



Efficacy of brassinosteroid analogues in the mitigation of toxic effects of salt stress in *Brassica juncea* plants

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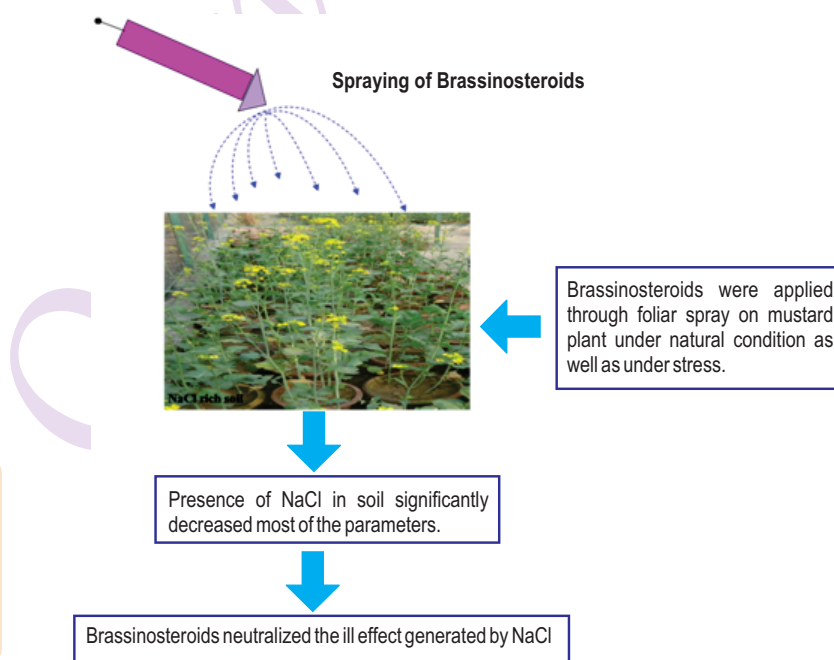
Abstract

Aim: Brassinosteroids have been recognized as sixth class of phytohormones which have the ability to confer tolerance against salinity stress. Brassinosteroids were applied through foliar spray on mustard plant under natural condition, as well as under stress.

Methodology: Analogues of Brassinosteroids (28-homobrassinolide, 24-epibrassinolide) were applied to the foliage of non-stressed and salinity stressed mustard plant under natural condition. Brassinosteroids neutralized the ill effect generated by NaCl. Presence of NaCl in soil significantly decreased most of parameters.

Results: The results showed that the presence of NaCl in soil significantly decreased most of the parameters in concentration dependent manner. Whereas, out of the two brassinosteroid analogues, 24-epibrassinolide excelled over 28-homobrassinolide and neutralized the ill effects generated by NaCl.

Interpretation: It can be inferred from the observations that brassinosteroids (in particular 24-epibrassinolide) can be used as an effective tool in the amelioration of salt stress.



Introduction

India is the second largest producer of *Brassicac*s supplying nearly 27.8% of world's total edible oil (Shekhawat *et al.*, 2012). However, this supply still remains insufficient to fulfill the daily needs of the people (Shekhawat *et al.*, 2012). This scarce production of *Brassicac*s has been attributed to various biotic and abiotic stresses in the environment, among which salt stress has appeared as one of the serious threat to crop productivity (Hasanuzzaman *et al.*, 2013). Uptake of essential minerals (K^+ , Ca^{2+} and Mn^{2+}) is reduced by excess accumulation of Na^+ and Cl^- in cells under salt stress due to ionic imbalances, which naturally distorts the physiology of plants (Almodares *et al.*, 2014). Salinity stress decreases the stomatal conductance, internal CO_2 pressure and stomatal opening which affects gaseous exchange leading to inhibition of photosynthesis (Wani *et al.*, 2013). This salinity stress induced loss in the photosynthetic rate is responsible for retarded plant growth and productivity at maturity (Yadav *et al.*, 2011). Osmotic stress, the primary symptom, causes leaf senescence during the phase of ion toxicity due to accumulation of excessive salts in the transpiring leaves (Munns, 2002). These factors altogether cause adverse pleiotropic effects that lead to loss of plant growth and yield, at harvest.

Brassinosteroids, a class of poly-hydroxysteroids, have been recognized as sixth class of phyto-hormones which have been associated with wide range of plant physiological and molecular responses in plants. They have the ability to induce cell elongation and cell division in stem, promote xylem differentiation and abscission (Coll *et al.*, 2016). They also have the ability to confer tolerance against osmotic stress, temperature stress, salinity stress, and heavy metals like, cadmium, nickel, aluminum and copper in plants (Coll *et al.*, 2016).

