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Protection of growth via antioxidants and endogenous hormones maintenance in response to 24-epibrassinolide in tomato (*Solanum Lycopersicum* L) growing under salt, drought and heavy metal stress

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Abstract
Brass inosteroids (BRs) are a group of plant growth steroidal hormones present in very low concentrations in seeds, pollen and young plant tissues, reported to be involved in plant development as well as protection of plants against various biotic and abiotic stresses. In this study, we report the amelioration of salt, drought and heavy metal stress by the application of of 24-epibrassinolide (EBR) on *in vitro* and *ex vitro* grown tomato (*Lycopersicon esculentum* L. cv. Pusa Ruby). A reduction in seed germination and growth parameters such as shoot length, total leaf area and plant dry weight was observed seen under these stresses. However, these parameters showed enhanced values with the application of EBR showing either *in vitro* or *ex-vitro* conditions. Significant increases in total chlorophyll, proline contents and nitrate reductase (EC 1.6.6.1) activities were observed in tomato treated with EBR when compared to their respective stress controls. The activities of antioxidant enzymes ascorbate peroxidase (EC 1.11.1.11), catalase (EC 1.11.1.6), dehydroascorbate reductase (EC 1.8.5.1), glutathione reductase (EC 1.6.4.2) and superoxide dismutase (EC 1.15.1.1) enhanced in tomato under NaCl, drought or heavy metal stresses and their activities further enhanced with the application of EBR. The endogenous levels of indole-3-acetic acid (IAA) and abscisic acid (ABA) elevated significantly in the leaves of tomato seedlings and plants treated with EBR compared to stress controls. This is the first comprehensive report confirming the protective role of EBR in imparting plant tolerance to major abiotic stresses under both *in vitro* and *ex vitro* conditions.

Keywords: Abiotic stress; antioxidants; Solanum Lycopersicum L.; 24-epibrassinolide; abscisic acid

1. Introduction

Brassinosteroids (BRs) belong to a class of plant polyhydroxy steroids, structurally similar to animal and insect steroid hormones. BRs are considered as sixth group of plant hormone present in very low concentrations in seeds, pollen, young tissues; imparting a variety of plant responses including cell division and expansion, xylem differentiation, ethylene biosynthesis, ion uptake, photosynthesis, seed germination, vegetative growth, apical dominance and gene expression (Sasse, 2003) [62]. In addition to their role in plant growth and development, BRs appear to protect plants from a variety of environmental stresses (Krishna 2003) [48]. Including high and low temperature stresses (He *et al*, 1991; Ogweno *et al*, 2008) [37, 58].drought (Behnamnia *et al*, 2009; Yuan *et al*, 2010). [13, 71]. Salinity (Anuradha *et al*, 2001; Hayat *et al*, 2010) [6, 35]. Heavy metal toxicity (Anuradha *et al* 2007; Choudhary *et al* 2010) [5, 17]. Herbicides (Pinol *et al*, 2009) [60]. And pathogen attack (Khripach *ET AL*, 2000; kagale *et al*, 2007) [47, 40]. However, the underlying mechanism for BR-mediated stress tolerance is still a matter of debate.

Drought, salinity and heavy metal toxicity are the major environmental stress factors affecting plant growth and productivity. Drought stress has been considered as one of the serious environmental factors affecting plant growth and development as well as crop production. It alters carbon fixation (Flexas *et al*, 2004) [27]. And the activities of PSII/Rubisco (Yordanov *et al*, 2003) [70]. Salinity stress also results in accumulation of toxic ions such as Na^+ and Cl^-, which results in imbalance of mineral nutrition leading to increased membrane permeability (Kaya *et al*, 2001) [44]. Reduced photosynthetic activity and stomatal conductance (Chartzoulakis *et al*, 2000; Martinez-Ballesta *et al*, 2004) [16, 52]. Similarly, the heavy metal stress being a vital component of many enzymes, structural stabilizer for proteins, membrane and DNA-binding proteins (Zn-fingers) (Vallee *et al*, 1990) [69]. Higher concentrations causes toxicity resulting decrease in growth and development, metabolic activity and induction of oxidative damage in various plant species (Panda *et al*, 2003) [57].

Tomato (*Solanum Lycopersicum* L*)* is a major vegetable crop which has now achieved tremendous popularity over the last century. Tomato aside from being tasty, are supposed to be good source of essential minerals, vitamin A, C and other antiioxidants. It is one of the most economically important crops, cultivated widely in America, east/south Asia as a favorite vegetable. However, tomato is susceptible to several environmental stresses like salinity, drought, and heavy metals affecting yields. Keeping in view the protective role of BRs to a broad range of abiotic stresses, we aimed to study the effect of 24-epibrassinolide (EBR) on tomato plants grown under salt,

drought and heavy metal stresses. In addition to plant growth parameters, we studied the influence of EBR on the levels of endogenous plant hormones, free proline, nitrate reductase activity, lipid peroxidation, antioxidant levels, and activity of antioxidative enzymes in tomato seedlings and plants grown under salt, drought and ZnCl2 stresses. Also, the differential influence of EBR foliar spray and EBR seed treatments on drought tolerance of rice plants in earlier study (Farooq *et al*, 2009) [26]. Compelled us to investigate the influence of EBR on stress tolerance in tomato under *in vitro* and *ex vitro* conditions.

