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Mitigation of Drought Stress by 24-Epibarassinolide and 28-Homobrassinolide in Pigeon Pea Seedlings

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Abstract

This article encompasses the results on the effects of 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) on germination, seedling growth and antioxidative defense of pigeon pea (Cajanuscajan(L.) Millsp.)under Polyethylene glycol 6000 (PEG) induced drought stress. Drought stress significantly decreased seed germination, growth and dry mass of pigeon pea. However, both EBL and HBL treatments alleviated the negative effects of drought on germination, growth and increased the dry mass accumulation. Drought stress induced hydrogen peroxide production, lipid peroxidation and electrolyte leakage was significantly counteracted by both EBL and HBL treatments. Supplementation of EBL and HBL enhanced the activities of antioxidative enzymes viz., catalase, peroxidase, superoxide dismutase glutathione reductase and ascorbate peroxidase in both unstressed and drought stressed seedlings. Further, BRs increased the proline, Glycine betaine and ascorbic acid levels in stressed seedlings. The present study demonstrated the ameliorating ability of EBL and HBL on the drought stress induced inhibition of germination and seedling growth of C. cajanby improving the antioxidative defense system.

Keywords: *24-epibrassinolide, 28-homobrassinolide, antioxidative enzymes, drought stress, osmolytes.*

1. Introduction

A lack of water (water deficit) in soil is one of the gravest problems in cultivation of crops on a global scale because there is precipitation lower than 500 mm on 61% of the area on the Earth (Deng *et al*., 2005). Drought is considered the single most devastating environmental stress, which severely affects plant growth and development with substantial reductions in crop growth rate and biomass accumulation more than any other environmental stress (Yordanov*et al*., 2003; Osakabe*et al*., 2014). The main consequences of drought in crop plants are reduced rate of cell division and expansion, leaf size, stem elongation and root proliferation, and disturbed stomatal oscillations, plant water and nutrient relations with diminished crop productivity, and water use efficiency (Chaves and Oliveira, 2004).

During a water deficit, overall plant development is delayed, and leaf size is reduced; anatomical changes due to modifications in cell size, senescence and, ultimately, plant death are also observed in several species (Morales *et al*., 2013) The low water availability in the soil decreases gas exchange in plants by reducing transpiration and the photosynthetic rate and carbohydrate accumulation, limiting overall plant growth (Jaleel*et al*., 2008; Chaves and Oliveira, 2004). Under severe drought stress conditions oxidative stress occurs by overproduction of reactive oxygen species (ROS). Due to their chemical properties, ROS are highly reactive and can damage proteins, chlorophylls, membrane lipids and nucleic acids (Sofo*et al*., 2005).

To prevent or alleviate these damages, plants possess a complex antioxidant system to detoxify ROS, including low-molecular mass antioxidants as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and enzymes involved in the ascorbate–glutathione cycle (Li *et al*., 2003; Gill and Tuteja 2010; Vradhini*et al*., 2010). In this context, it is believed that a simultaneous increase in several components of the antioxidative defense system would be necessary in order to obtain an increase in the plant protective mechanisms

Brassinosteroids are sixth group of plant specific hormones that promotes various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, seed germination, leaf bending and epinasty, proton pump activation, regulation of gene expression, nucleic acid and protein synthesis and photosynthesis (Vardhini*et al*., 2010; Clouse, 2011). BR binds to the plasma membrane localized receptor brassinosteroid-Insensitive (BRI1) triggers the BRs signaling cascade. Therefore, mutant alleles that are defective in BRI1 display severely growth

inhibited dwarf phenotypes, whereas BRI1 over expressing plants shows longer and narrower growth in their leaves and petioles (Yang *et al*., 2011; Kim *et al*., 2013). In addition to their roles in plant growth and development BRs also help to overcome stresses provoked by low or high temperature, drought, salt, infection, pesticides and heavy metals (Bajguz and Hayat 2009). However, the mechanisms underlying the regulation of stress tolerance by exogenous treatment of 24-epibrassinolide have not been elicited. The objective of this study was to investigate effects of 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) treatment for improving germination, seedling tolerance against drought stress and the role of the antioxidant defense system in this process.

2. Materials and Methods

2.1. Chemicals and plant material

Seeds of *Cajanuscajan* (l.) Millsp.were procured from National Seed Corporation, Hyderabad, India. Two active brassinosteroids (BRs): 28-homobrassinolide (HBL) and 24-epibrassinolide (EBL) employed in the present study were procured from Sigma chemicals.