Keeping in view the roles assigned to Brassinosteroids and the ever increasing salt stress in soil, the present experiment was designed with an aim to counteract the changes in growth and photosynthetic parameters in salinized plants of *Brassica juncea* (L.) Czern & Coss by inducing resistance, against the stress through Brassinosteroids application. Two varieties of *Brassica juncea* (L.) Czern & Coss namely Varuna and RH-30 were selected in this study based on their high and low photosynthesizing capacity, respectively.

Materials and Methods

Hormone solution : The stock solutions of 28-homobrassinolide (homobrassinolide) or 24-epibrassinolide (epibrassinolide) were prepared by dissolving the required quantity of homobrassinolide or epibrassinolide in 5 ml of ethanol, in 100 ml volumetric flasks. 5 ml of surfactant "Tween-20" was added to it and final volume was made up to the mark by using double distilled water. The desired concentrations (10^{-8} M) of homobrassinolide or epibrassinolide

were prepared by diluting the stock solution with double distilled water.

Plant material: Authentic and healthy seeds of *Brassica juncea* (L.) Czern & Coss cv. Varuna and RH-30 were procured from National Seed Corporation Ltd., New Delhi, India. The seeds were surface-sterilized with 0.01% mercuric chloride solution followed by repeated washing with double distilled water in order to remove adhered mercuric chloride on the seed surface. The experiment was conducted with 60 earthen pots in such a way that each treatment had five replicates and within each pot three plants were maintained under simple randomized block design. Surface sterilized seeds of *Brassica juncea* (L.) Czern & Coss cv. Varuna and RH-30 were sown in earthen pots (25×25 cm), filled with equal quantity of sandy loam soil mixed with farmyard manure (9:1) amended with different levels (2.8, 4.2 or 5.6 $ds\ m^{-1}$) of NaCl. These pots were placed in a net house of Botany department, Aligarh Muslim University, Aligarh, India under natural environmental conditions. At 29 days after sowing (DAS), the foliage of plants was sprayed with double distilled water, homobrassinolide (10^{-8} M) or epibrassinolide (10^{-8} M). Each plant was sprinkled three times at an interval of 10 min on the same day. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 ml of double distilled water or homobrassinolide or epibrassinolide solution in one sprinkle. The plants were then sampled at 60 DAS to assess various morphological and physiological parameters and at 120 DAS to study the yield parameters.

Growth characteristics: On the day of sampling, the plants were removed along with soil and dipped in water to dislodge the adhering soil particles while ensuring the integrity of roots. The plants were blotted and the lengths of root and shoot were measured, followed by their subsequent weighing to record fresh mass. The root and shoot were then oven dried (80 °C for 72 hr) and weighed to note their dry mass. Leaf area was ascertained by gravimetric method by tracing the outline of leaf on graph sheet and counting the squares covered by it on a graph paper.

Electrolyte leakage, leaf water potential and carbonic anhydrase activity: The electrolyte leakage, leaf water potential and carbonic anhydrase activity were determined as discussed in our previous study (Wani *et al.*, 2013).

Soil-Plant analysis development (SPAD) chlorophyll value, photosynthetic attributes and maximum quantum yield of PSII: The SPAD chlorophyll value, photosynthetic attributes [net photosynthetic rate (P_n), stomatal conductance (g_s), internal CO_2 concentration (C_i), and transpiration rate (E)] and maximum quantum yield of PS II (Fv/Fm) in the intact leaves were assessed as described in our previous study (Wani *et al.*, 2013).

Statistical analysis: Treatment means were compared by the analysis of variance using SPSS (SPSS, ver. 17, Chicago, United

