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## RESEARCH ARTICLE

## 24-epibrassinolide and spermidine modulate photosynthesis and antioxidant systems in *Vigna radiata* under salt and zinc stress

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Brassinosteroids and polyamines are widely used to overcome various abiotic stresses including salinity (NaCl) and heavy metal stresses in plants. In the present investigation, co-application efficacy of 24-epibrassinolide (EBL) and spermidine (Spd) on the salt stress in the form of NaCl (100 mM), and zinc toxicity in the form of zinc sulphate (200 mg kg<sup>-1</sup> soil) were evaluated in *Vigna radiata* (L.) Wilczek. At 15 d stage, the seedlings were sprayed with deionized water (control), 1mM Spd and/or 10<sup>-8</sup> M EBL and plant responses were studied at 30 days after sowing. The salinity and zinc stress significantly reduced the plant growth, gas exchange parameters but increased antioxidant enzyme activity, proline content and electrolyte leakage in the leaves. Furthermore, out of the two plant growth regulators (EBL/Spd), EBL excelled in its effects. Toxic effects generated by salinity and zinc metal stress were completely overcome by the combination of these two plant growth regulators.

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## INTRODUCTION

Salinity is one of the most serious problems posing a threat to sustained agricultural crop production (Wahid and Ghazanfar 2006), thereby limits food production worldwide. In both natural and agricultural conditions, plants are frequently exposed to environmental stresses. Some environmental factors, such as air and temperature, could become stressful in just a few minutes; others (such as soil salinity and zinc content) may take days or weeks to become stressful. High salinity together with higher concentration of metals like zinc is among the quite important abiotic stresses that usually become the limiting factors for normal plant growth and productivity (Duman et al. 2014). Soil salinity, predominantly sodium chloride (NaCl) is a major agricultural issue, particularly in irrigated areas (Zhu 2001). High levels of soluble salts including chlorides of sodium, calcium, and magnesium often cause many types of stress symptoms in plants, like potassium (K) deficiency (Coskun et al. 2013), osmotic stress (Shavrukov 2012) and soil sodicity, alkalinity and other soil problems. Soluble salts at higher concentrations lead to hyperosmolality and imbalance of nutrients in plant systems that harmfully decline plant growth (Turan et al. 2010). Salt stress and many other biotic and abiotic stresses, lead to the production of reactive oxygen species (ROS) like superoxide (O<sub>2</sub><sup>-</sup>), hydroxyl (·OH) free radicals, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and free singlet oxygen (Gill and Tuteja 2010; Sharma et al. 2012). Below a threshold level, ROS are not detrimental to plant cells, furthermore, these can act as a messenger molecules to initiate signal transduction cascades involving mitogen-activated protein kinase (MAPK) under various environmental cues (Opdenakker et al. 2012). However, the over-accumulated ROS trigger detrimental effects on cellular processes, including oxidative damage to nucleic acids, proteins, membrane lipids, pigments and as a result alter the activities of antioxidative enzymes (Wang et al. 2008; Sharma et al. 2012; Fariduddin et al. 2013). To cope with these damaging radicals, plants have developed enzymatic (e.g., superoxide dismutase, peroxidase, and catalase) and non-enzymatic (e.g., antioxidants and some secondary metabolites)

strategies to detoxify excessive ROS (Scandalios 2002; Mittler et al. 2004), in order to create a balance between ROS production and their utilization to maintain cellular redox homeostasis.

Heavy metals stress is another serious problem for the environment and is also one of the major environmental stresses for higher plants. Zinc (Zn) is an essential micronutrient for normal growth and development of plants. It is the second most abundant transition metal in organisms after iron (Fe) and is involved in various biological processes in organisms (Broadley et al. 2007). Large parts of agricultural soil are contaminated with zinc by both natural and anthropogenic activities like natural pedochemical background, atmospheric transport (Steinnes and Friedland 2006), old or recent pyrometallurgical slag weathering (Ettler et al. 2001), mining and industrial processes and agricultural practices, involving the use of fertilizers containing heavy metals (Singla-Pareek et al. 2006). Zn, as metal ion is easily assimilated by plants but it could also generate phytotoxicity at higher concentrations (Broadley et al. 2007; Muschitz et al. 2009). Zinc toxicity leads to inhibition of growth of roots and stems (Hagemeyer 2004), chlorosis and necrosis in leaves (Hermle et al. 2007), damage photosynthetic system (Cherif et al. 2011), significantly alter mitotic activity (Rout and Das 2009), affect membrane integrity and permeability (Stoyanova and Doncheva 2002), and even destroy cells (Chang et al. 2005). Plants have developed partial exclusion or specific complexation and compartmentation to alleviate zinc stress (Leitenmaier and Kupper 2013). However, because of the similarities in ion radius of different bivalent cations like Mn, Fe, Cu, Mg and Zn, excess zinc can shift certain physiological equilibria by local competition at various sites (De Fillipis and Ziegler 1993). Zn forms the cyclic 6-membered peripheral chelate with chlorophyll molecule, forming non-functional Zn-chlorophyll complexes (Petrovic et al. 2006). This results in the reduction of the maximum quantum yield of PSII, which can be closely linked with either a block of synthesis or an increase of degradation rate of PSII functional proteins, mainly the D1 protein (Ralph and Burchett, 1998). Zinc stress affects the intrinsic core D1 and D2 reaction centre proteins, with D1 as its main target. D1/D2 heterodimer is necessary for stable light-induced charge separation across the thylakoid membrane (Edelman and Mattoo 2008). Zinc also affect adversely the activation of the Calvin cycle key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, and the equilibrium between CO<sub>2</sub> and O<sub>2</sub> binding by this protein.

Among various compounds exploited to alleviate plant stress, the brassinosteroids (BRs) and polyamines (PAs) are recognized as group of plant growth regulators that regulate plant growth and productivity. BRs are steroidal plant hormones ubiquitously distributed in plant kingdom. These steroidal compounds occur in free form and conjugated to sugars and fatty acids. Till this date, up to 70 BRs have been isolated from plants. However, brassinolide (BL), 24 epibrassinolide (EBL) and 28 homobrassinolide (HBL) are three bioactive BRs being the most widely used in physiological studies (Khrpach et al. 2000). Ameliorative roles of BRs have been recognized in plants subjected to various biotic and abiotic stresses (Bajguz and Hayat 2009). Exogenous application of BRs increased tolerance to low and/or high temperature stress (Bajguz and Hayat 2009; Fariduddin et al. 2011) and heavy metal stress (Yusuf et al. 2012), drought stress (Fariduddin et al. 2009), salinity (Ali et al. 2007) and water logging (Bajguz and Hayat 2009). Another group of plant growth regulators used to alleviate stress conditions in plants are polyamines (PAs). These are small aliphatic nitrogenous compounds that are found in a wide range of organisms from bacteria to plants and animals. The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are low-molecular-weight organic cations. In plants, polyamines play significant roles in the regulation of cell division, embryogenesis, senescence, reproductive organ development, root growth and in alleviation of environmental stresses (Pang et al. 2007; Alcázar et al. 2010). Implication of PAs in amelioration of various abiotic and biotic stresses has made them an essential component of plant defense mechanism (Hussain et al. 2011).

