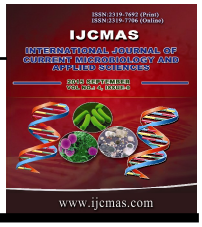
Int.J.Curr.Microbiol.App.Sci (2015) 4(9): 442-453

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 9 (2015) pp. 442-453**
http://www.ijcmas.com



Original Research Article

Germination, Growth and Oxidative Responses of Maize Seedlings to Salt and/or Cadmium Stress: Mechanism of Toxicity Amelioration by 28-Homobrassinolide

P. Anusha, Taheniyat Shahana and E. Sujatha*

Department of Botany, Osmania University, Hyderabad 500007, India

**Corresponding author*

Keywords

28-homo brassinolide, Antioxidative enzymes, Cadmium stress, Germination, Oxidative stress, Salt stress

A B S T R A C T

The effect of exogenous application of 28-homobrassinolide (HBL) on maize seed germination and seedling growth under salt and /or cadmium stress was studied. The seeds exposed to NaCl and/or Cd exhibited a significant decline in germination, seedling length and biomass growth. However, the follow up treatment with HBL detoxified the stress generated by NaCl and/or Cd and significantly improved the above parameters. The NaCl and/or Cd increased electrolyte leakage, lipid peroxidation and hydrogen peroxide content. However, HBL application significantly decreased the hydrogen peroxide levels and membrane damage in maize seedlings under salt and/or Cd stress. The antioxidative enzymes and the level of proline exhibited a significant increase in response to HBL as well as to NaCl and/or Cd stress.

Introduction

Salinity is one of the most important abiotic stresses reducing crop production. In the world, more than 20% of the irrigated arable land in arid and semi-arid regions has been already salt-affected (Huang *et al.,* 2007). In developing countries cadmium (Cd) contamination in arable soils and surface water has become severe due to improper management of waste and application of chemicals containing Cd, in addition to salinity (Helal *et al.,* 1999). In arid and semi-arid regions, bio-solids may be used on saline soils in order to improve soil quality which results in heavy metals contamination such as Cd, (Weggler-Beaton, *et al.*, 2000). Moreover, soil salinity has been shown to increase Cd concentration in crops grown on soils fertilized with phosphorous (P) fertilizers containing Cd (McLaughlin *et al.*, 1994). In the past two decades, phosphorous fertilizers were extensively applied to these saline soils to alleviate salt stress to crops (Deng *et al.,* 2002). As a result, Cd content in these soils has been dramatically increased. It has been well documented that salt and Cd stress in combination caused higher plasma membrane permeability and enhanced the production of reactive oxygen species in wheat (Muhling and Lauchli, 2003). Excess reactive oxygen species accumulation in cells may expose the plant to severe oxidative stress, causing growth inhibition even death. On the other hand, in plant cells, an antioxidant defense

mechanism has been developed for protection against reactive oxygen species (Ramakrishna and Rao, 2013). One of the protective mechanisms is the enzymatic antioxidant system, which involves the sequential and simultaneous action of a number of enzymes including superoxide dismutase (SOD) and peroxidase (POD). In fact, activities of antioxidant enzymes are inducible by oxidative stress due to exposure to abiotic or biotic stresses (Shafi *et al.,* 2010). Peroxidase and SOD activity increased distinctly in barley plants subjected to salinity stresses and/or cadmium (Huang *et al.,* 2006). Thus, interaction of Cd and salinity should be taken into consideration where both stresses are expected to impact crop growth and yield.

Brassinosteroids (BRs) are polyhydroxylated steroid hormones synthesized in all tissues that are involved in regulation of broad spectrum of processes i.e. cell expansion, pollen tube growth, vascular differentiation, senescence, photomorphogenesis and fruit development (Rao *et al.,* 2002; Vardhini *et al.,* 2010). BRs bind to the extracellular domain of a receptor kinase, brassinosteroid insensitive1 (BRI1), leading to sequential phosphorylation and dephosphorylation of signaling components and finally regulate gene expression (Clouse, 2011; Yang *et al.,* 2011). Recently, BRs have gained more attention as lots of studies indicated that BRs modulate plant metabolic response to environmental biotic and abiotic stresses, and these physiological processes include salt and drought stress tolerance, heavy metal stress resistance, pathogen resistance (Vardhini *et al.,* 2010; Ramakrishna and Rao 2014) as well as herbicide and pesticide tolerance (Bajguz and Hayat 2009; Xia etal., 2009). The question how BRs may impact such a diverse physiological processes remained unanswered. Owing to obvious evidence of adverse effects of salt and Cd stress on plant growth, it was hypothesized that the novel plant bio-regulator 28-homobrassinolide (HBL) used in this study can overcome damaging effects of salt and Cd stress on maize plants. Therefore, the study aims to examine whether or not could alleviate the injurious effects of salt and Cd stress on germination and growth by improving the antioxidant enzyme activities involved in stress tolerance.