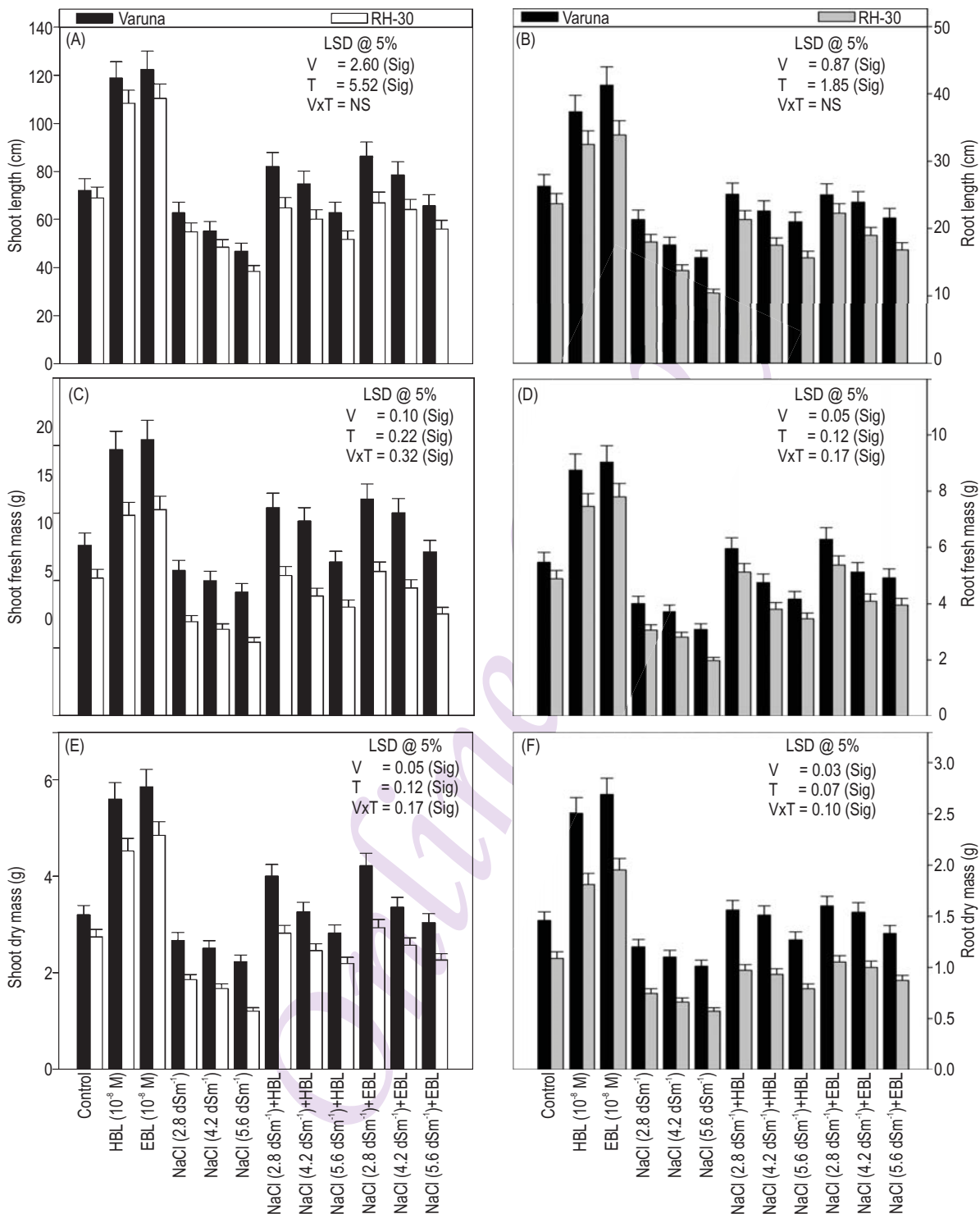


Fig. 1 : Effect of 28-homobrassinolide (HBL; 10^{-8} M) or 24-epibrassinolide (EBL; 10^{-8} M) as foliar spray (29 day stage) and/or soil applied sodium chloride (NaCl; 2.8, 4.2, or 5.6 dSm^{-1}) on (A) Shoot length, (B) Root length, (C) Shoot fresh mass, (D) Root fresh mass, (E) Shoot dry mass and (F) Root dry mass in two varieties (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS

States). Least Significant Difference (LSD) was calculated at the 5% level of probability. Standard error between the replicates was also calculated.

Results and Discussion

The plants raised in soil amended with different levels (2.8, 4.2, or 5.6 dSm^{-1}) of NaCl exhibited a linear decrease in the values of all the growth characteristics (length, fresh mass and dry mass of root and shoot and leaf area) in both the varieties (Figs. 1,

2A). Varuna variety was more tolerant to stress than RH-30, attributed to its differential regulation of physiological, biochemical and genetical processes. The reason behind the salt induced decrease in plant growth is reduction of cell division and elongation (Pitann *et al.*, 2009) which mainly occurs due to the alterations in the nutrient uptake, excess reactive oxygen species accumulation (Ashraf, 2009), cytoplasmic enzyme inhibition, turgor loss and hormonal imbalance (Iqbal and Ashraf, 2010) induced by the salt. The studies are further authenticated by the reports in sunflower (Akram and Ashraf, 2011) and *Ocimum*