The exposure of plants to salt (NaCl) and zinc stress is increasingly important problem due to the pollution of salinized lands with metals like zinc (Kholodova et al. 2010). Limited studies were carried out in exploring the combined effect of zinc and salinity stress. But the studies exploring roles of polyamines and brassinosteroids on the interactive effects of zinc and salinity stress, since they are interrelated, is completely lacking. Therefore, the present study was aimed to test the hypothesis that application of Spd (an active analogue of polyamines) and/or EBL (an active analogue of BRs) will induce tolerance to zinc and/or salt stress in *Vigna radiata* (L.) Wilczek and the induced tolerance correlated to the modulation of antioxidant system.

## Materials and Methods

### Biological material

The authentic seeds of *Vigna radiata* L. Wilczek cultivar T-44 were procured from National Seed Corporation Ltd., New Delhi. The healthy seeds were surface sterilized with 1% sodium hypochlorite solution for 10 min, followed by repeated washing with deionised water. The seeds were sown at the rate of 6 seeds per pot of 20 cm diameter, filled with sandy loam soil and farmyard manure (mixed in the ratio of 6:1). On germination, three plants per pot were maintained after thinning.

### EBL and Spd preparation

The stock solutions of EBL and Spd were prepared by dissolving the required quantity of EBL or Spd in 5 mL of ethanol, in 100 mL volumetric flasks and final volume was made up to the mark by using double distilled water (DDW). The desired concentrations of EBL or Spd were prepared by the dilution of stock solution with DDW. 5 mL of surfactant "Tween-20" was added to it just before application.

### Treatment pattern and experimental design

Surface sterilized seeds of *Vigna radiata* were sown in the earthen pots (20 cm in diameter, 20 cm in depth) filled with air dried sandy loam soil mixed with farmyard manure in the ratio of 6:1. The zinc as finely powdered ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) was thoroughly mixed with the soil (200 mg/kg). These pots were placed in the net house of the Botany department, Aligarh Muslim University, Aligarh, India ( $27^\circ 53' \text{N} / 78^\circ 5' \text{E}$ ) under natural environmental conditions, under 12 h natural photoperiod irradiance of  $1600\text{--}1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ , with day/night temperatures of  $35^\circ \text{C} / 28^\circ \text{C}$  and the relative humidity between 45 and 70%. Six days after sowing (DAS), NaCl treatment (100 mM) was given through soil. At 15 d stage, the seedlings were sprayed with deionized water (control), 1mM Spd and/or  $10^{-8}$  M EBL. Each seedling was sprinkled thrice. The nozzle of the sprayer was adjusted in such a way that it pumped out 1 mL in one sprinkle. Therefore, each seedling received 3 mL of DDW or Spd and/or EBL solution. The plants were then grown under natural environmental conditions. Plants were harvested at 30 d stage to assess the various growth and leaf gas exchange traits as well as biochemical parameters. The experiment was conducted under completely randomized block design. Each treatment was replicated three times and each replicate contained three plants.

### Plant growth analysis

The plants were removed from the pots along with the soil and were dipped in a bucket filled with water. The plants were moved smoothly to remove the adhering soil particles and the length of root and shoot were measured by using a meter scale. The plants were weighed to record their fresh mass. The samples were then placed in an oven run at  $80^\circ \text{C}$  for 24 h. These dried plants were weighed to record their dry mass. The leaf area was measured by leaf area meter (ADC Bioscientific, Hoddesdon, Herts, UK).

Determination of chlorophyll (SPAD) value, leaf water potential (LWP) and fluorescence i.e. Maximum quantum yield of PS II ( $F_v/F_m$ )

The SPAD value of chlorophyll in the fresh leaf was measured by using the SPAD chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan). Leaf water potential (LWP) was monitored with the help of Psypro water potential system (Wescor, Inc. USA) Chlorophyll fluorescence ( $F_v/F_m$ ) was measured by using a leaf chamber fluorometer (Li-COR 6400-40, Li-COR, and 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 ( $F_m$ ) 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  $F_v/F_m$ .

### Analysis of leaf gas exchange parameters

Gas-exchange parameters were determined on the third fully expanded leaves between 11:00 and 12:00 h by using an infrared gas analyzer (IRGA) portable photosynthetic system (Li-COR 6400, Li-COR, and Lincoln, NE, USA). To measure net photosynthetic rate ( $P_N$ ) and its related attributes [stomatal conductance ( $g_s$ ), internal  $\text{CO}_2$  concentration ( $C_i$ ), water use efficiency (WUE)], the air temperature, relative humidity,  $\text{CO}_2$  concentration and PPFD were maintained at  $25^\circ \text{C}$ , 85%,  $600 \mu\text{mol mol}^{-1}$  and  $800 \mu\text{mol mol}^{-2} \text{s}^{-1}$ , respectively.

### Determination of leaf electrolyte leakage

The total inorganic ions leaked out of the leaves were estimated by the method described by Sullivan and Ross (1979). Twenty leaf discs were taken in a boiling test tube, containing 10 mL of deionized water and electron conductivity (EC) was measured (ECa). The contents were heated at  $45^\circ \text{C}$  and  $55^\circ \text{C}$  for 30 min each in a water bath and EC was measured (ECb). Later the controls were boiled at  $100^\circ \text{C}$  for 10 min and EC was again recorded (ECc). The electrolyte leakage was calculated using the formula:

$$\text{Electrolyte leakage (\%)} = [(ECb - ECa)/(ECc)] \times 100$$

### Determination of nitrate reductase activity

The activity of nitrate reductase (NR) was measured following the method laid down by Jaworski (1971). The fresh leaf samples were cut into small pieces and transferred to plastic vials, containing phosphate buffer (pH 7.5),

KNO<sub>3</sub> and isopropanol which was incubated at 30 °C for 2 h. After incubation, sulfanilamide and N-1-naphthylethylenediamine hydrochlorides solutions were added. The absorbance was read at 540 nm on a spectrophotometer (Spectronic 20D; Milton Roy, USA).