Materials and Methods

Plant material and chemicals

The seeds of maize (*Zea mays* L.) were procured from National Seed Corporation, Hyderabad, India. 28-homobrassinolide (HBL) employed in the present study was procured from Sigma chemicals.

Standardization and selection of NaCl and Cadmium concentrations

To induce salt stress, NaCl salt was used. The experimental concentration was selected based on the IC_{50} value using different concentrations of NaCl *i.e.* 50, 100, 150, and 200 mM and 150 mM was selected as workable concentration. On the basis of screening experiments with varying concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mM) of cadmium [$CdCl_2.2H_2O$], 1 mM Cd^{2+} was selected based on IC_{50} values where the germination and seedling growth was found inhibited substantially but not completely and Cd dose is under the safe limit (WHO, 2007).

Growth conditions and treatments

Seeds of maize were surface sterilized with 0.5% (v/v) sodium hypochlorite from commercially available (4% NaOCl) and washed thoroughly with several changes of sterile distilled water. Preliminary experiments were conducted to choose

hormone concentration using a wide range of concentrations of HBL (0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 µM). Based on the growth response test two concentrations of HBL i.e., 1.0 and 2.0 µM were selected where significant growth promotion was observed (data not shown). The treatments were divided into eight groups: (i) Distilled water (Control) (ii) 1.0 µM and 2.0 µM 28-homobrassinolide solutions (iii) NaCl (150 mM) (iv) Cd Cl_2 (1mM Cd^{2+}) (v) NaCl+ $CdCl_2$ (vi) NaCl supplemented with 1.0 and 2.0 µM 28-homobrassinolide solutions (vii) $CdCl_2$ supplemented with 1.0 and 2.0 µM 28-homobrassinolide solutions (viii) NaCl+$CdCl_2$ supplemented with 1.0 and 2.0 µM 28-homobrassinolide solutions. Twenty seeds from each treatment were placed in each of 9 cm sterile petri dishes layered with Whatman No.1 filter paper. The petri dishes were supplied with 5 ml of respective test solutions. The seeds were allowed to germinate in dark at 20 $\pm 1^0$C. 3 ml more of test solutions were added on the 4th day of the experiment.

Germination percentage and morphological parameters

Number of seeds germinated was recorded at the end of 12, 24 and 48 hours under safe green light. Emergence of radicle was taken as the criteria for germination. The number of seeds germinated was counted in each treatment and percentage of seed germination was calculated by fallowing: No. of seeds germinated / total no. of seeds X100. The growth of 7 day-old maize seedlings was assessed by recording the length, fresh weight (FW) and dry weight (DW). Five seedlings one each from each Petri dish were selected randomly.

Lipid peroxidation: One gram of seedling material was macerated in 5 ml of 0.1% (w/v) TCA (Trichloroacetic acid). The homogenate was centrifuged at 10,000 X g for 5 minutes. For 1 ml of the aliquot of the supernatant, 4 ml of 20 % TCA containing 0.5% TBA was added. The mixture was heated at 95 ^{0}C for 30 minutes and cooled quickly in ice bath. The absorbance was measured at 532 nm and 600 nm. The concentration of malondialdehyde (MDA) was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Hydrogen peroxide: Isolation was made from 0.5 g of the treated seedling material in ice-cold acetone. By addition of 50% (w/ v) titanyl sulphate and conc. NH_4OH solution, the peroxide-titanium complex was precipitated. The precipitate was dissolved in 15 ml of 2M H_2SO_4, making the final volume to 20 ml in cold water. The absorbance of the resultant solution was read at 415 nm. The H_2O_2 content was calculated from a standard curve prepared in similar way (Mukherjee and Choudari, 1983).

Electrolyte leakage: was measured using electrical conductivity meter (Systronics-304, India) as described by Lutts *et al.* (1996). Seedlings were washed with deionized water to remove surface adhered electrolytes. After drying with filter paper, 1 g fresh weight of seedlings were immersed in 20 mL deionized water and incubated at 25 °C on rotary shaker for 24 hours. After 24 h, electrical conductivity (EC1) of the bathing solution was recorded. These seedling samples were then autoclaved at 120 °C for 20 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25 °C and the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was expressed following the formula:
EL (%) = (EC1/EC2) ×100.

Assay of the antioxidant enzymes

Fresh seedling material (1 g) was

homogenized in 50 mM Tris-HCl (pH 7.5) with addition of 40 mM phenyl methyl sulfonyl fluoride (PMSF) and 0.2 mM EDTA, 2% (w/v) polyvinyl pyro pyrolidone (PVPP). The extract was centrifuged at 15,000 X g for 20 min and the resultant supernatant was used for measuring the following enzyme assays. The amount of protein in the enzyme extract was calculated according to Lowry and others (1951).