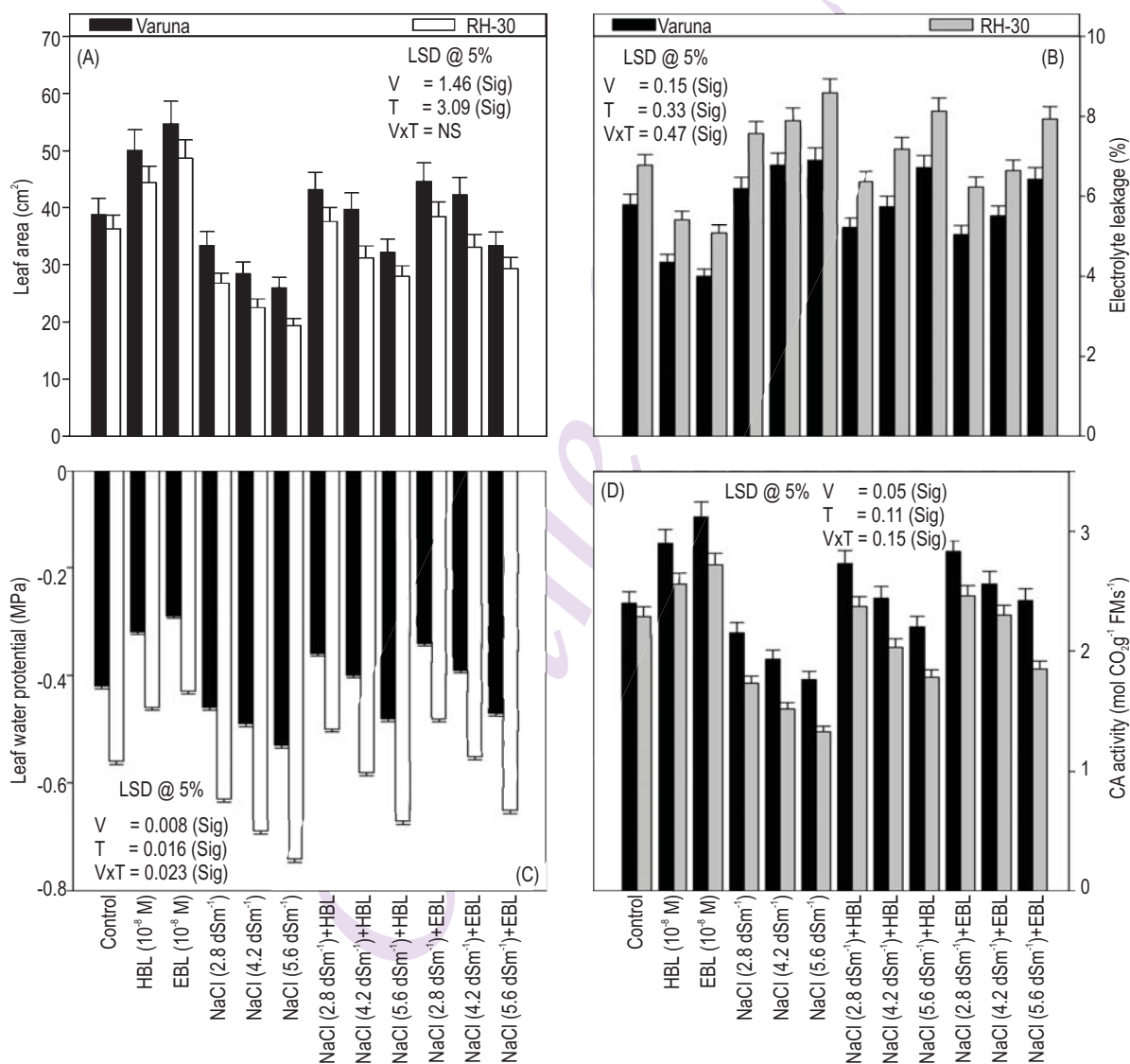


Fig. 2 : Effect of 28-homobrassinolide (HBL; 10^{-8} M) or 24-epibrassinolide (EBL; 10^{-8} M) as foliar spray (29 day stage) and/or soil applied sodium chloride (NaCl; 2.8, 4.2, or 5.6 dSm^{-1}) on (A) Leaf area, (B) Electrolyte leakage, (C) Leaf water potential and (D) Carbonic anhydrase (CA) activity in two varieties (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS

basilicum (Heidari, 2012) for similar responses. However, the foliage applied brassinosteroids (homobrassinolide/epibrassinolide) of stressed or non-stressed plants improved plant growth. Out of these two, epibrassinolide was more effective in generating favorable response and increased the length of shoot and root by 70% and 57% in Varuna and 60% and 43% in RH-30, over control plants (Fig. 1). Brassinosteroids are recognized to modulate a number of metabolic phenomena leading to plant resistance against the stress (Coll *et al.*, 2016). Amelioration of salt stress by brassinosteroids application is well documented in various plants such as *Cajanus cajan* (Dallo *et al.*, 2011), eggplant (Wu *et al.*, 2012) etc. Brassinosteroids involves regulation of cell division and elongation (Peleg and Blumwald, 2011), genes encoding xyloglucan endo-transglycosylase/hydrolase *i.e.*, enzymes responsible for the modification of cell wall activity and cell enlargement, cellulose synthase and sucrose synthase (Coll *et al.*, 2016) that play a vital role in plant growth and development. Exogenous application of brassinosteroids accelerates plant growth and development, however, the extent of their effects may vary with plant species and their concentration applied. Moreover, reduction in the values of growth characteristics caused by lower concentrations (2.8 or 4.2 dsm^{-1}) of NaCl was completely neutralized by brassinosteroids (10^{-8} M) spray in Varuna, where epibrassinolide was more effective than homobrassinolide. The foliage of *Brassica* plants exposed to brassinosteroids possessed larger leaves (Fig. 2A), which could be an expression of activated cell division and cellular enlargement (Bajguz and Tretyn, 2003). Similar observations have also been reported in *Brassica juncea*, under copper stress (Fariduddin *et al.*, 2009) and in wheat under stress free conditions (Shahbaz *et al.*, 2008).

