoot and blotted. The roots and shoot were weighed separately to record their fresh mass and placed in an oven at 80 °C for 72 h. The samples were weighed again to record their respective dry mass. Leaf area was ascertained by gravimetric method by tracing the outline of the fresh leaves on graph sheet and counting the squares covered by it on graph paper.

## 2.3. Leaf water potential

Leaf water potential (LWP) was measured in detached fresh leaves by using PSYPRO, water potential system (WESCOR Inc. Longman, USA). One leaf from each replicate was used for the measurement of LWP during 11:00–13:00 h. The leaf disc (5 mm) was placed in the chamber of the instrument for the measurement of LWP.

## 2.4. Soil plant analytical development (SPAD) chlorophyll value and photosynthetic attributes

SPAD chlorophyll meter (Minolta 502) was used to assess chlorophyll values in intact leaves. This instrument gives us the estimative values of chlorophyll content. The photosynthetic attributes [net photosynthetic rate ( $P_N$ ), stomatal conductance ( $g_s$ ), internal  $\text{CO}_2$  concentration ( $C_i$ ), and transpiration rate ( $E$ )] were measured by using an infrared gas analyser (IRGA) portable photosynthetic system (LI-COR 6400, LI-COR, Lincoln, NE, USA), under bright sunlight. All the measurements were done in well expanded three young leaves attached to the plants of each replicate, at least thrice during 11:00–13:00 h. The atmospheric conditions during the measurement were photosynthetically active radiation (PAR),  $1016 \pm 6 \mu\text{mol m}^{-2} \text{s}^{-1}$ , relative humidity  $60 \pm 3\%$ , atmospheric temperature  $22 \pm 1^\circ\text{C}$  and atmospheric  $\text{CO}_2$   $360 \mu\text{mol mol}^{-1}$ . The duration of the measurement of each sample was 10 min after the establishment of steady-state conditions inside the measurement chamber.

## 2.5. Maximum quantum yield of photosystem II (PSII)

The maximum quantum yield of PSII (Fv/Fm) was measured on the adaxial surface of the intact leaf using leaf chamber fluorometer (LI-COR 6400-40, LI-COR, Lincoln, NE, USA). All the measurements were carried out at a photosynthetic photon flux density (PPFD) of  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  with a constant airflow rate of  $500 \mu\text{mol s}^{-1}$ . The minimal fluorescence level ( $F_0$ ) was determined by modulated light, which was sufficiently low ( $< 1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) not to induce any

significant variable fluorescence. The maximal fluorescence (Fm) was determined by a 0.8-s saturation pulse at  $4200 \mu\text{mol m}^{-2} \text{s}^{-1}$  on dark-adapted leaves (30 min). The sampled leaf was dark-adapted for 30 min prior to measurement of Fv/Fm. Three measurements were done in each expanded upper young leaf, attached to the plants. Three leaves in each replicate were measured during 11:00–13:00 h.

## 2.6. Biochemical analysis

The activity of various enzymes [carbonic anhydrase (CA), peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD)], leaf electrolyte leakage (EL) and proline content were analysed as described in our previous study (Wani et al., 2013).

## 2.7. Statistical analysis

Treatment means were compared by the analysis of variance using SPSS (SPSS ver. 17, Chicago, United States). Least Significant Difference (LSD) was calculated at 5% level of probability. Standard error between the replicates was calculated.

## 3. Results

### 3.1. Growth attributes

Application of  $10^{-8}$  M EBL and/or 20 mM proline to the foliage of mustard plants increased their growth (length, fresh and dry mass of root and shoot, and leaf area) in both the cultivars (Varuna and RH-30) at 60 DAS (Figs. 1 and 2A). EBL and proline together proved best in increasing the values for all the above characteristics which in terms of percentage were 72% and 56% in length, 77% and 67% in fresh mass, 93% and 88% in dry mass of shoot and root respectively and 41% in leaf area in Varuna, compared with its control plants, under stress-free conditions. The plants raised in the soil fed with NaCl (78, 117, 156 mM) had significantly lesser values for all the attributes in both the cultivars. The damage was more pronounced in RH-30 than Varuna. However, the follow-up treatment of EBL and proline together to NaCl stressed plants, completely neutralised the toxic effects generated by the two lower concentrations of the salt and the values were, therefore, comparable with that of the controls.

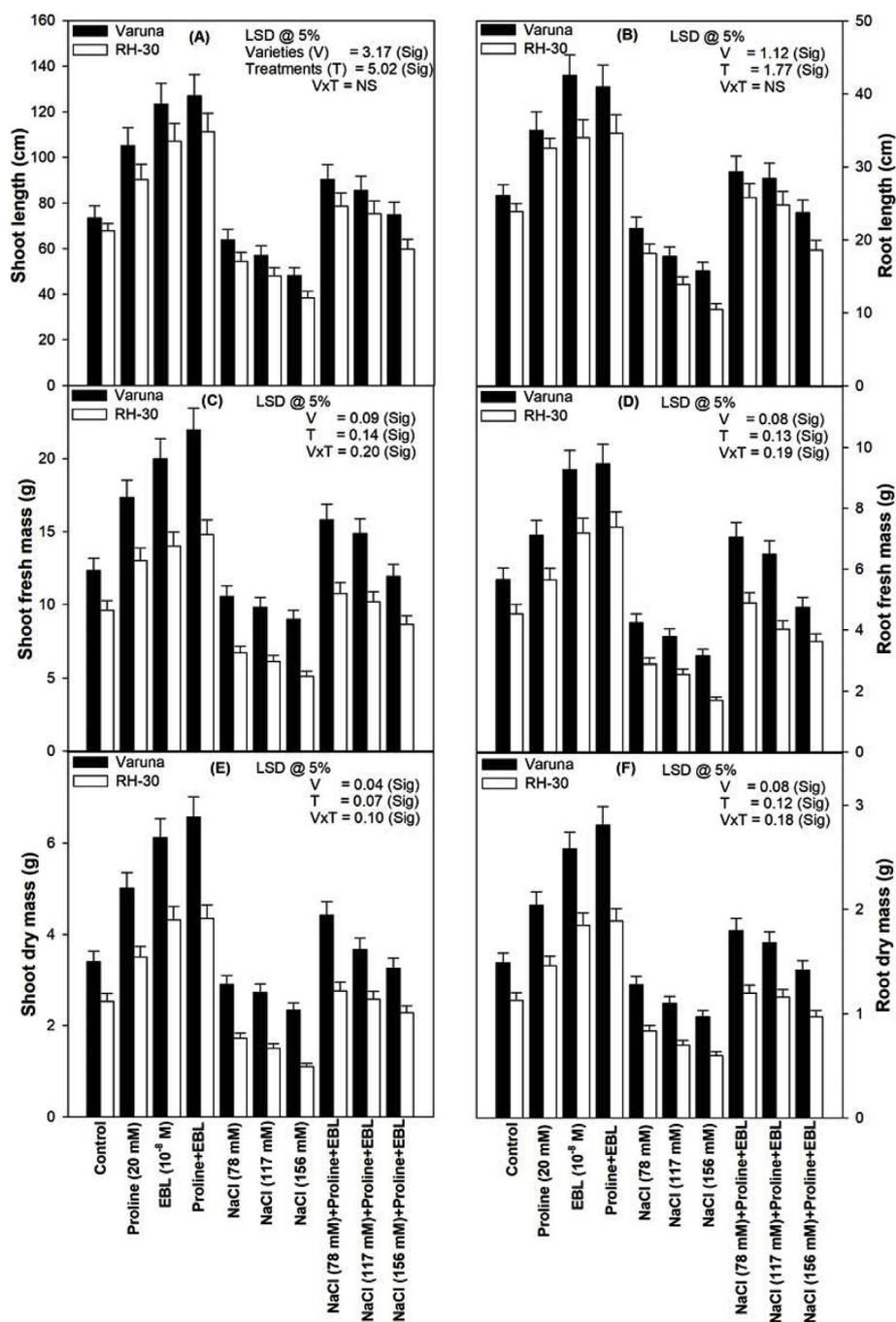
### 3.2. Electrolyte leakage and leaf water potential