Catalase: (CAT, E.C.1.11.1.6.) activity was determined following Aebi (1974). The rate of H_2O_2 decomposition at 240 nm was measured spectrophotometrically and calculated using a molar extinction coefficient of 45.2mM^{-1} cm^{-1}. The reaction mixture consisted of 50 mM phosphate buffer, 0.1mM H_2O_2 and enzyme extract. One unit of catalase activity was assumed as the amount of enzyme that decomposed 1 µmol of H_2O_2 per mg of soluble protein per minute at 30 ^{0}C.

Peroxidase: (POD, E.C.1.11.1.7) activity was assayed by employing the procedure of Kar and Mishra (1976). To 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7), 1 ml of 0.01 M pyrogallol and 1 ml of 0.005 M H_2O_2 were added. A blank was prepared with 0.5 ml of enzyme extract, 3.5 ml of 0.1 M phosphate buffer and 1 ml of 0.005 M H_2O_2. After 5 minutes of incubation at 25 ^{0}C, the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm against a blank. The enzyme activity was expressed as Units mg^{-1} protein.

Superoxide dismutase: (SOD, E.C 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT (Nitroblue tetrazolium) of Beauchamp and Fridovich (1971). A 3 ml of reaction mixture contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 0.1 ml of enzyme extract and 2 µM riboflavin. Riboflavin was added at the end. The reaction mixture was exposed to 15 watt fluorescent tubes and the decrease in the absorbance of the reaction mixture was read at 560 nm. Fifty percent inhibition was considered as one enzyme unit.

Ascorbate peroxidase (APX; E.C 1.11.1.11) was assayed by the method of Nakano and Asada (1981). The reaction mixture contained 1.5 ml of 50 mM sodium phosphate buffer (pH 7), 0.2 mM EDTA, 0.5 ml of 0.5 mM ascorbic acid, 0.5 ml 0.5 mM H_2O_2 and 0.5 ml of enzyme sample. The activity was recorded as the decrease in absorbance at 290 nm for 1 minute and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.6 mM^{-1}cm^{-1}.

Glutathione reductase (GR; EC 1.6.4.2) activity was performed according to Jiang and Zhang (2002). The reaction mixture contained 500 µl of sodium phosphate buffer (pH 7.0), 100 µl each of 10 mM GSSG, 1 mM NADPH and 180 µl of distilled water. The reaction was started by addition of enzyme extract and NADPH oxidation was recorded as the decrease in absorbance at 340 nm for 1 min. The activity was calculated using the extinction coefficient of NADPH 6.22 mM^{-1}cm^{-1}.

Free Proline

Seedling material (0.5 g) was homogenized with 10 ml of 3 % (w/v) sulfosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. The supernatant was taken for proline estimation. The reaction mixture was composed of 2 ml of plant extract, 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid. The test tubes containing above mixture were heated in a boiling water bath for one hour. The

reaction was terminated in an ice bath followed by addition of 4 ml of toluene. The contents were shaken vigorously and then allowed to separate into phases. The chromophase containing upper toluene phase was carefully taken out with the help of a pipette and the absorbance was taken at 520 nm. The amount of proline present was quantified with the help of proline standard graph (Bates *et al.*, 1973).

Ascorbic acid (AsA): and glutathione (GSH) levels

Fresh seedlings (0.2 g) were homogenized in 5 ml of 5% (v/v) *m*-phosphoric acid. The homogenate was centrifuged at 12,000 X g for 15 min. For determination of total ascorbate, 0.1 ml supernatant and 0.5 ml of 100 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA and 0.2 ml 10 mM DTT (dithiothreitol) were mixed and incubated at room temperature for 15 min. Then 0.2 ml 0.5% (w/v) N-ethylmaleimide was added to remove excess DTT, and then 0.8 ml 10% (w/ v) TCA, 0.8 ml 44% (v/v) *o*-phosphoric acid, 0.8 ml a,a´-dipyridyl in 70% (v/v) ethanol and 0.4 ml 30 g l^{-1} $FeCl_3$ were added and well mixed in sequence. AsA was assayed in a similar manner except that 0.2 ml of ddH_2O was substituted for DTT. The absorbance of the mixture at 525 nm was recorded after incubation at 40 ºC for 1 h (Hodges *et al.*, 1996).