Carbonic anhydrase enzyme is important in plant carbon acquisition. This enzyme catalyzes dehydration of HCO_3^- and provides CO_2 external to the plasma membrane of plant cells when HCO_3^- is available, but CO_2 is limited (Badger, 2003). In the present study, NaCl-induced stress caused a significant decrease in the activity of CA (Fig. 2D). It could be due the negative impact of salinity on gene expression of carbonic anhydrase (Liu *et al.*, 2012). Under comparable conditions, Idrees *et al.* (2012) also reported loss of carbonic anhydrase activity. However, leaf applied brassinosteroids (homobrassinolide/epibrassinolide) to stressed or non-stressed plants increased the carbonic anhydrase activity. Epibrassinolide proved best and increased the activity by 30% and 18% in Varuna and RH-30 respectively, over their control plants under stress-free conditions. This increase in the carbonic anhydrase activity is due to up-regulation of CO_2 assimilation rate by brassinosteroids (Yu *et al.*, 2004) that may be due to enhanced expression of genes that encode other enzymes of calvin cycle and also play an important role in regeneration of RUBP, thereby maximizing carboxylation rate of Rubisco (Xia *et al.*, 2009), supported by Hayat *et al.* (2010) in tomato and Swamy and Rao (2009) in *Pelargonium graveoleus*.

The plants, under salt stress loss a significant level of leaf chlorophyll (Wani *et al.*, 2013; Heidari, 2012), in a concentration and/or variety dependent manner (Fig. 3A). Presumably, salt stress either inhibits its synthesis or accelerates the degradation of chlorophyll molecules (Ashraf and Harris, 2013). However, this harmful effect of NaCl was completely overcome by brassinosteroids application to the foliage of plants of various crops (Hayat *et al.*, 2011; Yu *et al.*, 2004) because of their involvement in improving the related transcription and/or translation machinery (Bajguz and Asami, 2005) (Fig. 3A). Epibrassinolide established itself as the most effective brassinosteroids analogue in increasing SPAD chlorophyll by 30% in Varuna over control. Moreover, brassinosteroids also retard the rate of chlorophyll degradation and that of the proteins, in particular those associated with light-harvesting complexes in thylakoid membranes (Holla, 2011).

The stressed plants also exhibited a decrease in various photosynthetic attributes (P_n , g_s , C , and E) in both the varieties (Fig. 3B-E). The highest concentration (5.6 dsm^{-1}) of NaCl decreased the values of P_n , g_s , C , and E by 27%, 40%, 17%, and 18% in Varuna and 46%, 52%, 32% and 29% in RH-30 respectively, as compared with non-stressed control plants. NaCl induces stomatal closure due to decreased leaf turgor and atmospheric vapor pressure along with root-generated chemical signals (Chaves *et al.*, 2009) which is the main cause for the observed loss of g_s , C , and E . This decrease in g_s and C , directly results in the slow-down of net photosynthetic rate in stressed plants (Lu *et al.*, 2009; Fig. 3B). Thus, decrease in the net photosynthetic rate under salt stress conditions is attributed to suppression in mesophyll conductance and stomata closure. Decrease in SPAD chlorophyll value (Fig. 3A) and carbonic anhydrase activity (Fig. 2D) may also be assigned as reasons for lowering P_n in stressed plants. Wu *et al.* (2012) reported that salt stress decreased the photosynthetic rate in eggplants, whereas, Hakim *et al.* (2014) also noted that the decreased photosynthesis in rice plants under saline stress. However, brassinosteroids (homobrassinolide/epibrassinolide) alone or as a follow-up treatment to the stressed plants improved all the photosynthetic attributes in both the varieties (Fig. 3B-E). The two important enzymes that initiate the process of photosynthesis *i.e.*, carbonic anhydrase and Rubisco, are assigned to be initiated by brassinosteroids (Yu *et al.*, 2004). The higher carbonic anhydrase activity increases the carboxylation state of Rubisco (Bajguz and Asami, 2005), thereby improves P_n (Fig. 3B). It may, therefore be inferred that brassinosteroids improved the CO_2 concentration (Fig. 3D) by increasing g_s (Fig. 3C) and also the efficiency of light harvesting system by elevating the level of chlorophyll (Fig. 3A) whose cumulative action speeded up the net photosynthetic rate of the plants (Holla, 2011; Fig. 3B). Moreover, brassinosteroids improved the water relations *i.e.* leaf water potential (Fig. 2C), membrane structure and its stability (Yan *et al.*, 2011) also