#### Determination of carbonic anhydrase activity

The activity of carbonic anhydrase (CA) in the leaves was measured following the method described by Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces in cysteine hydrochloride solution. These leaf samples were blotted and transferred in a test tube, followed by the addition of phosphate buffer (pH 6.8), 0.2 M NaHCO<sub>3</sub>, bromothymol blue, and the methyl red indicator, at the last. This reaction was titrated against 0.5 N HCl. The activity of the enzyme was expressed on a fresh mass basis.

#### Antioxidative enzyme assay

For the assay of antioxidant enzymes, the leaf tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 27600 × g for 10 min at 4 °C and the supernatant was used as source of enzymes catalase, peroxidase and superoxide dismutase. Peroxidase and catalase were assayed following the procedure described by Chance and Maehly (1956). Catalase was estimated by titrating the reaction mixture, consisting of phosphate buffer (pH 6.8), 0.1 M H<sub>2</sub>O<sub>2</sub>, enzyme extract and 2% H<sub>2</sub>SO<sub>4</sub>, against 0.1 N potassium permanganate solution. The reaction mixture for peroxidase consisted of pyragallol, phosphate buffer (pH 6.8), 1% H<sub>2</sub>O<sub>2</sub> and enzyme extract. Change in absorbance due to catalytic conversion of pyragallol to purpurogallin, was noted at an interval of 20 s for 2 min, at 420 nm on a spectrophotometer. A control set was prepared by using DDW instead of enzyme extract. The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0–50 mL enzyme extract and was placed under 15 W fluorescent lamp. The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light. 50% inhibition by light was considered as one enzyme unit.

#### Determination of proline accumulation

The proline content in fresh leaf samples was determined by adopting the method of Bates et al. (1973). Sample was extracted in sulphosalicylic acid. To the extract an equal volume of glacial acetic acid and ninhydrin solutions were added. The sample was heated at 100 °C to which 5 mL of toluene was added. The absorbance of toluene layer was read at 528 nm on a spectrophotometer.

#### Statistical analysis

Data were statistically analyzed using SPSS, 17.0 for windows (SPSS, Chicago, IL, USA). Standard error was calculated and analysis of variance (ANOVA) was performed on the data to determine the least significance difference (LSD) between treatment means with the level of significance at  $P \leq 0.05$ .

## Results

### Growth biomarkers

Growth parameters (i.e. length, fresh and dry mass of root and shoot and leaf area) significantly decreased with the application of NaCl and Zn (Fig. 1 and 2A). The root and shoot length; root and shoot fresh mass; root and shoot dry mass and leaf area were decreased in the presence of NaCl by 43.6%, 35.1%, 52.3%, 47.3%, 47.2%, 43.4% and 40.7% whereas under Zn application these parameters decrease by 26.7%, 18%, 33.1%, 29.8%, 34.2% 31.7% and 24.9% respectively, as compared to the respective controls. Plants which received the foliage treatment of EBL (10<sup>-8</sup> M) or Spd (1mM) alone had more values than control plants, for the aforesaid growth biomarkers. The follow-up treatment to the stressed plants with EBL partially neutralized the deleterious effect of 100 mM of NaCl, and completely overcome the negative effect generated by Zn toxicity, in all the above mentioned parameters.

### Leaf water potential (LWP)

The plants raised in the presence of NaCl or Zn stress showed lower LWP as compared to unstressed control but maximum reduction was noticed in NaCl stressed plants (Fig. 2B). Under NaCl stress, the value of LWP was decreased by 33.2%, whereas the values decreased by 20% under zinc stress, as compared to the respective controls. However, under the combined stress of NaCl and Zinc, the decrease in the value LWP was 24.1%, which was more than Zinc alone but less than NaCl alone. Moreover, the follow-up treatment with EBL (10<sup>-8</sup> M) and Spd (1 mM) completely overcome the deleterious effect of NaCl and Zn stress in combination.

SPAD Chlorophyll value and fluorescence i.e. Maximum quantum yield of PS II ( $F_v/F_m$ )

Out of the two different stresses (NaCl and Zn), NaCl generated maximum decrease in both chlorophyll content and  $F_v/F_m$  (Fig. 2D and 3B) by 42.1% and 37.2% respectively, whereas the decrease by Zn stress in both the parameters were 26.3% and 25.6% lower than the control plants, respectively. The foliar application of EBL or Spd alone increased both chlorophyll content as well as  $F_v/F_m$  over the non-treated plants. Follow-up treatment of EBL completely overcomes the deleterious effects of NaCl and Zn stress, whereas the follow-up treatment of Spd to the stressed plants partially overcomes the deleterious effect of Zn stress only.

#### Leaf gas exchange parameters

The plants raised in the soil fed with NaCl or Zn had significantly decreased values for gas exchange parameters ( $P_N$ , gs, Ci and WUE) and the values decreased by 37.8% and 22.7%; 39.3% and 25%; 29.7% and 19.8%; and 23.6% and 14.3% respectively, as compared to their respective controls (Fig. 2E, F and 3A, C). The toxicity triggered by NaCl and Zn was effectively overcome by the follow-up treatment of EBL and Spd. The values of the above parameters were increased by 19.8%, 28.6%, 27.3% and 27.9% than their respective controls.

#### Electrolyte leakage

Plants subjected to NaCl stress had more electrolyte leakage (30.3%), than the plants exposed to Zn stress (19.8%) as compared to the control plants (Fig. 2C). However, the value for electrolyte leakage in the plants treated with NaCl and Zn in combination was 21.3% higher, over the control. The treatment of EBL and/or Spd to the stressed plants caused a remarkable decrease in the electrolyte leakage.

#### Carbonic anhydrase (CA) and nitrate reductase (NR) activity

Plants sprayed with either EBL or Spd possessed higher activity of these enzymes than their respective controls (Fig. 3D, E). The application of EBL increased NR activity by 30.6% and CA activity by 31.3%, whereas Spd application increased these enzyme activities by 18.2% and 16.7% respectively, than the control. Treatment of EBL alone or in combination with Spd overcomes the deleterious effect on the activities of these enzymes by NaCl or Zn given separately or in combination. However, the only treatment of Spd partially overcomes the toxicity generated by Zn stress.

#### Proline content

The level of proline exhibited an increase in relation to NaCl and/or Zn stress (Fig. 3F). The application EBL on the unstressed plants brought about a change in the level of proline. However, in association with NaCl or Zn, EBL further increased the quantity of proline. The maximum accumulation of proline (77.2%) was found in the plants which were subjected to combined stress of NaCl and Zn, and subsequently received EBL and Spd treatment, over the non-treated control plants.