2.2. Growth conditions and treatments

Seeds of pigeon pea were surface sterilized with 0.5% (v/v) sodium hypochlorite from commercially available (4% NaClO) and washed thoroughly with several changes of sterile distilled water. Polyethylene glycol 6000 (PEG 6000) [molecular formula $H(OCH_2CH_2)_n OH$] was used to induce water stress. The concentration of PEG was calculated by molecular weight and accordingly different concentrations were prepared. A dose-response curve with red gram was established for a workable concentration of PEG (using different concentrations of 10, 20, 30 and 40 %) and at an IC50 of 20% PEG-6000, the germination and seedling growth was found inhibited substantially but not completely. Similarly, to choose hormone concentration a dose response experiment was performed using a wide range of concentrations of BRs (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 µM). Based on the growth response test three concentrations of BRs (EBL/HBL) i.e., 0.5 µM, 1 µM and 2 µM were selected where significant growth promotion was observed. The treatments were divided into four groups: (i) Distilled water (Control) (ii) 0.5 µM, 1µM and 2 µM 24-epibrassinolide / 28-homobrassinolide solutions (iii) 20% PEGsolution (water stress) [equivalent to osmatic potential of -2.95 bars at 25° C] (iv) 20% PEGsolution supplemented with 0.5µM, 1µM and 2 µM 24-epibrassinolide / 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 25 $\pm 1^0$C. Three ml more of test solutions were added on the 4th day of the experiment. Experiment was repeated thrice with five replicates for each treatment. Number of seeds germinated was recorded at the end of 12, 24 and 36 hours under safe green light. Emergence of radicle was taken as the criteria for germination. On the 7 th day, five seedlings one each from each Petri dish were selected randomly and the length, fresh weight (FW) and dry weight (DW) were recorded.

2.3. Stress indices

2.3.1. Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content following the method of Heath and Packer (1968). One gram of seedling material was macerated in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). 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 MDA was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3.2. Hydrogen peroxide

Hydrogen peroxide was extracted and estimated according method of Mukherjee and Choudari (1983). Isolation was made from 0.5 g of the treated seedling material in ice-cold acetone. By addition of 50% (w/ v) titanylsulphate and concentrated 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.

2.3.3. Electrolyte leakage

The relative intactness of plasma membrane was measured as the leakage of electrolytes using electrical conductivity meter (Systronics-304, India), as described by Lutts*et al*. (1996). Seedlings were washed with deionized water. After drying with filter paper, 1 g fresh weight of seedlings were immersed in 20 ml deionized water and incubated at 25 °C for 24 h and initial electrical conductivity (EC1) of the bathing solution was recorded. These samples were then autoclaved at 120 °C for 20 min

to release all electrolytes, cooled and the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was expressed following the formula EL=EC1/EC2×100.

2.4. Antioxidant enzyme activities

Fresh seedling material (1 g) was homogenized in 50 mMTris-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 (Nitrobluetetrazolium) 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}.

2.5. 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 as described by Bates and others (1973).

2.6. Glycine betaine

To 0.1 g dried ground material, 5ml of toluene–water mixture (0.5% toluene) was added. All the test tubes were shaken mechanically for 24 h at 25 1C. The extract was filtered and made up to a volume of 100 ml. To 1ml of filtrate 1ml of 2N HCl solution was added. Then an aliquot of 0.5 ml from the earlier extract was taken and 0.1 ml of potassium triiodide solution was added. It was then shaken in an ice bath for 90 min and then ice-cooled water (2 ml) was added along with 4ml of 1,2 dichloroethane. By stirring, two layers were formed. The lower colored layer was taken for reading. The optical density was read at 365nm according to the method of Grieve and Grattan (1983).

2.6. Ascorbic acid (AsA)

AsA content was determined according to Hodges and others (1996). 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.

2.7. Data 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.

3. Results

PEG induced drought stress reduced the germination percentage at 12 hours (33.5%), 24 hours (39.6%) and 48 hours (28%) in comparison to their respective controls (Table 1). However, supplementation of BRs reduced the toxic effect of drought on seed germination and enhanced the germination. The percentage of seed germination increased gradually with increasing concentration of HBL and EBL from, 0.5µM to 2µM concentrations. In PEG treatments supplemented with BRs (at 2 µM concentration), the percentage of seed germination approached that of unstressed control treatments. The treatment of BRs 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 for both the brassinosteroids.