The electrolyte leakage from the leaves increased with an increasing level of NaCl in the soil. Out of the three concentrations (78, 117 or 156 mM), 156 mM of NaCl proved highly toxic that increased the leakage by 21% and 28% in Varuna and RH-30, over their respective control, at 60 DAS (Fig. 2B). However, proline and/or EBL treatment checked the loss of electrolytes significantly and the combination of the two proved best. Moreover, the follow-up application of proline plus EBL to salt stressed plants minimized the impact of stress and brought the values of electrolyte leakage below to that of the control plants.

The data in Fig. 2C revealed that the leaves of Varuna and RH-30, under NaCl stress possessed significantly lower leaf water potential (LWP) than their control plants in a concentration dependent manner. Foliar spray of proline and/or EBL to stress-free plants had significantly higher LWP values. Proline plus EBL proved best increasing the LWP by 39% and 31% in Varuna and RH-30 respectively, compared to their control plants. The decrease in LWP under stress was totally nullified by the follow-up treatment with EBL plus proline.

### 3.3. CA activity

The values of CA were higher in the leaves of both the cultivars which received proline and/or EBL as foliar application at 60 DAS (Fig. 2D). Moreover, the two together induced highest increase that was



**Fig. 1.** Effect of proline (20 mM) and 24-epibrassinolide (EBL;  $10^{-8}$  M) as foliar spray (28 and 29 d stages) and/or soil applied sodium chloride (NaCl; 78, 117 or 156 mM) on length (cm), fresh mass (g) and dry mass (g) of shoot and root in two cultivars (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS.

43% and 31% in Varuna and RH-30, over their respective control plants. Salt stress lowered CA values but supplementing them with proline plus EBL completely overcame the damaging effects of 78 and 117 mM of salt and the values were significantly higher than their controls.

### 3.4. SPAD chlorophyll values

The application of proline and/or EBL to the foliage increased the SPAD chlorophyll values and the combination of these two was most effective in both the cultivars (Fig. 3A). However, the plants grown in the soil amended with NaCl (78, 117 or 156 mM) showed a negative response where 156 mM triggered maximum loss which was 31% and 37% lower in Varuna and RH-30 respectively at 60 DAS, compared with

non-stressed control plants. The loss generated by NaCl (78 or 117 mM) was completely nullified by the follow-up treatment with EBL plus proline and the values were significantly higher than those of the control. The impact of 156 mM of NaCl was partially overcome by this combination.

### 3.5. Net photosynthetic rate and related attributes

The values for  $P_N$  and its related attributes i.e.,  $g_s$ ,  $C_i$  and  $E$  increased significantly with the foliar application of proline and/or EBL to the plants of both cultivars at 60 DAS (Fig. 3B–E). Proline plus EBL spray proved best and increased the  $P_N$  by 47% and 38%;  $g_s$ : 83% and 53%;  $C_i$ : 27% and 20% and  $E$ : 47% and 28% in Varuna and RH-30 respectively, over their control plants. NaCl solution at three levels (78, 117 or