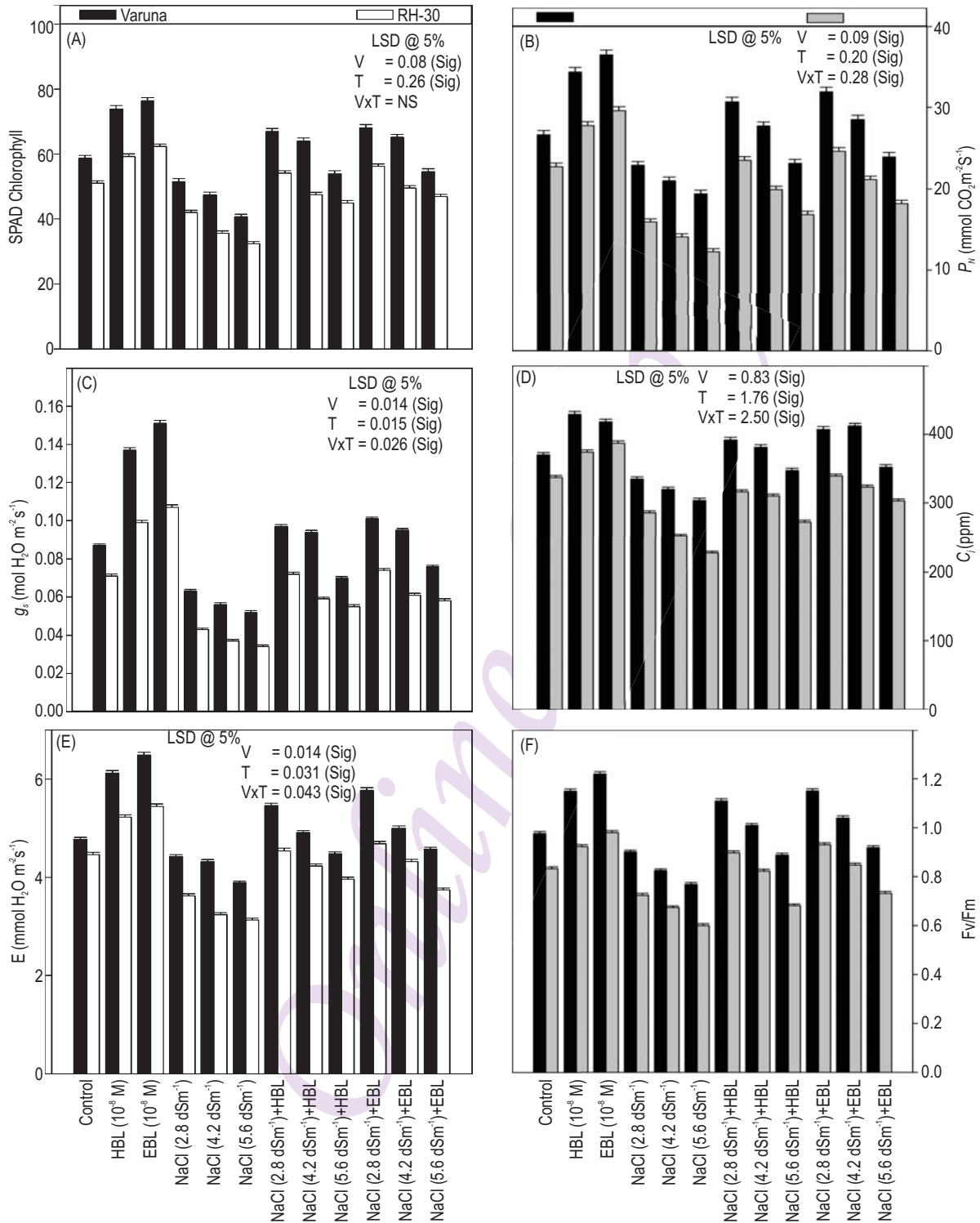


Fig. 3 : Effect of 28-homobrassinolide (HBL; 10^{-8} M) or 24-epibrassinolide (EBL; 10^{-8} M) as foliar spray (29 day stage) and/or soil applied sodium chloride (NaCl; 2.8, 4.2, or 5.6 dSm^{-1}) on (A) SPAD chlorophyll, (B) Net photosynthetic rate; P_n , (C) Stomatal conductance; g_s , (D) Internal CO_2 concentration; C_i , (E) Transpiration rate; E and (F) Maximum quantum yield of PSII; Fv/Fm in two varieties (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 60 DAS