Table 1 Effect of 24-epibrassinolide and 28-homobrassinolide on germination of *Cajanuscajan* seeds subjected to 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	12 hours	24 hours	48 hours
CONTROL	41.5 ±1.347e	81.2 ±2.436e	97.17 ±2.360d
0.5µM EBL	46.1 ±2.878c	83.8 ±3.391d	92.94 ±2.954f
1µM EBL	53.8 ±1.227b	88.5 ±2.545b	90.00 ±4.548h
2µM EBL	55.5 ±1.581a	89.3 ±2.494ab	99.00 ±2.260b
0.5µM HBL	44.5 ±1.789d	84.7 ±2.914d	96.79 ±1.870e
1µM HBL	52.8 ±2.521b	87.3 ±1.987c	98.82 ±1.870c
2µM HBL	54.7 ±1.257b	90.1 ±2.230a	100.00 ±2.549a
20% PEG *	27.4 ±2.638i	57.8 ±2.201k	68.47 ±2.551n
PEG+0.5µM EBL	34.7 ±3.301g	67.2 ±1.987i	77.84 ±3.102m
PEG+1µM EBL	37.6 ±2.012f	72.3 ±1.047g	83.56 ±3.478k
PEG+2µM EBL	51.7 ±2.004c	77.9 ±2.545f	89.76 ±4.125i
PEG+0.5µM HBL	31.3 ±3.672h	66.7 ±1.870j	81.75 ±4.012l
PEG+1µM HBL	34.9 ±3.316g	71.7 ±1.870h	87.86 ±3.001j 91.42
PEG+2µM HBL	46.8 ±1.870d	78.1 ±2.549f	±4.024g

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, *20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C

Table 2 Effect of 24-epibrassinolide and 28-homobrassinolide on *Cajanuscajan* seedling length, fresh weight and dry weight under 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	Seedling length (cm)	Fresh weight (mg)	Dry weight (mg)
CONTROL	11.3 ±0.854d	422.8 ±12.310f	36.2 ±4.280f
0.5µM EBL	13.9 ±0.663c	434.6 ±16.785e	37.6 ±4.854e
1µM EBL	14.5 ±1.291b	462.0 ±11.6524d	41 ±1.9748c
2µM EBL	15.6 ±0.367a	497.6 ±21.307b	43.6 ±5.067b
0.5µM HBL	13.7 ±1.663c	450.4 ±12.785e	38.8 ±3.854d
1µM HBL	14.1 ±0.291b	476.2 ±19.652c	42 ±5.974b
2µM HBL	15.2 ±1.367a	509.2 ±17.307a	45.2 ±5.067a
20% PEG *	4.4 ±0.430i	282.2 ±15.916i	28 ±3.707j
PEG+0.5µM EBL	8.1 ±0.620g	331.4 ±12.932h	30.2 ±1.435i
PEG+1µM EBL	10.7 ±0.841e	363.4 ±18.626g	34.6 ±4.969g
PEG+2µM EBL	11.6 ±1.061d	425.2 ±20.337ef	36.6 ±3.410f
PEG+0.5µM HBL	7.4 ±0.620h	336.0 ±14.932h	32.6 ±2.435h
PEG+1µM HBL	9.9 ±0.731f	373.0 ±16.626g	36.8 ±6.909f
PEG+2µM HBL	11.5 ±0.961d	431.2 ±18.337e	40.6 ±2.144c

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, *20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C.

Table 3 Effect of 24-epibrassinolide and 28-homobrassinolide on MDA, H_2O_2 and Electrolyte leakage levels in *Cajanuscajan* seedlings under 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	MDA (µmol g^{-1}FW)	H_2O_2 (µmol g^{-1}FW)	Electrolyte leakage
CONTROL	9.6 ±0.87 g	22.6 ±1.87 g	14.56±1.24 h
0.5µM EBL	9.1 ±0.59 g	21.4 ±1.77 h	14.25±0.97 i
1µM EBL	8.6 ±0.79 hi	21.2 ±1.58 h	13.12±1.34m
2µM EBL	8.3 ±0.74 i	20.4 ±2.34 i	12.98±0.88 n
0.5µM HBL	8.9 ±0.83 h	20.7 ±2.08 i	14.02±0.97 j
1µM HBL	8.1 ±0.65 i	21.7 ±1.47 h	13.66±0.87 l
2µM HBL	7.5 ±0.77 j	19.8 ±2.69 j	13.71±1.09 k
20% PEG *	16.4 ±0.84 a	43.8 ±2.45 a	23.94±1.24 a
PEG+0.5µM EBL	13.1 ±0.98 c	36.8 ±4.20 b	19.92±1.75 b
PEG+1µM EBL	12.4 ±0.75 d	27.6 ±3.89 d	16.32±0.89 d
PEG+2µM EBL	11.2 ±0.46 e	25.3 ±2.21 e	15.14±0.92 f
PEG+0.5µM HBL	14.5 ±0.81 b	34.2 ±2.11 c	17.88±0.75 c
PEG+1µM HBL	11.8 ±0.78 e	23.9 ±2.69 f	15.45±1.19 e
PEG+2µM HBL	10.2 ±0.98 f	23.7 ±3.14 f	14.72±1.42 g