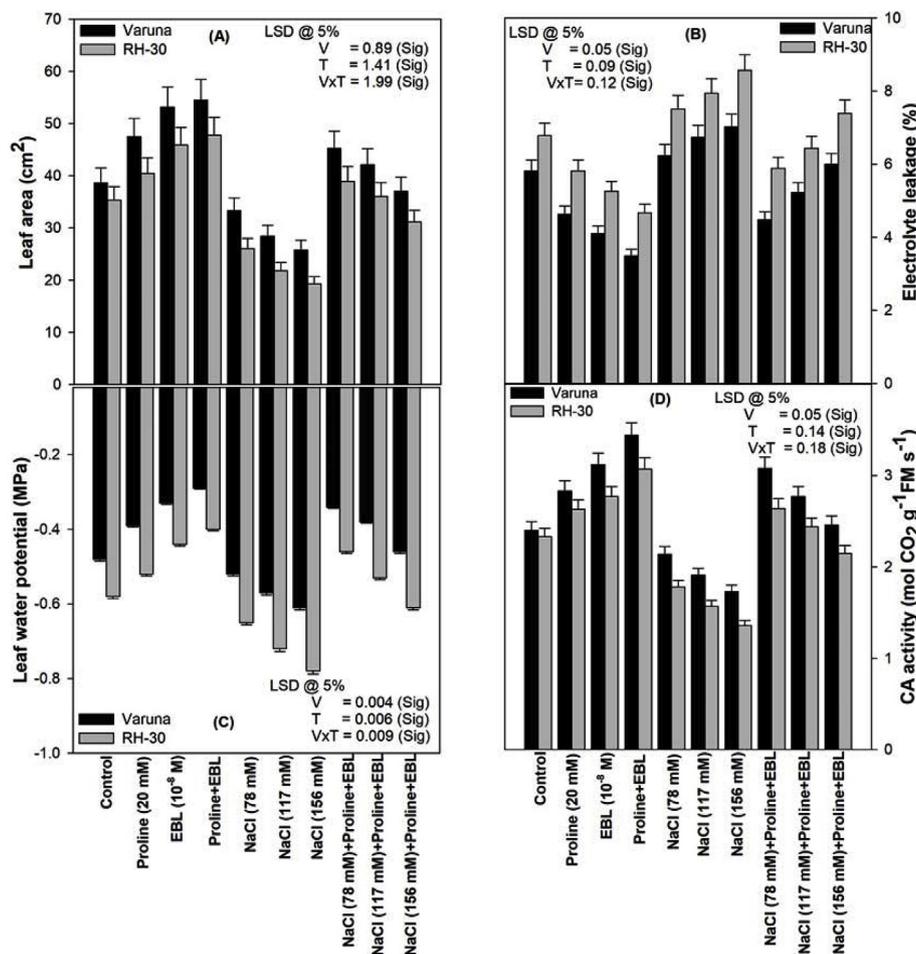


Fig. 2. Effect of proline (20 mM) and 24-epibrassinolide (EBL;  $10^{-8}$  M) as foliar spray (28 and 29 d stages) and/or soil applied sodium chloride (NaCl; 78, 117 or 156 mM) on (A) leaf area, (B) electrolyte leakage, (C) leaf water potential and (D) carbonic anhydrase (CA) activity in two cultivars (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS.

156 mM) administered through the soil generated stress which decreased  $P_N$  and all its related attributes significantly in a concentration dependent manner in both the cultivars, being more pronounced in RH-30 than Varuna. However, the toxicity generated by the two lower concentrations (78, or 117 mM) of the salt was completely overcome by the follow up application of proline plus EBL and the values were more than that of the control. Moreover, the treatment (proline + EBL) partially overcame the impact of the highest level of NaCl (156 mM).

### 3.6. Maximum quantum yield of PSII (Fv/Fm)

The values for Fv/Fm were inversely proportional to the concentration of NaCl (78, 117 or 156 mM) in Varuna and RH-30 (Fig. 3F). Moreover, the highest level of the salt was most toxic and reduced the values in Varuna and RH-30 by 22% and 28% respectively, compared to the corresponding control plants. However, proline and/or EBL given as a follow-up treatment to the leaves of stress free or NaCl-stressed plants, improved the values significantly. Moreover, the combination of proline and EBL together, completely overcame the impact of the two lower concentrations (78 or 117 mM) of NaCl and partially that of 156 mM.

### 3.7. Antioxidant enzymes

The activity of antioxidant enzymes (CAT, POX and SOD) increased in the presence of NaCl, proline and/or EBL (Fig. 4A–C). The maximum activity of these enzymes was recorded in Varuna, grown in the soil

amended with 156 mM of NaCl and sprayed with of proline plus EBL. In terms of percentage, the activity of CAT enzyme increased by 80% and 59%, POX by 130% and 95% and SOD by 118% and 95% in Varuna and RH-30 at 60 DAS respectively, over the control plants which possessed lowest values.

### 3.8. Proline content

Leaf proline content increased in both the cultivars fed either with NaCl (78, 117 or 156 mM), proline and/or EBL over the control plants (Fig. 4D). Out of the two cultivars, Varuna possessed higher proline content than RH-30. Moreover, the maximum values for proline content in both the cultivars were recorded in the plants fed with highest level of NaCl (156 mM) and supplemented with proline plus EBL. A maximum increase of 101% and 77% at 60 DAS, over the control plants, was noted in Varuna and RH-30, applied with EBL plus proline as a follow up treatment with NaCl (156 mM) as a soil amendment.

### 3.9. Yield characteristics per plant

With an increase of NaCl concentration from 78 to 156 mM, the number of pods per plant, number of seeds per pod, 100 seed mass and seed yield per plant decreased significantly in both the cultivars, at harvest (Fig. 5). The toxic effect of NaCl was more pronounced in cultivar RH-30 than Varuna. However, leaf-applied proline and/or EBL to stress-free plants significantly increased the number of pods per plant and seed yield per plant, over the control. Moreover, Varuna excelled in