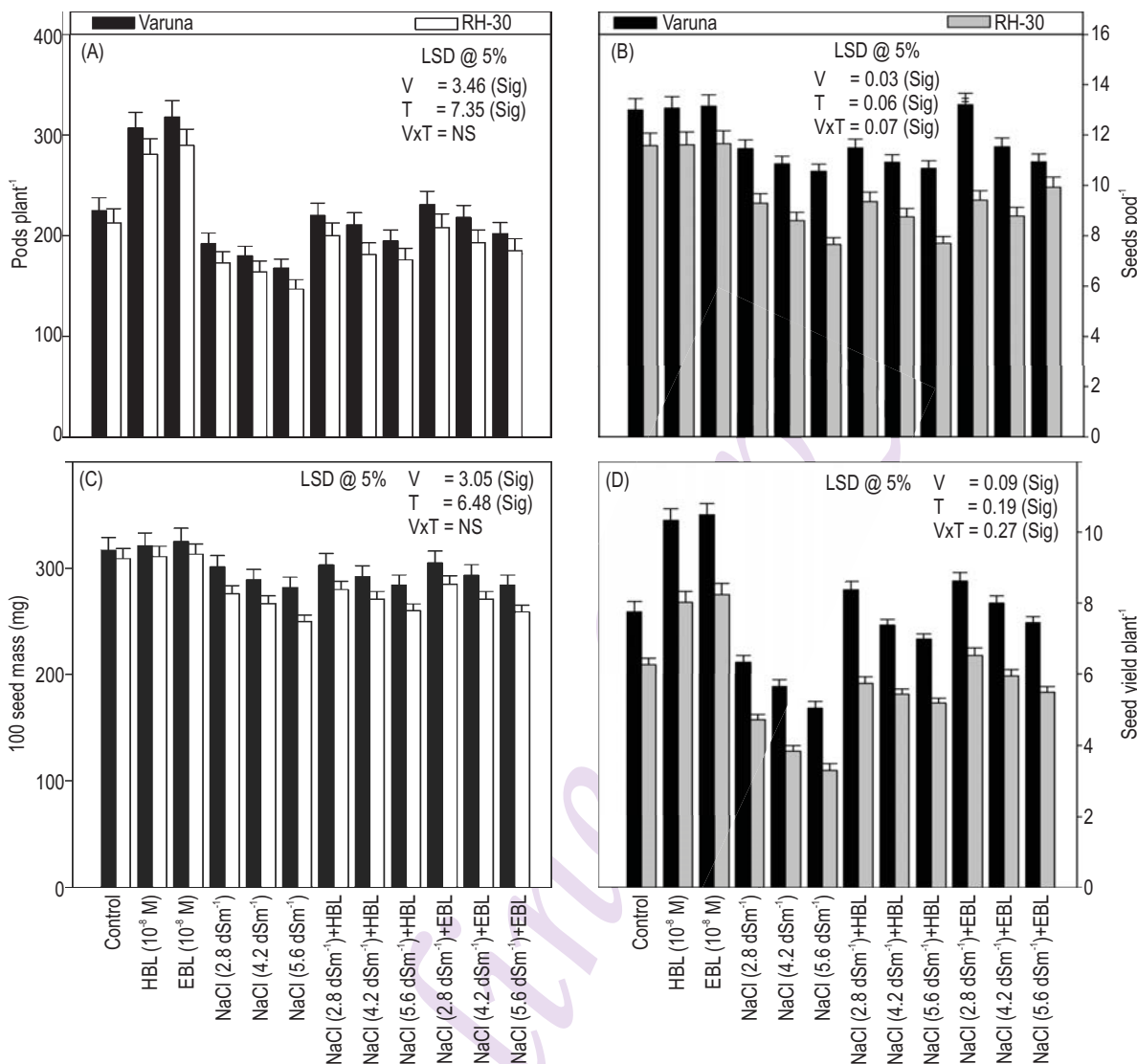


Fig. 4 : Effect of 28-homobrassinolide (HBL; 10⁻⁸ M) or 24-epibrassinolide (EBL; 10⁻⁸ M) as foliar spray (29 d stage) and/or soil applied sodium chloride (NaCl; 2.8, 4.2, or 5.6 dsm⁻¹) on (A) Pods plant⁻¹, (B) Seeds pod⁻¹, (C) 100 seed mass and (D) Seed yield plant⁻¹ in two varieties (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 120 DAS

expressed by the decreased electrolyte leakage in the present study (Fig. 2B).