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, *20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C

Table 4 Effect of 24-epibrassinolide and 28-homobrassinolide on the activities of CAT, POD and SOD in *Cajanuscajan* seedlings under 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	CAT (µmol H_2O_2 mg^{-1}protein min^{-1})	POD (U mg^{-1}protein min^{-1})	SOD (U mg^{-1}protein min^{-1})
CONTROL	9.8 ±0.95 j	0.813 ±0.052 f	31.8 ±1.25 k
0.5µM EBL	11.2 ±0.73 i	0.832 ±0.047 d	37.5 ±2.04 h
1µM EBL	13.7 ±0.82 h	0.858 ±0.023 c	35.6 ±1.80 i
2µM EBL	14.8 ±0.69 f	0.891 ±0.084 a	39.8 ±1.55 g
0.5µM HBL	13.5 ±0.85 h	0.828 ±0.081 e	33.4 ±2.47 j
1µM HBL	15.2 ±1.12 e	0.863 ±0.065 b	36.2 ±2.13 i
2µM HBL	11.8 ±0.76 i	0.887 ±0.038 a	40.7 ±1.04 f
20% PEG*	15.7 ±0.68 d	0.709 ±0.074 l	48.3 ±2.56 e
PEG+0.5µM EBL	17.8 ±0.71 c	0.735 ±0.057 k	49.6 ±1.02 d
PEG+1µM EBL	18.7±1.43 b	0.779 ±0.054 i	51.8 ±2.56 c
PEG+2µM EBL	19.4 ±0.58 a	0.796 ±0.081 g	53.9 ±2.87 b
PEG+0.5µM HBL	15.2 ±0.87 e	0.754 ±0.093 j	50.4 ±3.08 d
PEG+1µM HBL	18.3 ±1.11 b	0.789 ±0.067gh	54.1 ±1.77 a
PEG+2µM HBL	19.8 ±0.66 a	0.818 ±0.083 f	52.7 ±3.13 c

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, *20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C.

Table 5 Effect of 24-epibrassinolide and 28-homobrassinolide on the activities of APX and GR and ascorbate content (AsA) in *Cajanuscajan* seedlings under 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	APX (µmolAsA mg^{-1}protein min^{-1})	GR (µmolNADPH $min^{-1}mg^{1}$protein)	AsA content (µg g^{-1}FW)
CONTROL	3.9 ±0.11 k	0.478 ±0.012 c	322 ±9.921 k
0.5µM EBL	4.1 ±0.12 k	0.483 ±0.022 c	338 ±9.033 j
1µM EBL	4.7 ±0.21 j	0.491 ±0.010 b	356 ±11.728 i
2µM EBL	5.2 ±0.32 h	0.504 ±0.031 a	369 ±8.052 h
0.5µM HBL	4.1 ±0.41 k	0.481 ±0.052 c	341 ±12.915 j
1µM HBL	4.9 ±0.32 i	0.494 ±0.012 b	362 ±11.019 h
2µM HBL	5.5 ±0.18 f	0.510 ±0.040 a	378 ±10.012g
20% PEG*	7.6 ±0.47 e	0.293 ±0.013 h	403 ±9.122 f
PEG+0.5µM EBL	8.6 ±0.24 c	0.322 ±0.021 g	422 ±9.910 e
PEG+1µM EBL	9.8 ±0.27 a	0.338 ±0.033 f	447 ±10.631 c
PEG+2µM EBL	9.4 ±0.13 b	0.356 ±0.028 e	481 ±11.352 a
PEG+0.5µM HBL	8.1 ±0.17 d	0.329 ±0.052 g	438 ±10.112 d
PEG+1µM HBL	9.2 ±0.18 b	0.371 ±0.015 d	450 ±8.640 c
PEG+2µM HBL	10.2 ±0.44 a	0.361 ±0.019 e	464 ±9.340b

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.*20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C.