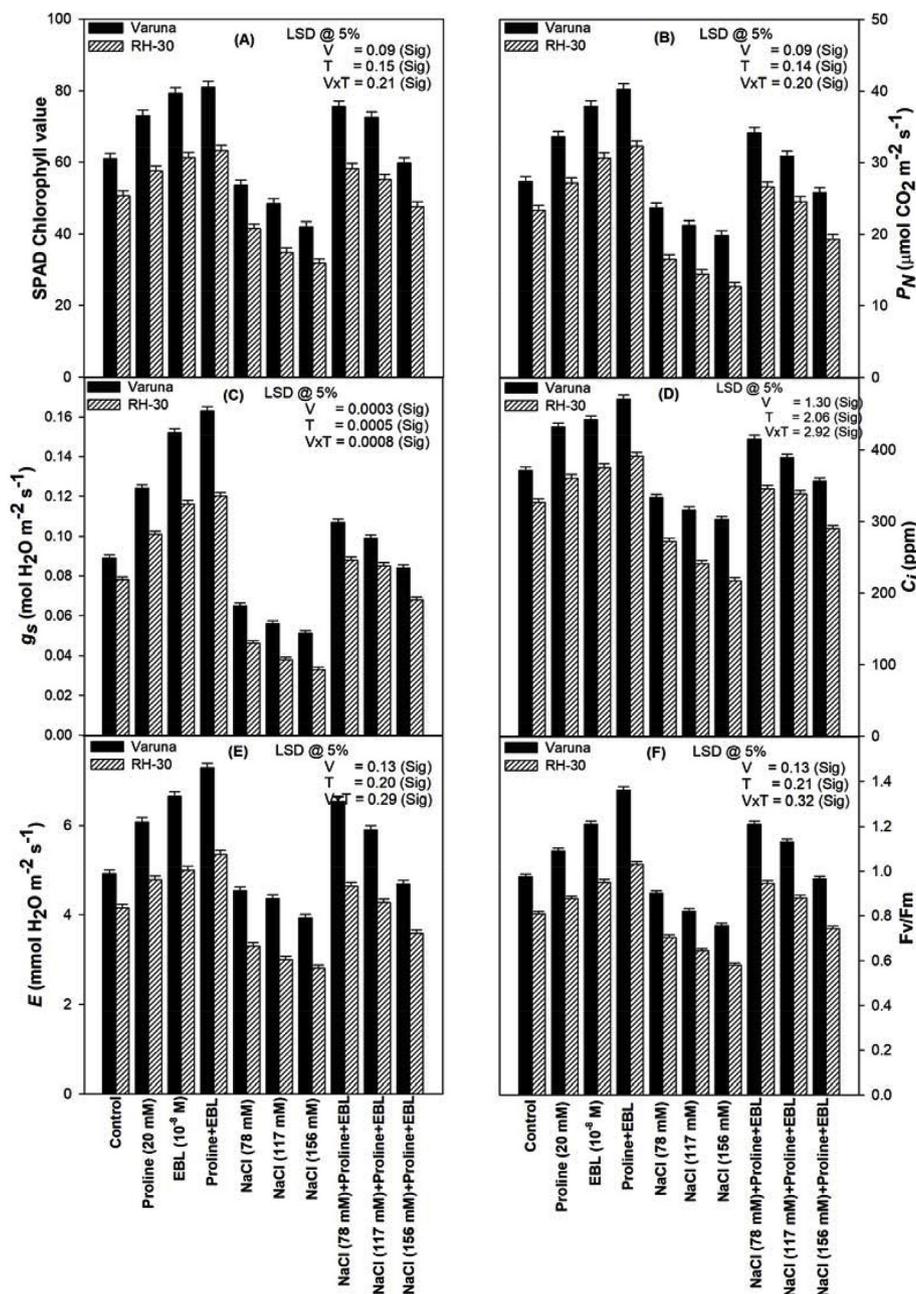


Fig. 3. Effect of proline (20 mM) and 24-epibrassinolide (EBL;  $10^{-8}$  M) as foliar spray (28 and 29 d stages) and/or soil applied sodium chloride (NaCl; 78, 117 or 156 mM) on (A) SPAD chlorophyll value, (B) net photosynthetic rate;  $P_N$ , (C) stomatal conductance;  $g_s$ , (D) internal  $CO_2$  concentration;  $C_i$ , (E) Transpiration rate;  $E$  and (F) maximum quantum yield of PSII;  $F_v/F_m$ , in two cultivars (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS.

its response, generating maximum values for both the parameters being 50% and 44% more than the control. The concentration, proline plus EBL proved best in nullifying the toxicity caused by 78 or 117 mM of NaCl, particularly in Varuna where these values were significantly higher than the control.

#### 4. Discussion

The NaCl (stress) taken up by the plants from the soil decreased the activity of CA enzyme (Fig. 2D) in the leaves because of the negative regulation of gene expression as NaCl hinders the carbon availability leading to the reduction in photosynthetic efficiency (Liu et al., 2012). Similar observations have also been noted earlier by Hayat et al. (2011), Liu et al. (2012) and Wani et al. (2013). However, in a natural course proline interferes with the hydrophobic/hydrophilic interactions

between the side chains of the constituent amino acids of the protein that maintain their 3D structure that plays the protective role to maintain enzyme activity (Hayat et al., 2012). This may be the reason to explain the positive effect of proline on CA activity (Fig. 2D). Moreover, the foliar application of EBL increased CA activity (Fig. 2D and Fariduddin et al., 2014; Wani et al., 2017) by elevating  $CO_2$  assimilation rate (Yu et al., 2004) through enhanced expression of genes that encode other enzymes of the Calvin cycle to regenerate RUBP, thereby maximizing the carboxylation rate of Rubisco (Xia et al., 2009). Therefore, a combination of proline plus EBL imparted an additive effect on CA activity (Fig. 2D).

The leaves of stressed plants lose a significant quantity of chlorophyll (Yadav et al., 2011; Wani et al., 2013 and Fig. 3A) in a salt concentration dependent manner, possibly as a consequence of inhibited chlorophyll synthesis and/or acceleration in its denaturation