#### Antioxidant enzymes

The antioxidative enzymes, catalase, peroxidase and superoxide dismutase exhibited an increasing trend in response to EBL, Spd and stress (NaCl and/or Zn) (Fig. 4A–C). EBL alone caused a significant increase in the activities of all the enzymes. However, the combined treatment of NaCl and EBL further enhanced the activities of these antioxidative enzymes. Moreover, the maximum activities of antioxidative enzymes [CAT (73.2%), POX (77.2%), and SOD (83.8%)] were recorded in the plants exposed to the combination of NaCl and Zn stress, and subsequently received treatment of EBL and Spd.

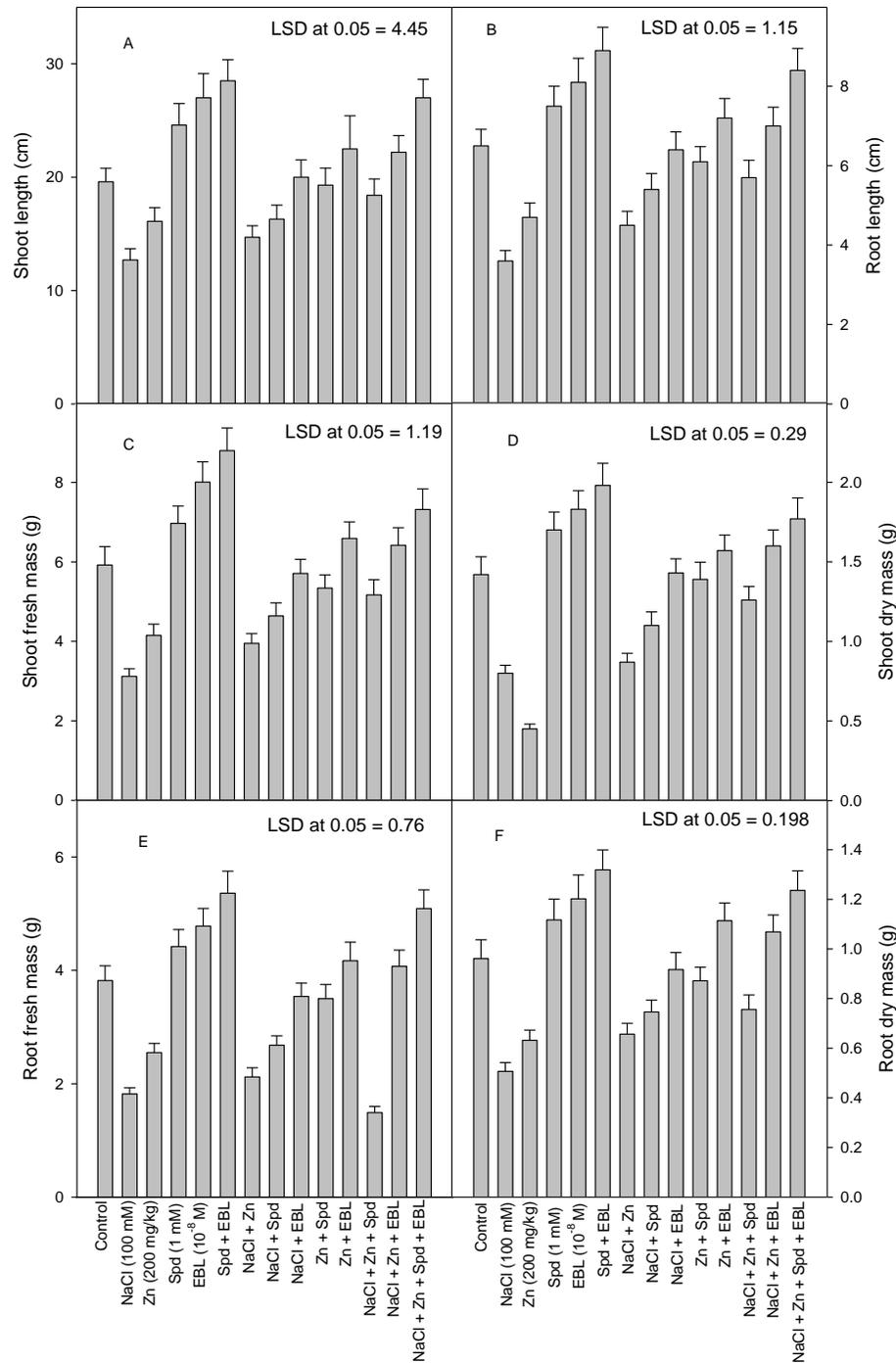


Fig. 1: Effect of 24-epibrassinolide (EBL  $10^{-8}$  M) and/or spermidine (Spd 1 mM) on the NaCl and/or Zn induced changes in the (A) Shoot length (B) Root length (C) Shoot fresh mass (D) Shoot dry mass (E) Root fresh mass (F) Root dry mass of *Vigna radiata* (L.) plants at 30 DAS.