Table 6 Effect of 24-epibrassinolide and 28-homobrassinolide on the levels of proline and glycine betaine in *Cajanuscajan* seedlings under 20% PEG imposed drought stress (OP= -2.95 bars) and stress free conditions

Treatments	Proline(mg g^{-1} FW)	Glycine betaine(mg g^{-1} FW)
CONTROL	6.34 ±0.75 k	2.45 ±0.24 h
0.5µM EBL	6.78 ±0.61 j	2.51 ±0.43 h
1µM EBL	8.54 ±0.45 g	2.91 ±0.21 f
2µM EBL	8.91 ±0.98 e	2.78 ±0.37 g
0.5µM HBL	7.26 ±0.64 h	2.63 ±0.42 e
1µM HBL	6.89 ±0.57 i	2.89 ±0.13 f
2µM HBL	8.84 ±0.37 f	2.79 ±0.78 g
20% PEG *	8.94 ±0.28 e	3.23 ±0.04 e
PEG+0.5µM EBL	10.22±0.80cd	3.38 ±0.26 d
PEG+1µM EBL	10.67 ±0.81 b	3.42±0.12 c
PEG+2µM EBL	10.19 ±0.96 d	3.78 ±0.21 d
PEG+0.5µM HBL	10.38 ±0.73 c	3.71 ±0.08 b
PEG+1µM HBL	11.09 ±0.65 a	3.53 ±0.17 c
PEG+2µM HBL	10.74 ±0.59 b	4.01 ±0.38 a

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, *20% PEG is equivalent to osmatic potential of -2.95 bars at 25° C.

PEG induced drought stress was reflected on seedling length, fresh and dry weight as they were significantly reduced in comparison to control (Table 2). However, BRs (EBL/HBL) application to drought stressed seedlings improved the seedling growth, fresh weight and dry weight. There was progressive increase in seedling length, fresh weight and dry weight with an increase in applied BRs concentration and maximum increase was seen at 2 µM. Similarly, BRs alone treatments also accounted for marked enhancement of seedling growth compared to control plants.

PEG induced drought stress markedly increased the levels of H_2O_2 (93.8%), MDA content (70.8%) and electrolyte leakage (64.4%) in pigeon pea seedlings (Table 3). Foliar application of BRs to stressed plants led to considerable decrease in H_2O_2, MDA and ELP levels. Seedlings with HBL application under drought stress showed maximum decrease in H_2O_2, MDA and ELP levels by 42.2, 31.7 and 36.7% and with EBL application, which were decreased by 45.8, 38.2 and 38.5% respectively at 2µM concentration relative to that of stressed control. There was no significant change in the levels of H_2O_2, MDA, ELP in seedlings receiving BRs alone treatments compared to the unstressed control seedlings.

Pigeon pea seedlings growing under drought stress conditions, exhibited significant increase in the activities of CAT, APX and SOD (Table 4). Exogenous application BRs to stressed plants caused further increase in the activity of CAT, APX and SOD enzymes. BRs alone treatments also considerably increased the CAT, APX and SOD activities but not to the extent as in drought stressed seedlings. A marginal decrease in POD activity in drought stressed plants was observed which was restored to the normal levels with foliar application of BRs (at 2µM concentration). The activity of GR found drastically reduced in radish plants subjected to drought stress. BRs application to plants under drought stress resulted in increase in the activity of GR. Plants receiving only BRs application exhibited higher GR activities in comparison to unstressed as well as BRs supplemented drought stress seedlings. Ascorbate content was increased by 19.4% under drought stress in seedlings (Table 5). Exogenous supplementation of both BRs further improved the ASA content in both stressed and unstressed seedlings.

Drought stress significantly increased the free proline (41%) and glycine betaine (31.8%) content of Pigeon pea seedlings compared to the control (Table 6). Interestingly, seedling supplemented with BRs led to further enhancement of proline and glycine betainelevels in stressed plants. A pronounced increase in free proline levels was observed in stressed seedlings for both BRs at the 1 µM concentration (with EBL by 75 % and HBL by 68%) over the unstressed control plants. Application of BRs further increased the glycine betainelevels and maximum increase was observed at 2 µM concentration (17.2 and 24.5% by EBL and HBL respectively) in drought stressed seedlings. BRs alone application also resulted in increase in free proline and glycine betaine levels but not to the levels observed in BRs supplemented drought stressed treatments.