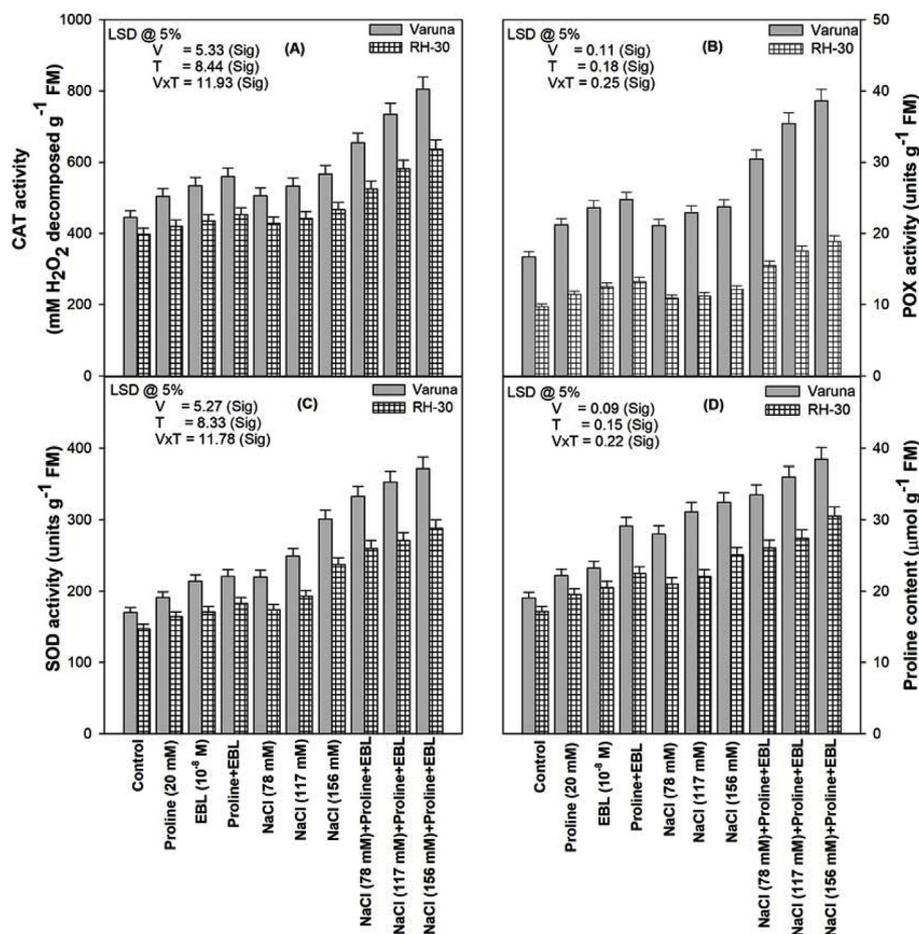
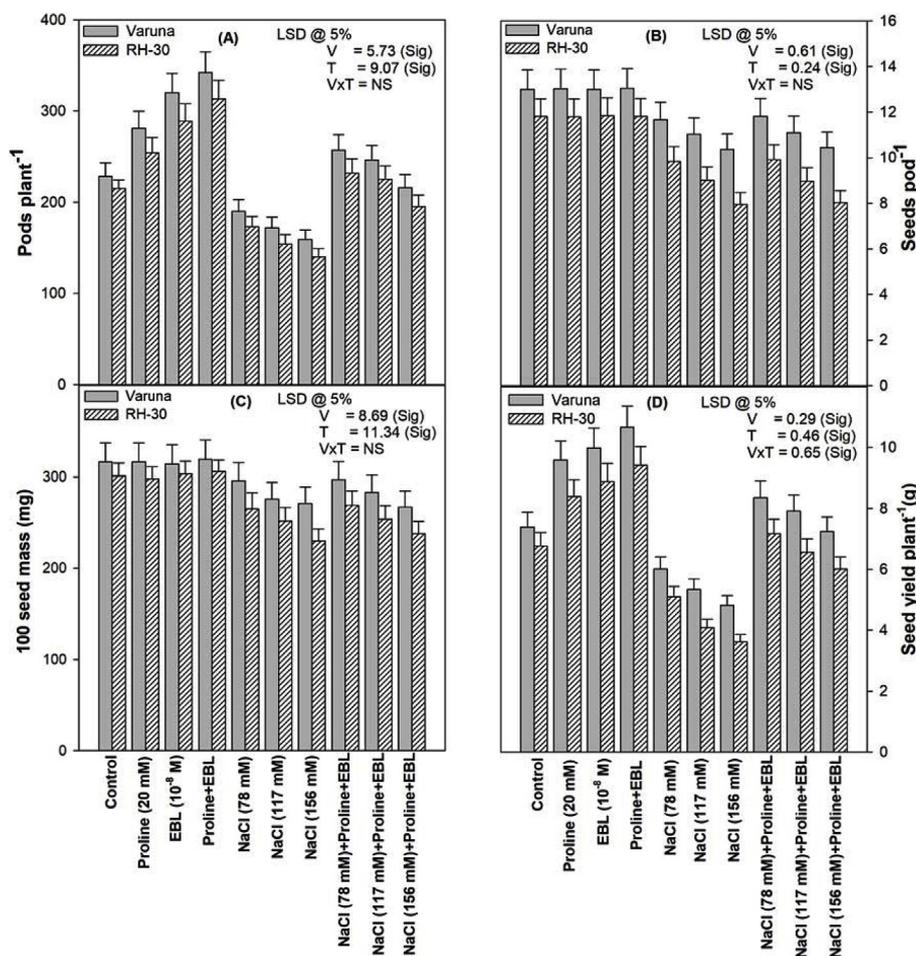


Fig. 4. Effect of proline (20 mM) and 24-epibrassinolide (EBL;  $10^{-8}$  M) as foliar spray (28 and 29 d stages) and/or soil applied sodium chloride (NaCl; 78, 117 or 156 mM) on activities of (A) catalase; CAT, (B) peroxidase; POX, (C) superoxide dismutase; SOD enzymes, and (D) proline content in two cultivars (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS.

(Yadav et al., 2011). The stability of chlorophyll molecules depends largely on the membrane integrity which has possibly been maintained in our study by the applied proline (Hayat et al., 2012; Wani et al., 2016). Moreover, EBL like other BRs enhanced the SPAD chlorophyll value through its involvement at the transcription and/or translation level (Bajguz and Asami, 2005) therefore, enhanced the chlorophyll level (Fig. 3A) and in other crops as well (Yu et al., 2004; Fariduddin et al., 2014; Wani et al., 2017). Brassinosteroids also reduce the activity of chlorophyllase which is responsible for the chlorophyll degradation under stress (Sharma et al., 2017). The cumulative favorable effects of proline plus EBL, therefore maintained chlorophyll level in the leaves (Fig. 3A). The NaCl-stress causes closure of stomata due to salt-induced ABA accumulation (Niu et al., 2018), that leads to a decrease in partial pressure of CO<sub>2</sub> in the stroma (Yadav et al., 2011) that becomes the main reason for the observed loss in  $g_s$ ,  $C_b$ , and  $E$  (Fig. 3C–E). Together, these factors lead to the decrease in  $P_N$  (Fig. 3B) which is positively correlated with  $g_s$  and  $C_i$  (Lu et al., 2009). Moreover, the stress-induced activation of senescence, changes in cytoplasmic structure, negative feedback of reduced sink activity (Yadav et al., 2011), the decrease in SPAD chlorophyll values (Fig. 3A) and CA activity (Fig. 2D) are the additional reasons to justify the lower  $P_N$  values. A positive correlation between  $P_N$  and SPAD chlorophyll value (Fig. 6A–B) as well as between  $P_N$  and CA (Fig. 6C–D) further corroborate the present investigations. Additional support is also gained from others (Wu et al., 2012; Wani et al., 2013). However, the recovery in  $P_N$  and its related attributes ( $g_s$ ,  $C_i$  and  $E$ ) in the salt-stressed plants was attained by spraying their foliage with EBL and/or proline as a follow-up treatment (Fig. 3B–E). As photosynthesis is mainly dependent on the stomatal movement and metabolism of mesophyll cells (proteins associated with PSI, PSII and chlorophyll) (Athar and Ashraf, 2005) it may, therefore be inferred from the present observations that proline application caused an

increase in stomatal conductance by maintaining appropriate cellular turgor (Hayat et al., 2012) thereby facilitating sub-stomatal accumulation and assimilation of CO<sub>2</sub> at a higher pace. Moreover, higher chlorophyll values (Fig. 3A) and CA activity (Fig. 2D), under proline would also have resulted in higher  $P_N$  (Fig. 3B). The two important enzymes, (CA and Rubisco) that initiate the process of photosynthesis are activated by BRs (Yu et al., 2004). Higher CA activity increases the carboxylation state of Rubisco (Bajguz and Asami, 2005), which in turn improves  $P_N$  that gives positive correlation with CA (Fig. 6C–D). The present study also revealed that BRs increased CO<sub>2</sub> concentration (Fig. 3D) by increasing  $g_s$  (Fig. 3C) that improved the efficiency of light harvesting system by increasing chlorophyll level (Fig. 3A). Therefore, in a cumulative action the net photosynthetic rate of treated plants was speeded (Fig. 3B Fariduddin et al., 2014; Wani et al., 2017) similar reasons have. Besides this, BRs and/or proline also improved the cell water relations i.e. leaf water potential (Fig. 2C) and membrane structure and its stability (Slathia et al., 2012) so as to decrease the electrolyte leakage (Fig. 2B) in both stress-free and stressed plants which could have been helpful in maintaining normal cellular structure and metabolism.