One gram of seedlings were homogenized with 10 ml Tris EDTA (pH 8.2) and centrifuged at 25,000 X g for 30 minutes at 4^0 C. From the homogenate 300 µl was pipetted into 1 ml of tube to which 60 µl of 25% phosphoric acid is added and kept in ice for 5 minutes, centrifuged at 25,000 X g for 30 minutes at 4^0 C. Supernatant was collected for the estimation of GSH. For measurement of GSH**,** 450 µl of cold phosphate EDTA buffer (pH 8) was added to 50 µl of supernatant and mixed thoroughly Aliquots (25 and 50 µl) was taken into 5 ml test tube and made up to 100 µl with cold glass distilled water. Phosphate EDTA buffer (1.8 ml) was added to the tube and mixed and 100 µl of OPT (*O*-phthalaldehyde) solution was then added and after thorough mixing, incubated at room temperature (25^0 C) for 15 minutes (Hissin and Hilf 1976).

Statistical analysis

The results presented are the mean values of 5 replicates. The data analyses were carried out using one-way analysis of variance (ANOVA) followed by Post Hoc Test (Multiple Comparisons) using SPSS (SPSS Inc., Chicago, IL, USA). The differences were considered significant if p was ≤ 0.05. The mean values were compared and lower case letters are used in figures/table to highlight the significant differences between the treatments.

Results and Discussion

The stress generated by NaCl and/or Cd significantly reduced the germination percentage of maize seeds in comparison to their respective controls (Table 1). However, the inhibitory effect of NaCl and/or Cd stress on seed germination was significantly ameliorated by addition of HBL. The NaCl and/or Cd stressed seedlings treated with HBL, the percentage of seed germination approached that of unstressed control treatments at 2 µM concentration. The treatment of HBL alone exhibited enhanced germination percentage as compared to the untreated control, with maximum increase in seed germination percentage was noted for seedlings applied with 2µM concentrations.

The presence of NaCl and/or Cd significantly reduced the growth (seedling length, fresh weight and dry weight in maize seedlings (Table 2). The combined effect of

these two stress factors was more toxic compared to their individual ones. Compared to control, the above mentioned parameters were decreased by 47.2, 33.2 and 53.3% respectively. However, the application HBL, in the absence of NaCl and/or Cd stress increased the growth that was significantly higher than the control seedlings. HBL treatment also enhanced the growth of maize seedlings grown under NaCl and/or Cd stress and the values were considerably higher than those of the seedlings grown under stress alone.

In the present study, NaCl and/or Cd stress resulted in oxidative stress as reflected by increased levels of H_2O_2 (33.8%), MDA content (90.8%) and electrolyte leakage (48.7%) in maize seedlings (Table 3). Supplementation of HBL alleviated the oxidative stress by significantly depressing the H_2O_2, MDA and ELP levels by 22.3, 40 and 25.2% respectively. There was no significant change in the levels of H_2O_2, MDA, ELP in seedlings receiving HBL alone treatments compared to the unstressed control seedlings.

The antioxidative enzymes, CAT, SOD, POD, APX and GR exhibited an increasing trend in response to HBL and NaCl and/or Cd treatment (Table 4 and 5). The HBL treatment caused a significant increase in the activities of all the enzymes. The seedlings exposed to NaCl alone stress also possessed an increased level of these enzyme activities but Cd reduced the GR activity (Table 5). However, in association with NaCl or HBL treatment, it also improved the activities of all the above mentioned enzyme activities. Maximum activities of these enzymes recorded in maize seedlings exposed to combined NaCl and/or Cd stress and subsequently received HBL treatment at 1 µM concentration.

The level of proline exhibited an increase in response to NaCl and/or Cd stress, in seedlings compared to control (Table 5). Application of HBL to NaCl and/or Cd stressed seedlings, elevated the quantity of proline in maize with maximum quantity being at 2 µM concentrations. HBL alone treatments also resulted in considerable increase in the quantity of free proline in maize seedlings.

In the presence of NaCl and/or Cd stress the content of ASA was reduced considerably, where as GSH content was increased significantly in maize seedlings (Table 6). However, the exogenous application of HBL to maize seedling under NaCl and/or Cd stress, the contents of both ASA and GSH were improved significantly. HBL alone treatments also accounted for significant enhancement of ASA and GSH contents in maize seedlings.

The present investigation shows that, the germination of maize seeds was dramatically suppressed by NaCl and/or Cd stress (Table 1). Further, the results showed that NaCl and/or Cd stress exerted strong inhibiting effect on growth and development as reflected by significant reduction of seedling length, fresh weight and dry weight as compared to the control (Table 2). However, HBL application reduced the toxic effect of NaCl and/or Cd stress on seed germination and growth.