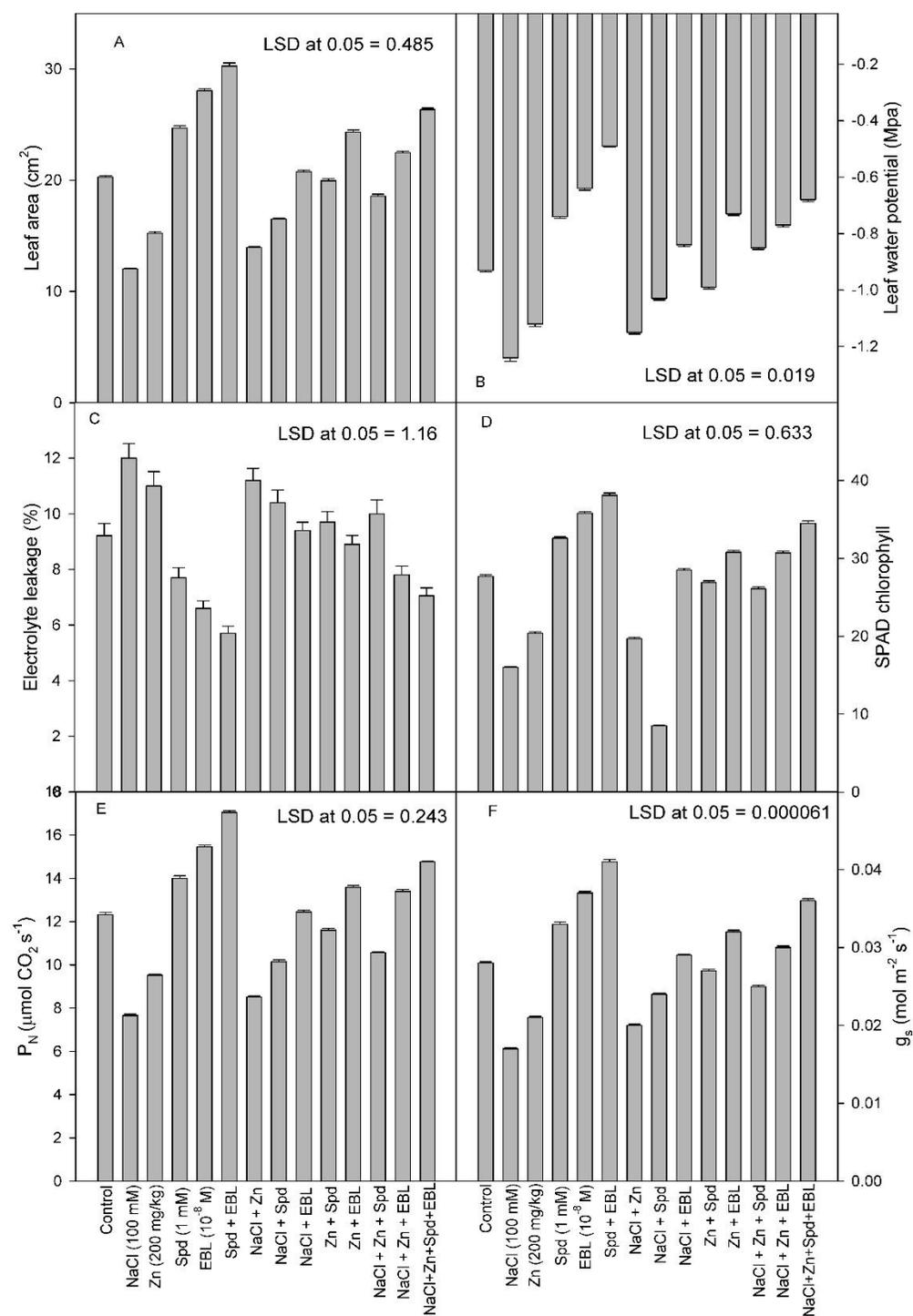


Fig. 2: Effect of 24-epibrassinolide (EBL 10<sup>-8</sup> M) and/or spermidine (Spd 1 mM) on the NaCl and/or Zn induced changes in the (A) Leaf area (B) Leaf water potential (C) Electrolyte leakage (D) SPAD Chlorophyll content (E) Net photosynthetic rate (P<sub>N</sub>) and (F) Stomatal conductance (g<sub>s</sub>) of *Vigna radiata* (L.) plants at 30 DAS.

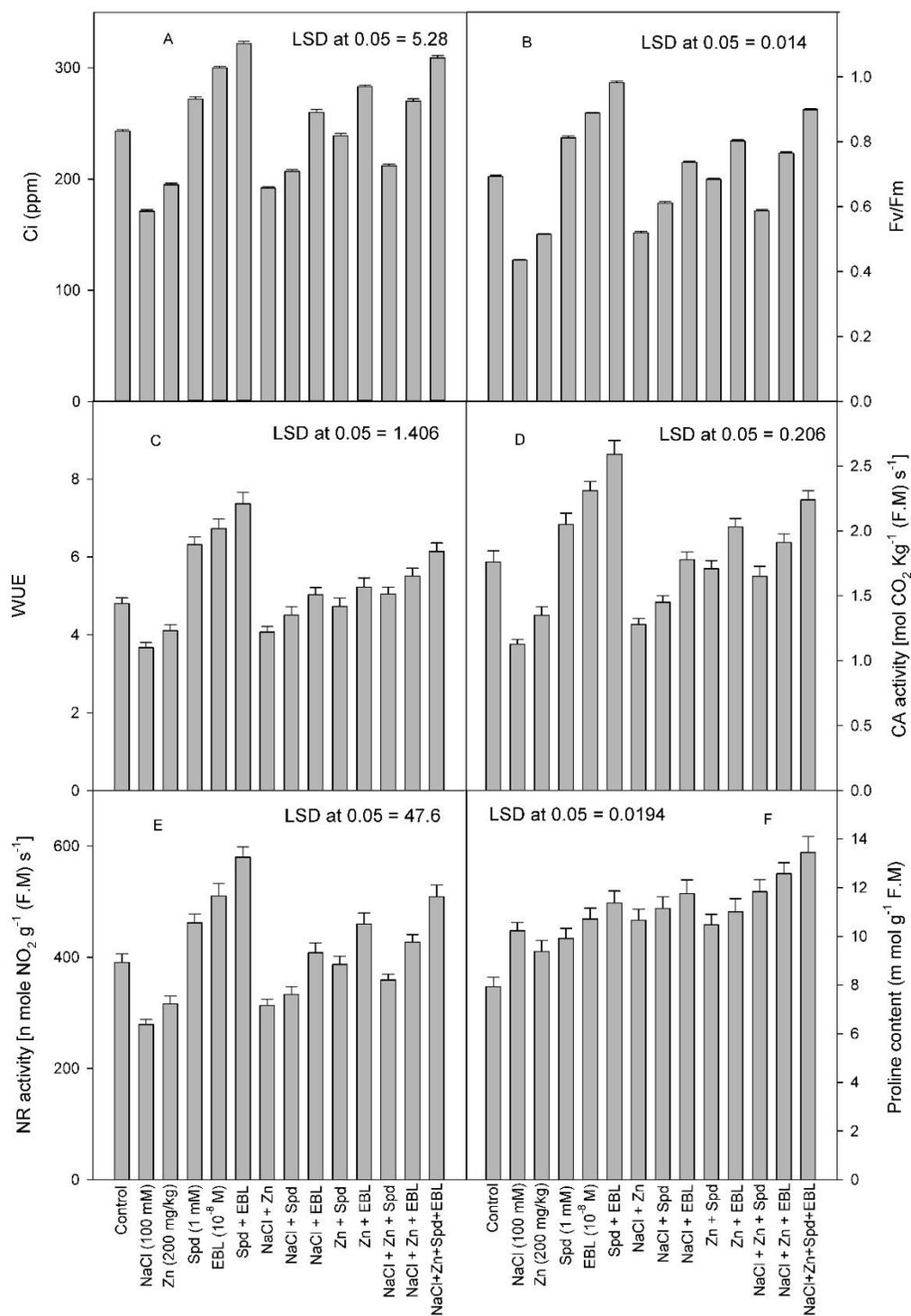


Fig. 3: Effect of 24-epibrassinolide (EBL 10<sup>-8</sup> M) and/or spermidine (Spd 1 mM) on the NaCl and/or Zn induced changes in the (A) internal carbon dioxide concentration (Ci) (B) maximum quantum yield (Fv/Fm), (C) water-use efficiency (WUE) (D) carbonic anhydrase (CA) activity, (E) nitrate reductase (NR) activity and (F) proline content, of *Vigna radiata* (L.) plants at 30 DAS.