The plants exposed to salt stress had significantly lower values for quantum yield of PSII (Fig. 3F) because of the damage in PSII electron transport system (Megdiche *et al.*, 2008). The highest concentration of salt (5.6 dsm⁻¹) decreased Fv/Fm values by 21% and 28% in Varuna and RH-30 respectively, when compared with control plants. NaCl decreases the photochemical efficiency leading to suppression of PSII activity (Mehta *et al.*, 2010). It also blocks the electron transfer from primary acceptor,

plastoquinone (QA) to the secondary acceptor plastoquinone (QB) at the acceptor side of PSII which leads to decrease in Fv/Fm values (Shu *et al.*, 2012). Similar observations have also been reported in other crops, *Solanum melongena* (Wu *et al.*, 2012) and *Cucumis sativus* (Shu *et al.*, 2012) on being exposed to salt stress. However, the brassinosteroids applied to foliage of *Brassica* improved the values of Fv/Fm both in the presence or absence of NaCl stress (Fig. 3F). Out of two Brassinosteroids analogues epibrassinolide proved best and completely neutralized the damage caused by two lower concentrations of NaCl (2.8 or 4.2 dsm⁻¹) in Varuna. Brassinosteroids protect PSII

against over-excitation, under salt stress that otherwise could have caused the loss of integrity of thylakoid membrane (Wu *et al.*, 2012). Brassinosteroids induced protection of Fv/Fm, under salt stress, is also reported earlier in *Solanum melongena* (Wu *et al.*, 2012) and wheat (Shahbaz *et al.*, 2008).

The yield characteristics (number of pods per plant, number of seeds per pod, 100 seed mass and seed yield per plant) decreased significantly with the increase in NaCl level in soil (Fig. 4). The highest NaCl level (5.6 dsm⁻¹) generated maximum damage to number of pods per plant (25%, 31%), number of seeds per pod (18%, 34%), 100 seed mass (11%, 19%) and seed yield per plant (35%, 47%), in Varuna and RH-30, respectively, as compared with non-stressed plants. Loss in the seed yield, under salt stress can be attributed to the observed poor plant growth (Fig. 1) resulting largely from the lower pace of photosynthesis (Fig. 3B, Chen *et al.*, 2009). These results are in conformity with those of Asgari *et al.* (2012) who noted that salt stress decreased the growth and leaf ion conc. corresponds to decreased yield under salt stress in wheat plant. Later Wani *et al.* (2013) also reported the decreased seed yield in the presence of NaCl in mustard plant. Under salt stress, the thickness of assimilate conducting pathway gets reduced (Aldesuquy and Ibrahim, 2001), and the leaves start behaving as sink rather than source (Arbona *et al.*, 2005). This causes inhibition of assimilate movement towards the developing reproductive organs, which might be the reason for the observed decrease in the yield characteristics. However, stress-free and stressed plants, applied with brassinosteroids to their foliage, exhibited an improvement in the values for yield characteristics, at harvest (Fig. 4). The possible reason for increase in the crop yield might be due to BR-induced action on senescence of plant organs (leaves and flowers) (Iwahori *et al.*, 1990) that will automatically increase the life span of photosynthetically active sites. This consequently resulted in the observed increase in the number of pods per plant and ultimately increased seed yield per plant (Fig. 4). The observed increase in photosynthetic CO₂ assimilation rate i.e., P_n, under brassinosteroids (Fig. 3B) will naturally result in the availability of more carbohydrates for metabolism and export to the sink (Bajguz and Asami, 2005). Therefore, availability of additional photosynthates at the sink in association with induced expression of genes encoding enzymes for carbohydrate metabolism (Roitsch, 1999) lead to the improved seed yield (Fig 4D). Similarly, higher biological yield in passion fruit, correlating with higher photosynthetic CO₂ assimilation, under brassinosteroids (Gomes *et al.*, 2003) was reported. Brassinosteroids favored improvement in the yield characteristics in *Cicer arietinum* exposed to cadmium stress (Hasan *et al.*, 2008) has also been reported earlier.

Out of the two analogues of brassinosteroids (homobrassinolide/ epibrassinolide) used in the study, epibrassinolide proved more effective in improving the values of most of the parameters studied both in stress-free and stressed

plants possibly due to differences in the structure and stability (Khrupach *et al.*, 2003). In almost all brassinosteroids, S-oriented alkyl (methyl or ethyl) group at C-24 of side chain is present while epibrassinolide and CS (another brassinosteroids analogue) being exceptions, have R-oriented alkyl group on the side chain of the steroid nucleus. The attachment of epibrassinolide to its plasma membrane receptor leads to more distorted three dimensional conformational states as compared to homobrassinolide. This thermodynamically acquired new stable state of epibrassinolide seems to be more actively involved in triggering wide array of favorable signal cascades than homobrassinolide. However, more research is required to know about the transcription factors that are involved in BRI-1 kinase inhibitor-1 (BKI-1) dissociation with BRI-1 associated kinase 1 (BAK-1) to avail the binding in brassinosteroid insensitive-1 (BRI-1) at membrane (Hategan *et al.*, 2011; Codreanu and Russinova, 2011).

It can be concluded from the present study that brassinosteroids can be used as stress alleviators of NaCl. Brassinosteroids (especially epibrassinolide) may, therefore, be applied to the plant foliage to improve the biological yield, and to ameliorate the toxic effects of stress in *Brassica juncea*.

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