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds of tomato (*Lycopersicon esculentum L.* Pusa Ruby) obtained from vegetable seed division of IARI, New Delhi, Pusa. Seeds were surface sterilized with 2% (v/v) sodium hypochlorite for 20 min and washed thoroughly with several changes of sterile distilled water. Seeds were soaked for 24 h, either in distilled water or in NaCl (150 mM) or $ZnCl_2$ (25 mM) or mannitol as drought stress agent (100 mM), with or without epibrassinolide (1 - 2 µM). 24-epibrassinolide (EBR, E1641 Sigma-Aldrich) was purchased from Sigma-Aldrich chemicals, USA. Stock solution (0.1M) was prepared by dissolving required quantity of the EBR in 5.0 ml of ethanol. 5.0 ml surfactant Tween-20 was added to it and final volume was made up to the mark by using double distilled water (DDW).

The required concentration of EBR was prepared by the dilution of stock solution. The soaked seeds were then placed on sterile filter paper (whatmann No 3) moistened with respective solutions as above and incubated in dark for germination at 24±2 ºC temperature. After germination, the seedlings were shifted to plant growth chamber (Matrix Eco-solutions, New Delhi, India) maintained at 16 h light and 8 h dark period and 24 ºC temperature. Seeds germination and growth were recorded after two weeks.

For *ex vitro* studies, seeds were directly sown in peat for germination in small pots (6 cm in size) containing sand, loam and peat (2:1:1). After the appearance of second leaf, seedlings were planted in bigger pots (10 cm diameter and 15 cm deep) and transferred to plant growth chamber. The seedlings were irrigated with Hoagland's solution (Hoagland *et al*, 1933) [38]. once a day (for few days) to prevent mineral deficiency. Seedlings at four leaf stage were given stress treatments by irrigating with 150 mM NaCl, 25 mM $ZnCl_2$ and withheld water for 5 days to impart salt, heavy metal and drought stresses, respectively. Simultaneously plants were sprayed with 1 - 2 µM EBR for one week, while control plants were sprayed with water alone. Growth characteristics were recorded after two weeks of stress treatments.

2.2. Plant growth characteristics and relative water content

The individual and interactive effects of stress with EBR on seed germination, seedling growth, plant height, shoot and root dry masses were analyzed at 3 weeks after germination *in vitro* and *ex vitro* conditions, respectively.

Total leaf area was calculated using leaf area meter. The relative water content (RWC) was determined and calculated as per standard formula. The leaf were weighed and immediately floated on double distilled water in petri-dishes to saturate them with water for the next 24 h in dark. The leaves were blotted on filter paper and turgor weights were measured. Dry mass of these leaves were obtained after drying them in oven at 80 ◦C for 48-72 h. RWC was calculated according to following formula: RWC= (fresh weight – dry weight)/ (turgor weight – dry weight).

2.3. Chlorophyll estimation

Total chlorophyll was estimated using the method reported by Arnon (Arnon *et al*, 1949). Ca. 100 mg of leaf tissue was suspended in 10 ml of 80% acetone, mixed well and kept at 4 °C overnight in dark. Supernatant was withdrawn after centrifugation at 5000 rpm and absorbance of the supernatant was recorded at 663 and 645 nm in spectrophotometer (Model: Pharmaspec UV-1700, Shimadzu, Japan). Chlorophyll content was estimated using the formula:

Chlorophyll content: (20.2 x OD_{645}nm + 8.02 x OD_{663}nm) x Dilution Factor

2.4. Lipid peroxidation

Lipid peroxidation in leaf tissue was measured by determination of melondialdehyde (MDA) (Heath *et al*, 1969) [36]. Using thiobarbituric acid (TBA) reaction. About 0.2 g leaf tissue was homogenized in a solution of 0.5% (w/v) TBA in 20% (w/v) TCA. The homogenate was boiled at 95 °C in a water bath for 30 min followed by cooling on ice. Samples were centrifuged at 12000 rpm for 10 min and the absorbance of resultant supernatant was taken at 532 and 600 nm wavelength. The non-specific absorbance of the supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm for MDA content. The level of lipid peroxidation was expressed as µmol of MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5. Proline estimation

Proline was estimated by the method described by Bates *et al* (1973) [12]. With minor modification. Leaf tissues (1.0 g) were homogenized in 5 ml of 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 rpm for 20 min. To 0.5 ml supernatant, 2 ml reagent mixture (30 ml glacial acetic acid, 20 ml water and 0.5 g ninhydrin) was added and the mixture was boiled for 1 h in a boiling water bath. After cooling the mixture to room temperature, proline was extracted with 6 ml toluene and the absorbance was taken at 546 nm using spectrophotometer. Proline content was estimated using a proline standard curve prepared by dissolving the known quantities of proline standard in 3% sulfosalicylic acid.