In NaCl and/or Cd stressed treatments supplemented with HBL, the percentage of seed germination approached to that of unstressed control treatments. Application of HBL significantly alleviated suppression of seedling growth caused by NaCl and/or Cd stress resulted in improved seedling length and biomass indicating the stress alleviation capability of BRs (Table 2). Furthermore, the stress amelioration capability of HBL was found to be dose dependent. Exogenous application of 24-epibrassinolide up

regulated a particular cyclin gene, *CycD3*, which was involved in the cell cycle of *Arabidopsis*, leading to enhanced seed germination by stimulating cell expansion and cell proliferation (Nakaya *et al.*, 2002). Recently, Wang *et al.* (2011) reported that the inhibitory effect of salinity on seed germination of cucumber (*Cucumis sativus*) seeds was significantly alleviated by addition of 24-EBL. Anuradha and Rao (2007) also observed that exogenous application of 24-EBL and 28-HBL improved the seed germination in radish under Cd toxicity dose dependently. The growth promoting effects of BRs on seedlings under stress conditions might be attributed to their involvement in cell elongation and cell cycle progression as well as regulation of genes encoding xyloglucan endotransglucosylase/hydrolases (XTHs), expansions, glucanases, sucrose synthase and cellulose synthase or by activating the H^+-ATPase activity (Clouse, 2011).

Table.1 Effect of 28-Homobrassinolide on maize seed germination under NaCl and /or Cd stress and stress free conditions

Treatments	12 hours	24 hours	48 hours
Control	45.5 ±1.347c	81.2 ±2.436c	96.17 ±2.360 c
1µM HBL	48.9 ±2.878b	83.8 ±3.391b	98.94 ±2.954b
2 µM HBL	52.8 ±1.227a	88.5 ±2.544a	99.80 ±4.548 a
NaCl	37.6 ±2.012f	49.3 ±2.494i	63.00 ±2.260h
Cd	31.3 ±3.102i	44.7 ±2.914j	56.79 ±1.470 i
NaCl+ Cd	27.4 ±2.638j	38.3 ±1.987k	47.82 ±1.850 j
NaCl +1µM HBL	39.5 ±1.581f	66.1 ±2.230g	79.87 ±2.549f
NaCl+2µM HBL	42.5 ±1.789d	79.8 ±2.201d	91.47 ±2.551d
Cd+1µM HBL	38.8 ±2.521gh	61.2 ±1.507h	77.84 ±3.102 fg
Cd+2µM HBL	41.3 ±3.672e	72.2 ±1.922e	89.2 ±1.983e
NaCl+Cd+1µM HBL	33.3 ±2.172g	67.2 ±2.187g	79.2 ±2.947f
NaCl+ Cd+2µM HBL	40.7 ±1.272e	70.2 ±1.687f	87.2 ±1.217e

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test.

Table.2 Effect of 28-Homobrassinolide on seedling length, fresh weight and dry weight of maize seedlings under NaCl and /or Cd stress and stress free conditions

Treatments	Seedling length (cm)	Fresh weight (mg)	Dry weight (mg)
Control	8.9 ±1.620c	422.8 ±12.310c	37.4 ±4.854c
1µM HBL	10.7 ±0.844b	438.6 ±16.785b	41.2 ±1.974b
2 µM HBL	11.6 ±1.061a	467.0 ±11.652a	43.6 ±5.067a
NaCl	7.3 ±0.854g	353.4 ±18.626h	28.8 ±3.854g
Cd	5.8 ±0.530i	321.4 ±12.932i	25.8 ±5.974h
NaCl+ Cd	4.7 ±1.291j	282.2 ±15.916j	21.2 ±3.707i
NaCl +1µM HBL	7.6 ±2.367fg	397.6 ±21.307f	30.2 ±1.435f
NaCl+2µM HBL	8.4 ±1.663d	415.4 ±12.785d	36.6 ±4.969d
Cd+1µM HBL	6.4 ±0.791h	376.2 ±19.652g	31.2 ±1.207g
Cd+2µM HBL	8.2 ±1.367e	398.2 ±17.307f	34.2 ±7.117e
NaCl+Cd+1µM HBL	6.4 ±0.891h	347.2 ±17.307h	30.7 ±1.307f
NaCl+ Cd+2µM HBL	7.9 ±0.291f	405.2 ±20.337e	33.6 ±3.410e

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test.

Table.3 Effect of 28-Homobrassinolide on MDA, H_2O_2 and Electrolyte leakage levels in maize seedlings under NaCl and /or Cd stress and stress free conditions

Treatments	MDA (µmol g^{-1}FW)	H_2O_2 (µmol g^{-1}FW)	Electrolyte leakage
Control	7.6 ±0.57 g	18.5 ±1.87 f	16.32±1.29h
1µM HBL	6.4 ±0.75 i	18.2 ±2.77fg	15.14±0.92i
2 µM HBL	6.1 ±0.48 i	17.8 ±2.58h	14.88±1.75j
NaCl	11.8 ±0.45c	21.4 ±2.34 c	23.94±1.24b
Cd	12.2 ±0.76b	22.8 ±2.08 g	21.56±1.24c
NaCl+ Cd	14.5 ±0.88a	24.7 ±1.45 a	24.25±0.59a
NaCl +1µM HBL	8.5 ±0.65 f	19.8 ±2.69 d	18.16±1.34f
NaCl+2µM HBL	7.9 ±0.27g	18.8 ±2.45 f	16.81±1.81h
Cd+1µM HBL	10.6 ±0.67 d	21.8 ±4.20 c	19.02±0.85e
Cd+2µM HBL	9.3 ±0.66 e	19.6 ±3.89 d	17.01±0.97g
NaCl+ Cd+1µM HBL	10.2 ±0.73d	21.3 ±2.19 c	20.71±1.39d
NaCl+ Cd+2µM HBL	8.7 ±0.55 f	19.2 ±3.17de	18.12±1.24f