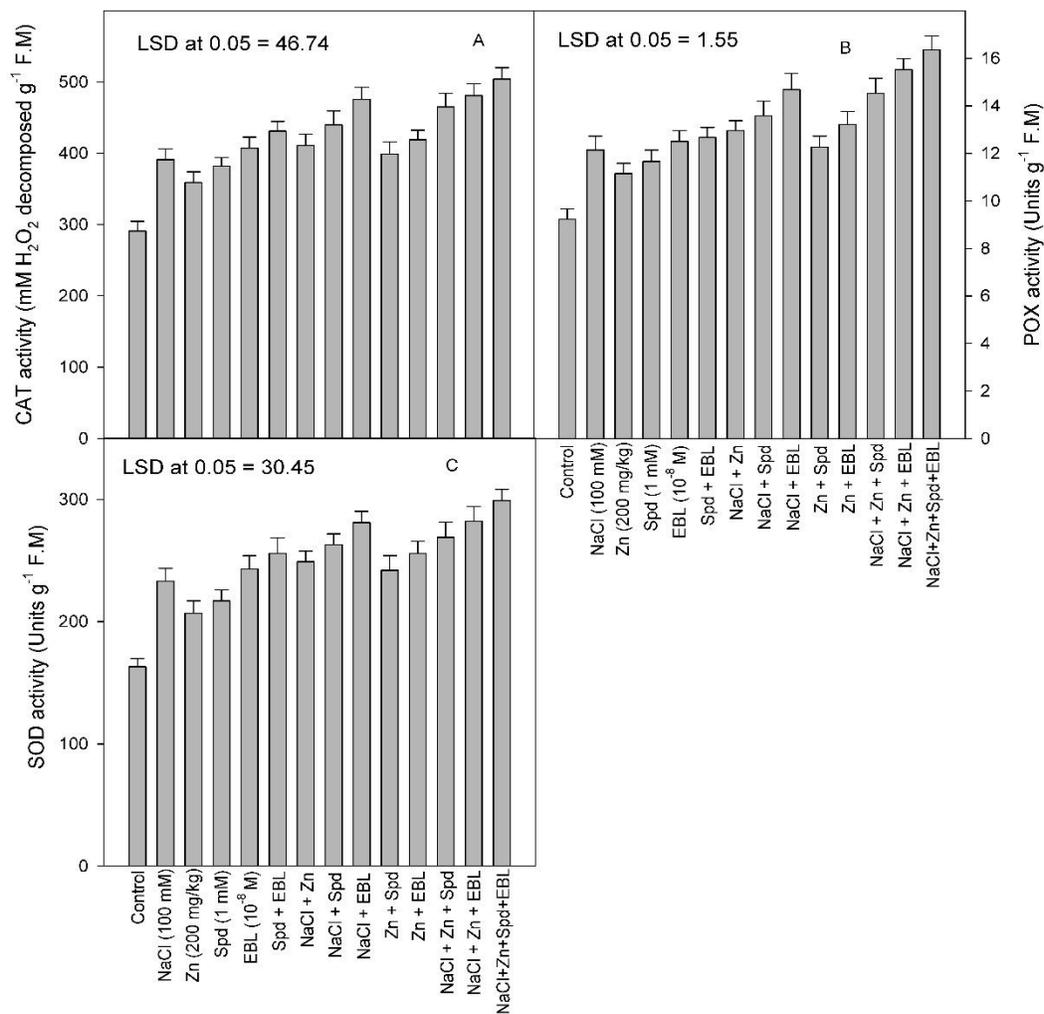


Fig. 4: Effect of 24-epibrassinolide (EBL  $10^{-8}$  M) and/or spermidine (Spd 1 mM) on the NaCl and/or Zn induced changes in the (A) catalase (CAT) activity, (B) peroxidase (POX) activity, and (C) superoxide dismutase (SOD) activity of *Vigna radiata* (L.) plants at 30 DAS.

## Discussion

Environmental stresses, like salinity, drought, heat, chilling, heavy metals or the micronutrients as stresses, are among the major factors limiting crop productivity. One of the important consequences of all the environmental stresses including salinity and zinc stresses is the overproduction of reactive oxygen species (ROS), which include superoxide radical hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $OH^\bullet$ ), and singlet oxygen species ( $^1O_2$ ). Exposure of plants to NaCl and Zn stress has now become a common phenomenon due to their environmental pervasiveness and consequently cause oxidative stress by formation of free radicals (Youssef and Azooz 2013). These free radicals generated under stress conditions trigger deleterious effects in the plants by oxidizing proteins, lipids and nucleic acids resulting in abnormalities both at cellular and individual level. In order to counteract these reactive oxygen species, plants induce the synthesis of antioxidant metabolites like proline, ascorbate, glutathione etc. and enhance the activity of antioxidant enzymes like catalase, peroxidase, superoxide dismutase etc, that neutralize the toxic effects of ROS generated through stress.

Mung bean (*Vigna radiata*) plants grown under salinity and zinc stress possessed elevated activity of antioxidant enzymes viz. catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) (Fig. 4A-C). Similar responses were obtained by Mittal et al., (2012) in *Brassica juncea* on exposure to salt stress and by Yusuf et al. (2011) in *Vigna radiata* on exposure to boron toxicity. Moreover, the follow-up treatment of EBL and/or Spd to the plants grown under stress conditions further enhanced the activity of antioxidant enzymes (CAT, POX and SOD) to reduce the level of ROS generated by salt and zinc stress. EBL regulate the activity of antioxidant enzymes in the tissues where accumulation of free radicals is very high. The similar effects of EBL were obtained by Ramakrishna and Rao 2013. EBL mediated tolerance could be due to the expression of regulatory genes involved in defence, like *RBOH* (Respiratory burst oxidase homologue), *MAPK1* (Mitogen-activated protein kinase 1), and *MAPK3* (Mitogen-activated protein kinase 3) (Xia et al. 2009). Similarly the polyamines especially Spd are implicated in abiotic stress tolerance including both salt and zinc stress by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation (Tang and Newton 2005; Wen et al. 2010). Spd is involved in the stress tolerance by preventing the formation of ROS through induction of antioxidant and antioxidants enzymes, by modulating the CAT, POX and SOD activities, which might be involved in the simultaneous induction of the antioxidant defense and by avoiding lipid peroxidation reactions (Yang et al. 2011; Mostofa et al. 2014). It is also well established that the levels of PAs, such as Put and Spd, in a plant tissue increase under various abiotic stresses (Xia et al. 2009; Choudhary et al. 2012). The exogenous application of Spd may have acted as a supplement to enhance the overall level of Spd to mitigate the NaCl or Zn induced oxidative stresses. Spd is mostly expressed and synthesized in higher quantities under stress conditions and is restored to normal level upon alleviation of stress (Choudhary et al. 2012). In the present study, the application of EBL and/or Spd may be associated with the effort to combat or neutralize ROS by activating the antioxidant system.