4. Discussion

Results from this study showed that PEG induced drought stress significantly decreased the pigeon pea seed germination as compared to the control (Table 1). However, seeds supplemented with BRs (EBL and HBL) improved the germination percentage and the percentage of seed germination was near to the level of control at 2 µM BRs concentration, reflecting the stress mitigation capability of BRs. The present study also point out that inhibitory effects of drought stress on seedling length, and biomass production (fresh and dry mass) of pigeon pea was significantly restored to nearly that of

unstressed seedlings upon BRs application (Table 2). In accordance with our results, exogenous application of brassinosteroids significantly improved seed germination and seedling growth of *Medicago sativa* under salt stress (Zhang *et al*. 2007) and radish under cadmium stress (Anuradha and Rao 2007). 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 xyloglucanendotransglucosylase/hydrolases (XTHs), expansions, glucanases, sucrose synthase and cellulose synthase or by activating the H^+-ATPase activity (Clouse 2011).

Excess production of reactive oxygen species (ROS) is a common phenomenon of drought stress in plants, which cause oxidative damage to proteins, lipids and nucleic acids, leading to irreparable metabolic dysfunction and cell death (Zhu, 2001). In the present study, PEG induced drought stress resulted in oxidative injury, as evidenced by an increase of H_2O_2 and MDA contents in radish plants. As a consequence, the membranes become leaky as reflected by increased electrolyte leakage measured indirectly as electrical conductivity (Table 3). However, application of EBL/HBL dramatically depressed H_2O_2 and, MDA accumulation there by electrolyte leakage in drought stressed pigeon pea seedlings, suggesting that BRs helps in the maintenance of membrane integrity against oxidative damage induced by drought stress. Similarly, BRs treatment significantly declined the H_2O_2 and MDA contents, and electrical conductivity under drought stress in *Robiniapseudoacacia* (Li *et al*., 2008) and soyabean (Zhang *et al*., 2008) plants.

Plants have several efficient enzymatic and non enzymatic antioxidant defence systems that allow scavenging of ROS and protection of plant cells from oxidative damage (Gill and Tuteja 2010). Superoxide radicals are dismutated to H_2O_2 and water by SOD in the first line of defence (Alscher*et al*., 2002).Subsequently, removal of H_2O_2 is achieved by CAT and POD (Gill and Tuteja 2010). POD scavenges the H_2O_2 in the presence of ascorbate as electron donor. Under drought stress an increase in the activities of SOD, CAT and APX were observed whereas POD and GR activities were decreased in pigeon pea seedlings. Supplementation of pigeon pea seedlings with BRs (HBL and EBL) increased the all enzyme activities (CAT, POD, APX, SOD and GR) in both stressed and unstressed conditions (Table 4 and 5). Yuan *et al*. (2010) reported that the activities of SOD, APX, and CAT significantly increased in water stressed tomato plants treated by EBR than those of controls. It was suggested that increase in activities of antioxidant enzymes, at least in part, was responsible for amelioration of the drought tress of pigeon pea seedlings. Interestingly both BRs (EBL and HBL) have enhanced the osmolytes levels i.e. proline and glycine betaine and ascorbate content under drought stressed and unstressed conditions in red gram seedlings (Table 6). Upon supplementation of EBL improved the proline and AsA levels in *Chorisporabungeana* under PEG induced drought stress (Li *et al*., 2012). Similarly, exogenous application of BRs enhanced the cellular osmolytes (proline and glycine betaine), antioxidants levels and antioxidative enzyme activities under different stress conditions in *Phseolusvulgaris* (Rady 2011), rice (Farooq*et al*., 2009) and radish (Vardhini and Rao, 2003). The enhanced antioxidativedefense 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 againstoxidative stress.

Summing up, application of EBL and HBL increased the germination, length and dry mass of pigeon pea seedlings under PEG induced drought stress. Exogenous application of HBL and EBL significantly enhanced the antioxidative enzymes activities (SOD, CAT, POD, APX and GR) and levels of osmolytes (proline and glycine betaine) as well as ascorbate content, while it decreased MDA, H_2O_2 content and electrolyte leakage in pigeon pea seedlings. The most effective dose of BRs under stress conditions was found to be 2 µMconcentration. Overall, the results indicated that treatment with BRs could reduce the negative effects of drought stress in red gram seedlings.

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