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test

Table.4 Effect of 28-Homobrassinolide on the activities of CAT, POD and SOD in maize seedlings under NaCl and /or Cd stress and stress free conditions

Treatments	CAT (U mg^{-1}protein min^{-1})	POD (U mg^{-1}protein min^{-1})	SOD (U mg^{-1}protein min^{-1})
Control	21.8 ±0.45f	0.473 ±0.052j	6.8 ±1.25 k
1µM HBL	24.1 ±0.23e	0.489 ±0.047i	7.4 ±2.04 h
2 µM HBL	24.9 ±0.84cd	0.501 ±0.023gh	8.2 ±1.80 i
NaCl	23.7 ±0.61e	0.548 ±0.084g	9.8 ±1.55 g
Cd	24.5 ±0.85d	0.567 ±0.081f	10.4 ±2.47 j
NaCl+ Cd	25.2 ±1.01c	0.572 ±0.065e	12.2 ±2.13 i
NaCl +1µM HBL	25.7 ±0.75c	0.582 ±0.038d	13.2 ±1.04 f
NaCl+2µM HBL	21.7 ±0.78f	0.599 ±0.074c	14.3 ±2.56 e
Cd+1µM HBL	24.8 ±0.78d	0.615 ±0.057b	13.9 ±1.02 d
Cd+2µM HBL	26.4±1.23b	0.629 ±0.054a	16.8 ±2.56 c
NaCl+Cd+1µM HBL	27.4 ±0.68a	0.591 ±0.081c	17.9 ±2.87 b
NaCl+ Cd+2µM HBL	26.9 ±0.58b	0.624 ±0.093a	18.4 ±3.08 d

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test.

Table.5 Effect of 28-Homobrassinolide on the activities of APX and, GR and free proline levels in maize seedlings under NaCl and /or Cd stress and stress free conditions

Treatments	APX (µmol AsA mg^{-1}protein min^{-1})	GR (nmol NADPH $min^{-1}mg^{1}$protein)	Proline (mg g^{-1} FW)
Control	0.578 ±0.042h	21.9 ±0.82g	4.34 ±0.27j
1µM HBL	0.583 ±0.052 g	22.6 ±0.92f	5.47 ±0.48i
2 µM HBL	0.591 ±0.030 g	23.7 ±0.81e	6.13±0.57 g
NaCl	0.624 ±0.072 f	25.5 ±0.72c	6.31 ±0.38f
Cd	0.681 ±0.074d	19.1 ±0.54h	5.76 ±0.44 h
NaCl+ Cd	0.694 ±0.024 c	24.9 ±0.42d	6.79 ±0.58e
NaCl +1µM HBL	0.637 ±0.030 e	27.3 ±0.28b	7.54 ±0.37b
NaCl+2µM HBL	0.693 ±0.021c	26.6 ±0.32b	7.96 ±0.23a
Cd+1µM HBL	0.702 ±0.021b	23.1 ±0.84 f	6.30±0.22f
Cd+2µM HBL	0.718 ±0.023 a	23.9 ±0.84 e	6.87 ±0.21e
NaCl+Cd+1µM HBL	0.706 ±0.087b	25.4 ±0.67 c	7.39 ±0.37c
NaCl+ Cd+2µM HBL	0.726 ±0.027 a	28.1 ±0.93a	7.11 ±0.26cd

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test.

Table.6 Effect of 28-Homobrassinolide on ascorbate and glutathione content of maize seedlings under NaCl and /or Cd stress and stress free conditions

Treatments	Ascorbate (nmol g^{-1} FW)	Glutathione (nmol g^{-1} FW)
Control	634 ±11.75c	15.4 ±2.26 g
1µM HBL	678 ±12.61b	17.8 ±1.12f
2 µM HBL	704 ±10.45 a	18.6 ±2.21 f
NaCl	581 ±10.98 f	19.7 ±1.08 e
Cd	514 ±10.64h	21.8 ±1.24 d
NaCl+ Cd	486 ±09.57i	24.1 ±3.43b
NaCl +1µM HBL	614 ±11.37e	22.7 ±1.21d
NaCl+2µM HBL	628±10.37d	23.1 ±3.37 c
Cd+1µM HBL	589 ±11.28 f	24.3 ±4.42 b
Cd+2µM HBL	621±12.80d	26.9 ±2.13 a
NaCl+Cd+1µM HBL	523 ±10.96h	24.8 ±4.78 b
NaCl+ Cd+2µM HBL	576 ±9.96g	27.6 ±3.58a