Accumulation of proline is a widespread plant response to environmental stresses, including salt stress. Proline has a clear role as an osmoticum, and acts as a compatible solute (Sasakai et al. 2007) because of its zwitterionic, high hydrophilic characteristics and can be accumulated to high concentrations in the cell cytoplasm without interfering with cellular structure or metabolism. Proline acts as an osmoprotectants (Sawahel and Hassan 2002), membrane stabilizer (Mansour 1998) and ROS scavenger (Filippou et al. 2014). Therefore, the increase in proline content under stress conditions in the present study was obvious. We also have found that the treatment of plants with EBL and/or Spd both in absence or presence of stress enhanced the proline content. The increase in proline content on application of EBL may be due to the induction expression of the genes of proline biosynthetic pathway (Ozdemir et al. 2004) and it has also been previously demonstrated that the exogenous treatment with BR and Spd elevated proline content in plants (Vardhini and Rao 2003; Ding et al. 2010). The increased pool of proline on BR and/or Spd application resulted in increased capacity of tolerance to NaCl which could be manifested in terms of improved growth and photosynthesis.

Plants grown in the soil amended with NaCl or Zn exhibited a significant decline in all the growth parameters compared to the control plants. Growth reduction by salinity or zinc stress could partly be due to the shortage of energy because processes involved in salt transport and to repair the damage on membrane/ proteins are energy consuming. Our results are in agreement with that of Manaa et al. (2011) and Cherif et al. (2011) who showed marked reduction in growth parameters of tomato plants under salt and zinc stress, respectively. Ghoulam et al. (2002) and Yusuf et al. (2012) also showed marked reduction in growth parameters in plants subjected to salt and heavy metal stress, respectively.

Plants exposed to saline or excess zinc conditions inevitably absorb a large amount of Na<sup>+</sup> or Zn<sup>2+</sup>, which could result in a limited assimilation, transport and distribution of important mineral nutrients. This also induces nutrient imbalance within the plant organs, i.e increase in the content of Na<sup>+</sup> or Zn<sup>2+</sup> and decrease in the essential mineral content, like that of K<sup>+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup> (Yao et al. 2010; Zhao et al. 2012). NaCl or Zn excess cause reduction in nutrient uptake due to the competition of Na<sup>+</sup> and Zn<sup>2+</sup> with other nutrient ions like K<sup>+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup> (Yao et al. 2010; Zhao et al. 2012). Another reason for the reduction in uptake of mineral nutrients under saline conditions may be due to the Na<sup>+</sup> induced blockage or reduced activity of the transporters, resulting in ionic imbalance of K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> as compared to Na<sup>+</sup> (Chen et al. 2005). The decrease may result due to the inhibition of cell division and elongation because of salinity mediated modulation on expansin transcripts (Geilfus et al. 2010). The positive effect of Zn treatment on plants grown under salinity could be explained by the decreases in uptake of Na<sup>+</sup> and its translocations from the old leaves to young leaves as a result of increased membrane integrity by Zn treatment (Alpaslan et al. 1999). However, in the present study spray of EBL and/or Spd increased the growth of plants (Fig. 1 and 2A). BRs are known to improve the growth of root and/or shoot in various groups of plants (Fariduddin et al. 2014; Zhou et al. 2013). Similarly, the leaves of BR treated plant possessed more surface area which could mainly be an expression of activated cell division and cellular enlargement induced by the BR application (Clouse et al. 1998) which is further supported by Yusuf et al. (2011) who also reported the increase in

leaf area by hormone treatment. The treatment of stressed plants with Spd also increased the fresh and dry mass as well as root and shoot length and leaf area, compared to those grown without Spd. A similar increase by Spd treatment has been reported by Roychoudhury et al. (2011). It has also been mentioned that Spd enhanced growth of tomato plants under salinity and alkalinity mixed stresses (Hu et al. 2012), activate defence protein biosynthesis to ameliorate salt stress in cucumber (Li et al. 2013).

Reduction in leaf water potential in the leaves of plants grown under stress (NaCl or Zn) is mainly due to the increased osmotic potential in the surrounding medium and decreased water absorption (Paranychianakis and Chartzoulakis 2005). It could also be attributed due to increase in electrolyte leakage in the leaves subjected to stress conditions as observed in the present study. However, treatment of EBL and Spd to stressed and non-stressed plants improved the values for LWP (Fig. 2B). EBL probably generated such a response due to its involvement in ATPase pump activation (Khrupach et al. 2003) and also due to the decrease in electrolyte leakage across the membrane. It can be inferred from the present study that Spd generated such impact similar to that of BR in relation to water potential.

There are many reports of salt and zinc damage to photosynthetic machinery at multiple levels such as pigment content, stomatal functioning and gaseous exchange, structure and function of thylakoids, electron transport and enzymes (Tiwari et al. 2005; Geissler et al. 2009). Salinity induced decrease in chlorophyll content may either be through inhibition of new chlorophyll biosynthesis or accelerated degradation of existing chlorophyll molecules (Iyengar and Reddy 1996). These results are in conformity with Heidari (2012), Chakraborty et al. (2012) and Fariduddin et al. (2013). Microelements, like Cu, Mn and Fe, are involved in many essential biological processes. These elements participate in the synthesis of Chl and are components of photosynthetic electron transport (Hänsch and Mendel 2009). Deficits or excesses of these elements affect normal metabolic processes (Hänsch and Mendel 2009; Pilon et al. 2011). Zn is a competitor for Fe, Cu, Mg and Mn ions, therefore, its accumulation results in an ion deficit and further affects photosynthesis related parameters (De Phillipis and Ziegler 1993, Rashid et al. 1994). All these cumulative effects generated could have triggered a decrease in the SPAD value of chlorophyll, as in the present study. Similar decrease in chlorophyll content was also observed by Hayat et al. (2012). However, a foliar spray of EBL and/or Spd, improved the SPAD chlorophyll value significantly (Fig. 2D). EBL applied to the non-stressed plants also increased the chlorophyll content duly supported by other workers (Yusuf et al. 2012; Fariduddin et al. 2014). The most convincing evidence for the BRs induced impact on the chlorophyll content is transcription and/or translation (Bajguz 2007) by involving the expression of specific genes responsible for synthesis of enzymes determining chlorophyll synthesis. Moreover, spray of Spd also increased the SPAD chlorophyll. It has been reported that Spd increased the chlorophyll content in rice under salt stress (Roychoudhury et al. 2011).