The salt stress induced loss in the photochemical efficiency i.e. decreased Fv/Fm values (Fig. 3F) and in other crops (Wu et al., 2012; Wani et al., 2013) which is proposed to be due to the suppression of PS II activity (Mehta et al., 2010). Here, salt stress interferes with the electron transport system by blocking the electron transfer from primary acceptor, plastoquinone (QA) to the secondary acceptor, plastoquinone (QB) at the acceptor side of PSII resulting in the suppression of its activity (Megdichea et al., 2008). However, the spray of EBL and/or proline to the stressed/stress-free plants improved the values of Fv/Fm (Fig. 3F). This can be attributed to the fact that EBL increases the photochemical quenching with no change in the efficiency of energy



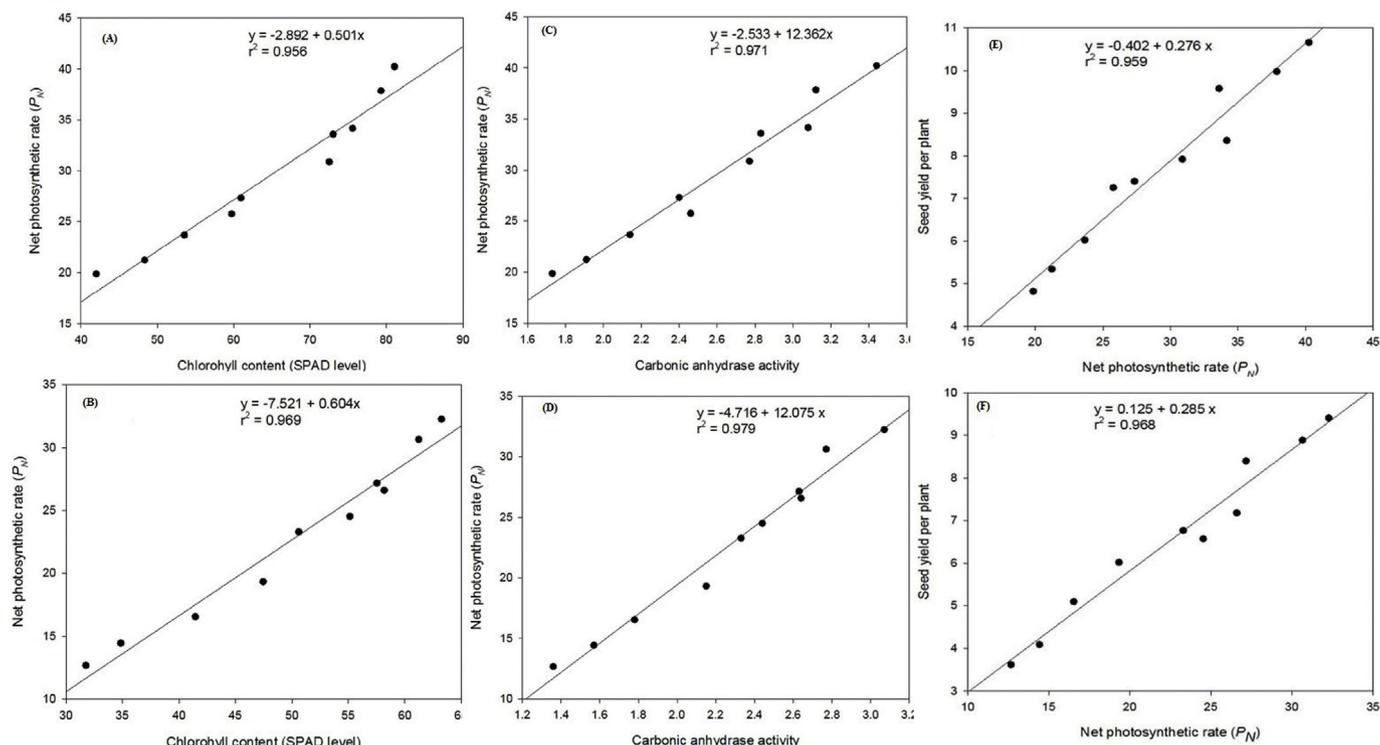
**Fig. 5.** Effect of proline (20 mM) and 24-epibrassinolide (EBL;  $10^{-8}$  M) as foliar spray (28 and 29 d stages) and/or soil applied sodium chloride (NaCl; 78, 117 or 156 mM) on (A) pods plant<sup>-1</sup>, (B) seeds pod<sup>-1</sup>, (C) 100 seed mass, and (D) seed yield plant<sup>-1</sup> in two cultivars (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS.

capture by PSII (Yu et al., 2004). Moreover, under salt stress, BRs protect PSII against over-excitation that otherwise could have caused the loss of integrity of thylakoid membranes (Ogwenio et al., 2008). Similarly, PSII machinery gets a similar type of protection from applied proline, under stress and stress-free conditions (Fig. 3F) which gets the support from Oukarroum et al. (2012) and Moustakas et al. (2011).