The values are means ±SE (n = 5); mean followed by the same alphabet in a column is not significantly different at p=0.05 according to Post Hoc test

A common consequence of most abiotic stresses, including salinity (Hayat *et al.*, 2010) and heavy metal stress (Yadav *et al.*, 2010) is an increased production of reactive oxygen species (ROS). These ROS namely superoxide radical ($O_2^{\cdot-}$), hyroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are extremely toxic to plants that cause oxidative damage to proteins, lipids and nucleic acids, leading to irreparable metabolic dysfunction and cell death (Sharma and Dietz, 2009). To combat oxidative damage of ROS, plants have antioxidant defense system comprising of enzymes of SOD, CAT, APX and GR, and the non-enzymic constituents such as reduced glutathione (GSH) and ascorbate (ASA) that remove, neutralize and scavenge ROS (Gill and Tuteja 2010). SOD dismutates $O_2^{\cdot-}$ to H_2O_2 and this is decomposed to water by CAT and POD. GR maintains the pool of glutathione in reduced state, thereby enhances tolerance to oxidative stress (Alscher *et al.*, 2002). In the present study, NaCl and/or Cd stress resulted in oxidative stress as evidenced by increased H_2O_2 generation, MDA content and electrolyte leakage in maize seedlings (Table 3). However, exogenous application of both HBL dramatically depressed H_2O_2 and, MDA accumulation there by electrolyte leakage in NaCl and/or Cd stressed maize seedlings, suggesting that BRs helps in the maintenance of membrane integrity against oxidative damage under NaCl and/or Cd stress. The acceleration of the activities of SOD, CAT, POD and APX were observed in NaCl and/or Cd stressed seedlings. But, the activity of GR exhibited differential response to stress i.e. decreased under Cd stress and increased under NaCl and Cd stress. The supplementation of HBL to NaCl and/or Cd stressed seedlings increased the all enzyme activities (CAT, POD, APX, SOD and GR) in both stressed and unstressed conditions (Table 4 and 5). Interestingly, exogenous application of HBL increased the ascorbate and glutathione content as well as frees proline levels in maize seedlings under NaCl and/or Cd stress and stress free conditions (Table 6).

The elevation of both enzymatic and non-enzymatic levels upon HBL treatment conferred stress tolerance which manifested in terms of improved growth and biomass production. In consistent with these observations Ali *et al.*, (2008) reported that exogenous application of 24-epibrassinolide enhanced the antioxidatve enzymes and proline levels under salinity and/or nickel stress in *Brassica juncea*. Similarly, enhanced the proline, antioxidant levels and antioxidative enzyme activities in *Phseolus vulgaris* under salt and cadmium (Rady, 2011), *Vigna radiata* under salt and temperature (Hayat *et al.*, 2010) and radish under zinc stresses (Ramakrishna and Rao, 2012) upon exogenous application of brassinosteroids were observed. The enhanced antioxidative defense system seems to be the result of *de novo* synthesis and/or activation of the enzymes, mediated through transcription and/ or translation of specific genes that has added more strength to drought stressed seedlings against oxidative stress.

In conclusion, 28-Homobrassinolide enhanced the level of antioxidant system (SOD, CAT, POD, APX and GR, and levels of proline, ascorbate and glutathione) both under stress and stress free conditions. The influence of HBL on antioxidant system was more pronounced under NaCl and/or Cd stress condition, suggesting that the elevated level of antioxidant system, at least in part, increased the tolerance of maize seedlings to NaCl and/or Cd stress, thus protected from oxidative injury. This further manifested in increased germination, growth and dry mass production of maize seedlings.

References

Ali, B., S. Hayat, Q. Fariduddin, A. Ahmad. 2008. 24-Epibrassinolide protects against the stress generated by salinity and nickel in Brassica juncea. *Chemosphere,* 72: 1387–1392

Alscher, R.G., N. Erturk, L.S. Heath. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.,* 53: 1331–1341

Anuradha, S., S.S.R. Rao. 2007. The effect of brassinosteroids on radish (*Raphanus sativus* L.) seedling growth under cadmium stress. *Plant Soil Environ.,* 53: 465–472

Bajguz, A., S. Hayat. 2009. Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiol. Biochem.,* 47: 1–8

Clouse, S.D. 2011. Brassinosteroids. The Arabidopsis Book 9, e0151, http://dx.doi.org/10.1199/tab.0151.