It has been reported that NaCl causes the closure of stomata (Zheng et al. 2012) thereby decreasing the partial CO<sub>2</sub> pressure, as well as internal CO<sub>2</sub> concentration (Li et al. 2013) and consequently availability of CO<sub>2</sub> for CA activity (Hayat et al. 2011). The observed decrease in CA activity is in agreement with other reports (Ali et al. 2008; Fariduddin et al. 2013). The damage caused by salt stress could also be attributed to water stress or a kind of physiological drought generated by NaCl (Hopkins 1999) as evident from the decrease in WUE in present study. Decrease in all gas exchange parameters (P<sub>N</sub>, g<sub>s</sub>, C<sub>i</sub>, and WUE) due to salinity has also been reported in *B. juncea* (Hayat et al. 2011; Wani et al. 2013). Other reasons for decrease in P<sub>N</sub> under NaCl are enhanced senescence and changes in the activity of enzymes induced by cytoplasmic structural shift and negative feedback by reduced sink activity (Iyengar and Reddy 1996). BRs improved the photosynthesis by positively regulating synthesis and activation of a variety of photosynthetic enzymes including ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a key enzyme in photosynthetic fixation (Xia et al. 2009). This could be the reason for the improvement of photosynthetic activity in plants supplemented with BRs. Higher P<sub>N</sub> and related attributes by BRs under differential abiotic stresses have been reported by other workers also (Ali et al. 2008; Kocova et al. 2010; Fariduddin et al. 2013).

The physiological drought caused by salinity could have increased the turnover of D2 protein of PS II leading to the decreased quantum yield of PS II (Fv/Fm) as observed in the present study (Fig. 3B). Plants grown under the Zn stress also showed the similar response, which is due to the effect of Zn excess on the chlorophyll synthesizing system and on the donor side of PS II by photoinhibition (Castiglione et al. 20007; Michael and Krishnaswamy 2012). Similar results were obtained in *Triticumaestivum* (Shahbaz et al. 2008), when exposed to salinity stress and *Glycine soja*, *Glycine max* (Qu et al. 2009) and radish (Ramakrishna and Rao 2013) when exposed to Zn stress. However, foliar spray of EBL and/or Spd improved the values of Fv/Fm in stressed plants indicating that EBL and Spd helped in the protection of PS II against over-excitation under stress that could have caused damage perhaps from the loss of integrity in thylakoid membrane (Haldimann and Feller 2005) which could also be true in the case of salinity and/or zinc stress. Shahbaz et al. (2008) also showed similar results of BR protecting the quantum yield of PS II under salt stress in wheat. Moreover, Spd treatment also improved the values

of Fv/Fm in the plants exposed to abiotic stress, indicating Spd takes a positive role in protecting the PS II machinery. These results are in conformity with that of Anjum (2009).

Salt stress markedly reduced the CA activity in *Vigna radiata*; however application of EBL improved its activity, possibly by accelerating the assimilatory rate of CO<sub>2</sub> (Talaat and Shawky 2012) and through the expression of specific genes (Khrpach 1999), especially in the stressed plants. The stress generated by salinity resulting in the loss of CA activity might be explained as the result of decrease in partial pressure of CO<sub>2</sub> in stroma by inducing the stomatal closure (Bethke and Drew 1992), an inhibition and/or metabolic dysfunction of the enzyme protein (Hopkins 1995). However, the involvement of BRs in regulating transcription and/or translation (Bajguz 2000) and their impact on the membrane to facilitate the uptake of CO<sub>2</sub> (Yu et al. 2004) could have favoured synthesis and activation of the enzyme. Exogenous application of Spd was also effective in maintaining the photosynthetic efficiency under salt stress, by stabilizing the photosynthetic enzyme, pH and enhancing the carboxylase activity (Fig. 3D). The similar results were obtained by Shu et al. (2012).

Decrease in NR activity under salt and/or zinc stress may be an after effect of inhibition and/or metabolic dysfunction of this enzyme (Panda and Choudhury 2005; Solanki et al. 2011). Salt stress restricts the uptake of nitrate; the inducer and substrate for NR (Campbell 1999), by distorting the structure and fluidity of membrane and adversely affecting the inbuilt ATPase activity (Feng et al. 2002; Munns 2005). Moreover, the level of NR drastically decreased by salinity (Ali et al. 2007; Hayat et al. 2010) mainly because of a lower flux of NO<sub>3</sub><sup>-</sup> from soil to roots as NR is a typical substrate-induced plant enzyme (Hoff et al. 1992) and a reduction in gene expression and NR-protein synthesis (Wang et al. 2004). The decreased NR activity under Zn stress may be due to the displacement of an essential metal ion from the central and functional part of the enzyme protein and secondly due to its interference with sulphhydryl (-SH) groups which often determine the secondary and tertiary structure of proteins. Besides, a reduced energy supply due to the inhibition of CO<sub>2</sub> incorporation, photosynthetic electron transport and an indirect inhibition of uptake of substrate (NO<sub>3</sub><sup>-</sup>) of enzyme may be other important reason (Luna et al. 2000; Panda and Choudhury 2005). However, the enhanced activity of NR, in both stressed and non-stressed plants could be explained, as a result of the impact of BRs on translation and/or transcription (Khrpach et al. 2000) and at the level of membrane to facilitate the uptake of NO<sub>3</sub> (Hayat and Ahmad 2003). Moreover, increased activity of NR by Spd is attributed to the fact that Spd stabilizes the plasma membrane hence preventing damage by salt stress (Kubis 2006) which could facilitate the increased uptake of nutrients including nitrate thereby increasing NR activity (Kucera 2003).

## Conclusion

From the present study, it can be concluded that NaCl and/or zinc stress significantly decreases the growth, gas exchange and biochemical parameters in the *Vigna radiata*. Based on the results obtained in this study, both EBL and Spd individually and in combination play an important role in the protection of crop plant against salt and zinc stress. Out of the two plant growth regulators, EBL excelled in its effects than Spd, furthermore the combination of both was most effective. The toxic effects generated by excess NaCl and zinc were completely overcome by the combination of EBL and Spd. The protection may mainly be conferred to the activation of antioxidant system including antioxidant enzymes and antioxidants. The present study revealed that EBL and Spd could be used as a potential growth regulators to improve plant growth and biochemical parameters under salt and/or zinc stress. However, it should be noted that these results came from laboratory practices; therefore, it would be necessary to carry out further experiments under field conditions which would be quite important research for sustainable crop production under salt and zinc stress.

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