Plants possess complex antioxidative defense system comprising of non-enzymatic (such as proline) and enzymatic components (such as CAT, POX, SOD) to scavenge reactive oxygen species (ROS), produced under stress. However, in the natural system, ROS are generated at a very slow pace and an appropriate balance is maintained between their production and quenching. However, various environmental stresses disturb this balance as they give rise to rapid increases in the intra and inter-cellular ROS levels (Sharma et al., 2012) which brings about oxidative damage to lipids, proteins and nucleic acids (Sharma et al., 2012). In order to avoid this damage, plants raise the level of endogenous enzymatic and non-enzymatic scavenging components (Sharma et al., 2012 and Fig. 4). Salt-induced increase in endogenous proline content (Fig. 4D) could have been due to the increased rate of hydrolysis of proteins (Irigoyen et al., 1992) as protein synthetic machinery is diverted towards proline accumulation (Claussen, 2005). Secondly, an enhanced level of proline could be due to its slower rate of degradation (Hayat et al., 2012). Similar observations have also been reported earlier by various authors (Yadav et al., 2011; Wani et al., 2013). Out of the two cultivars tested, Varuna possessed higher proline content and the activity of CAT, POX and SOD enzymes than RH-30. Such types of responses differing in salt tolerance have been reported

earlier in other cultivars which may be because of their genetic variations (Hayat et al., 2011). We, however noted that the treatment of stress-free and stressed plants with EBL and/or proline improved their antioxidant enzymes activity and proline content (Fig. 4). Being a membrane stabilizer, proline application and its rapid uptake coupled with de novo synthesis (Hayat et al., 2012), increased total endogenous level of proline (Fig. 4D) whose action in plant system is carried over through its involvement at transcription and/or translation level (Hayat et al., 2012). Furthermore, higher proline content improves water uptake (Jain et al., 2001) thereby maintains higher leaf water potential (Fig. 2C).

EBL like other BRs regulates the antioxidant enzyme activity in the tissues where free radical accumulation occurs (Ashraf et al., 2010). BRs confer the tolerance through the increased activity of membrane bound NADPH oxidase enzyme and elevate the level of H<sub>2</sub>O<sub>2</sub> in the apoplast to initiate protein phosphorylation cascade. Transcription factors may be activated via a phosphorylation cascade by MAPKs (Mitogen-activated protein kinases). Finally, the products of target genes participate directly in cellular protection against the stress (Xia et al., 2009). In addition to this, on the basis of molecular, physiological and genetic studies it is reported that the enhanced expression of *det-2* genes results in an increase in the activity of antioxidant enzymes that provide resistance to oxidative stress in *Arabidopsis* (Cao et al., 2005). Moreover, BRs also induce the expression of proline biosynthetic genes (Ozdemir et al., 2004) which results in the accumulation of proline in stressed plants (Fig. 4D). Proline is designated as a natural cytosolic osmoticum which scavenges free radicals, interacts with



**Fig. 6.** Correlation coefficient values between net photosynthetic rate and chlorophyll content (SPAD level) (A, B); net photosynthetic rate and carbonic anhydrase activity (C, D) and seed yield per plant and net photosynthetic rate (E, F) in Varuna and RH-30 respectively.

macromolecules of the cells such as enzymes, DNA and membranes to stabilize their structure and function (Hayat et al., 2012). Among various compatible solutes, only proline has the property to protect plants from singlet oxygen and free radical damages, that results from the stress (Hossain et al., 2014). Therefore, application of EBL plus proline to the leaves elevated the endogenous proline content and the activity of antioxidant enzymes (CAT, POX and SOD) possibly through its action at transcription and/or translation, thereby prevented the mustard plants against the damage caused by the salinity stress.

Plants exposed to soil amended NaCl, had significant reduction in growth traits (length, fresh and dry mass of shoot and root and leaf area) (Figs. 1 and 2A) as the salt causes reduction in cell division and elongation (Naz et al., 2018). Moreover, it also induces alterations in the nutrient uptake, reactive oxygen species accumulation (Yadav et al., 2011), inhibition of the activity of cytoplasmic enzymes, turgor loss (Naz et al., 2018) and hormonal imbalance (Ashraf et al., 2010) which in a natural course impair plant growth and biomass production. Similar responses to salt stress are also reported by others (Yadav et al., 2011). The toxic effects generated by the salt stress can, however, be overcome by the application of BRs and/or proline, as a follow-up treatment to the plants. The observed increase in the endogenous proline content (Fig. 4D) protects the cellular enzymes and 3-D structure of proteins, cell organelles and membranes by checking lipid peroxidation and facilitates the supply of energy, thereby brings about corrective measures in plant metabolism (Hayat et al., 2012 and Fig. 1). On the other hand, BRs modulate a number of metabolic phenomena in plants to generate tolerance against the stress (Ashraf et al., 2010). The amelioration of salt stress by BRs application is well documented in by various authors (Fariduddin et al., 2014; Wani et al., 2017). The hormone has a positive impact on cell division and cell elongation (Peleg and Blumwald, 2011), regulation of genes encoding XTHs (xyloglucan endo-transglycosylase/hydrolase) i.e., the enzymes responsible for the modification of cell wall activity and cell enlargement, cellulose synthase and sucrose synthase (Ashraf et al., 2010) that play a vital role in growth and development of plants. The plants sprayed with EBL,

therefore possessed larger leaves (Fig. 2A) which could have been an expression of activated cell division and cellular enlargement (Bajguz and Tretyn, 2003). Similarly, BRs have improved the leaf area in *Vigna radiata*, under aluminum stress (Ali et al., 2008) and *Brassica juncea*, under cadmium stress (Hayat et al., 2014). The combination of proline plus EBL had an additive effect on growth and development in *Brassica* (Figs. 1 and 2A), that looked quite obvious in the light of the above statements.

The yield characteristics, at harvest, decreased significantly under salt-stress in a concentration dependent manner (Aldesuquy and Ibrahim, 2001; Wani et al., 2013 and Fig. 5) where cultivar, Varuna expressed slight resistance to stress, compared with RH-30. The most prominent reasons that could have led to the loss in grain yield are poor vegetative plant growth (Fig. 1), limited supply of photosynthates (Fig. 3B and Chen et al., 2009), reduction in the thickness of the assimilate conducting pathway (Aldesuquy and Ibrahim, 2001) and leaves behaving as sinks rather than a source (Arbona et al., 2005). These factors, altogether caused an inhibition of assimilates free movement towards the developing reproductive organs, expressed in the form of decrease in pods plant<sup>-1</sup>, seeds pod<sup>-1</sup>, 100 seed mass and seed yield plant<sup>-1</sup> (Fig. 5D). In contrast to the above, EBL and/or proline improved the values for yield characteristics both in stressed and stress-free plants (Fig. 5). The extended life span and area of vegetative and reproductive organs under the impact of proline (Balestrasse et al., 2004) and/or BRs (Iwahori et al., 1990) could be the reason for the improved yield characteristics. Moreover, increased seed yield, under EBL (Fig. 5D) may be an expression of higher rate of photosynthesis (Fig. 3B) that facilitated the availability of more carbohydrates at the sink (Bajguz and Asami, 2005) for their healthy growth. Gomes et al. (2003) has reported higher biological yield similarly in passion fruit, correlating with higher photosynthetic CO<sub>2</sub> assimilation, under BRs. To strengthen our statement a positive correlation was observed between  $P_N$  and seed yield (Fig. 6E–F). From the above discussion it is quite evident that individually both proline and BRs have a positive effect on most of the characteristics determining plant growth and biological