Deng, L.Q., Z.P. Liu, A.W. Cheng, Q.R. Sheng, M.D. Chen. 2002. Influence of N and P fertilizers on sunflower grown in different saline soil on coast. *Chin. J. Oil Crop Sci.,* 24: 61–64

Gill, S.S., N.Tutej. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.,* 48: 909–930.

Hayat, S., S.A. Hasan, M. Yusuf, Q. Hayat, A. Ahamad. 2010. Effect of 28-homobrassinolode on photosynthesis, flurorescence and antioxidant system in the presence of salinity and temperature in *Vigna radiata*. *Environ. Exp. Bot.,* 69: 105–112.

Helal, H.M., A. Upenov, G.J. Issa. 1999. Growth and uptake of Cd and Zn by *Leucaena leucocephala* in reclaimed soils as affected by NaCl salinity. *J. Plant Nutri. Soil Sci.,* 162: 589–592.

Huang, Y., G. Zhang, F. Wu, J. Chen, Y. Xiao. 2006. Interaction of salinity and cadmium stresses on antioxidant enzymes, sodium, and cadmium accumulation in four barley genotypes. *J. Plant Nutri.,* 29: 2215–2225.

Huang, Y.Z., K. Wei, J. Yang, F. Dai, G.P. Zhang, 2007. Interaction of salinity and cadmium stresses on mineral nutrients, sodium, and cadmium accumulation in four barley genotypes. *J. Zhejiang Univ. Sci. B,* 8: 476–485

McLaughlin, M.J., K.G. Tiller, T.A. Beech, M.K. Smart. 1994. Soil salinity causes elevated cadmium concentrations in field-grown potato tubers. *J. Environ. Qual.,* 23: 1013–1018.

Muhling, K.A., A. Lauchli. 2003. Interaction of NaCl and Cd stress on compartmentation pattern of cations, antioxidant enzymes, and proteins in leaves of two wheat genotypes differing in salt tolerance. *Plant Soil,* 253: 219–231.

Nakaya, M., H. Tsukaya, H. Murakami, M. Kato. 2002. Brassinosteroids control the proliferation of leaf cells on *Arabidopsis thaliana*. *Plant Cell Physiol.,* 43: 239–244.

Rady, M.M. 2011. Effect of 24-epibrassinolide on growth, yield, antioxidative system and cadmium content of bean (*Phaseolus vulgaris* L.) plants under salinity and cadmium stress. *Sci. Horticult.,* 129: 232–237.

Ramakrishna, B., S.S.R. Rao. 2012. 24-Epibrassinolide alleviated zinc-induced oxidative stress in radish (*Raphanus sativus* L.) seedlings by enhancing antioxidative system. *Plant Growth Regul.,* 68: 249–259.

Ramakrishna, B., S.S.R. Rao. 2013a. Preliminary studies on the involvement of glutathione metabolism and redox status against zinc toxicity in radish seedlings by 28-Homobrassinolide.

Environ. Exp. Bot., 96: 52–58

Ramakrishna, B., S.S.R. Rao. 2013b. 24-epibrassinolie maintains eleated redox state of AsA and GSH in radish (*Rapahanus sativus* L.) seedlings under zinc stress. *Acta Physiol. Plant*, 35: 1291–1302

Shafi, M.D., Z. Guoping, J. Bakht, M.D. Aman khan, Ejaz-ul-Islam, M.D. Dawood khan, Raziuddin. 2010. Effect of cadmium and salinity stresses on root morphology of wheat. *Pak. J. Bot.*, 42: 2747–2754

Sharma, S., K.J. Dietz. 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci.*, 14: 43–50.

Vardhini, B.V., S. Anuradha, E. Sujatha, S.S.R. Rao. 2010. Role of brassinosteroids in alleviating various abiotic and biotic stresses - a review. *Plant Stress*, 4: 55–61.

Wang, B., Zhang, J., Xia, X., Zhang, W.H. 2011. Ameliorative effect of brassinosteroid and ethylene on germination of cucumber seeds in the presence of sodium chloride. *Plant Growth Regul.*, 65: 407–413.

Weggler-Beaton, K., M.J. McLaughlin, R.D. Graham. 2000. Salinity increases cadmium uptake by wheat and Swiss chard from soil amended with biosolids. *Aust. J. Soil Res.*, 38: 37–45.

Xia, X.J., Y. Zhang, J.X. Wu, J.T. Wang, Y.H. Zhou, K. Shi, Y.L. Yu, J.Q. Yu. 2009.Brassinosteroids promote metabolism of pesticides in cucumber. *J. Agric. Food Chem.*, 57: 8406–8413

Yadav, S.K. 2010. Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South Afr. J. Bot.*, 76: 167–179.

Yang, C.J., C. Zhan, Y.N. Lu, J.Q. Jin, X.L. Wang. 2011. The mechanism of brassinosteroids action: from signal transduction to plant development. *Mol. Plant*, 4: 588–600.