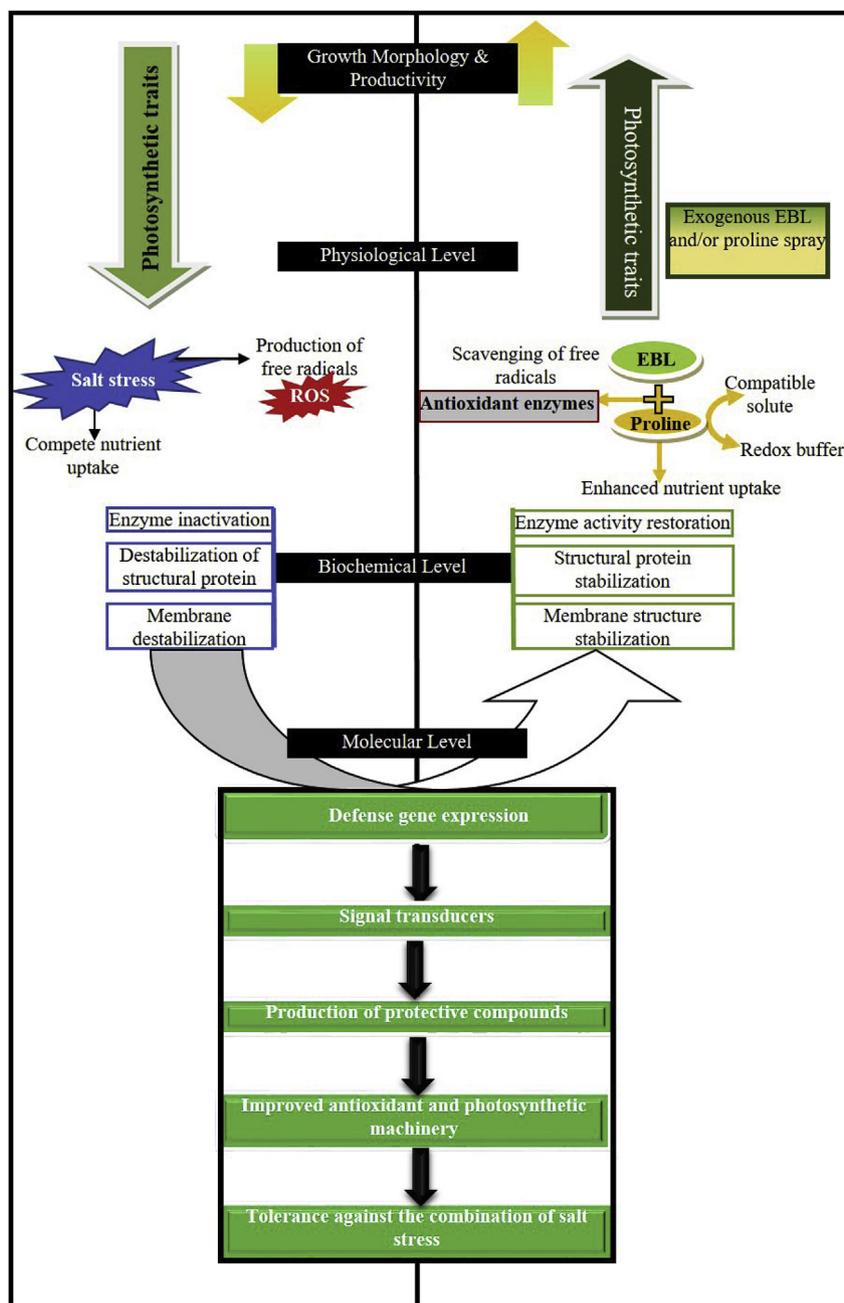


Fig. 7. Diagrammatic representation of the possible effects of 24-epibrassinolide (EBL) and/or proline on the salinity induced changes in *Brassica juncea* (L.) Czern & Coss.

yield. Therefore, it may not be a surprise if the combination of these two increased the values further (Fig. 5) to give better yield both under stress and stress-free conditions.

The leaf-applied combination of EBL and proline improved almost all the growth and yield parameters both in the presence or absence of the salt stress. A diagrammatic summary of the effect of EBL and/or proline on the salinity induced changes in mustard plants is shown in Fig. 7. It is now well documented that under various stresses, BRs induce the accumulation of compatible solutes which are associated with the stress tolerance (Sharma et al., 2017). Moreover, it is reported that BR tyrosine mutant enhances the production of proline under stress conditions. It is therefore suggested that both proline and EBL in combination act as signaling molecules for the modulation of gene expression to increase the ability of plants to tolerate various stresses. However, more study is needed at molecular level to disclose the

crosstalk between BRs and proline and with other phytohormones in providing plant tolerance against the stress.

### 5. Conclusion

The present study leads us to conclude that the application of proline or EBL improved plant growth and seed productivity, both under salt stress and stress-free conditions. Moreover, additional gains may be obtained in case both are given in a combination as the photosynthetic rate and the level of antioxidant system in plants is elevated further. These findings have huge agricultural implications with focus on NaCl induced stress as amelioration is feasible by using bio-friendly and cost-effective strategies. Further study will be required to identify the mechanisms of action of exogenously applied EBL and proline that will certainly promote their efficient use in crop production under stressful

conditions.

#### Author contribution

**Arif Shafi Wani:** Main author, carried out overall research during his PhD.

**Aqil Ahmad:** Supervised the PhD research.

**Shamsul Hayat:** Helped in experimentation, data collection and statistical analysis.

**Inayatullah Tahir:** helped in evaluating manuscript.

#### Conflicts of interest

The authors declare that they have no conflict of interest.

#### Acknowledgements

The authors are thankful to the Chairman, Dept. of Botany, AMU, for providing the necessary facilities to carry out this work. Arif Shafi Wani also gratefully acknowledges the financial assistance rendered by the SERB (SB/YS/LS-389/2013), DST, New Delhi, India.

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