2.6. Nitrate reductase (NR) activity

Nitrate reductase (NR) activity was determined following the *in vivo* method (Jaworski *et al* 1971) [39]. Leaf samples of same size were collected from different treatments and cut into small pieces. Ca. 500 mg of leaf pieces were incubated separately in a medium containing 1 ml of 1 M potassium nitrate, 2 ml of 0.2 M phosphate buffer (pH 7.5) and 2 ml of 0.5% Triton-X-100 for 1 h. One ml of the reaction mixture was transferred to another test tube containing 1.0 ml of 1% sulphanilamide in 2 N HCl and 1 ml of 0.2% NEEDA (N-1- napthyl ethylene diamide dihydro chloride). The absorbance of the mixture was recorded at 540 nm. A standard curve was prepared using different concentrations of potassium nitrite as substrate and NR activity is expressed as µmol of NO2 liberated h^{-1} g^{-1} FW.

2.7. Determination of antioxidants content

Ascorbic acid (AsA) was extracted from frozen leaves collected from different stress treatments according to the method described earlier (Han *et al*, 2004) [32]. Approx 0.5 g of frozen leaf tissue was ground using a mortar and pestle in 5% metaphosphoric acid and centrifuged at 13000 rpm for 10 min at 1 ºC. The pellet was re-suspended three times in metaphosphoric acid and the resulting supernatants from each spin were collected and mixed. The homogenate was then filtered through Whatman No. 1 filter paper and centrifuged at 17000 rpm for 10 min at 1 ºC. The supernatants (20 µl) were analyzed by HPLC using an Atlantis dC18 column (4.6 mm x 250 mm, 5 µm) with a mobile phase of 0.2% TFA (A) and 100% methanol (B) at 1 ml/min retention time. The eluents were monitored at 254 nm. Three independent analyses were carried out with each sample.

For determination of reduced glutathione (GSH) content, the leaves were homogenized in an ice bath with 5% (w/v) sulfosalicylic acid and centrifuged at 10000 rpm for 10 min. The supernatant was used for reduced glutathione (GSH) estimation and the GSH content was assayed following the change in absorbance at 412 nm after the addition of 5,5'-dithio-2,2'-dinitrobenzoic acid (DTNB) according to the method of Griffith (Griffith *et al*, 1980) [29].

2.8. Estimation of antioxidant gene expression by quantitative real-time PCR

Quantitative real-time PCR analysis was performed at mRNA expression levels to establish a correlation with the expression of the antioxidant gene. Total RNA was isolated from the leaves of *in vitro* and *ex vitro* tomato seedlings grown under different stress conditions using TRI reagent (Invitrogen, USA). First-strand cDNA was synthesized using SuperScript™ Reverse Transcriptase (Invitrogen, USA).

Samples were amplified using thermal cycler (Bio-Rad). The real-time PCR amplification of ROS pathway genes (APX, CAT, DHAR, GR and SOD) were carried out using cDNA specific primers as mentioned in Table 1. RT-PCR was performed using a SYBR green PCR kit (Bio-Rad, USA) with Actin serving as an internal control. Comparative threshold (Ct) values were normalized to actin control and compared to obtain relative expression levels.

Table 1: Primer sequence of genes used for real time PCR analysis

Name	NCBI Acc. No.	Primer sequence (5'-3')
Actin	X55749	F-CTGGTGGTGCAACAACCTTA
		R-GAATGGAAGCAGCTGGAATC
APX	AB041343	F-ACCAATTGGCTGGTGTTGTT
		R-TCACAAACACGTCCCTCAAA
CAT	AY442179	F-TGCCCTTCTATTGTGGTTCC
		R-GATGAGCACACTTTGGAGGA
DHAR	DQ512964	F-AGGTGAACCCAGAAGGGAAA
		R-TATTTTCGAGCCCACAGAGG
GR	X76533	F-GGATCCTCATACGGTGGATG
		R-TTAGGCTTCGTTGGCAAATC
SOD	AF354748	F-GTTTGTGGCACCATCCTCTT
		R-GTGGTCCTGTTGACATGCAG

2.9. Assay of antioxidative enzymes

Frozen leaf samples (0.5 g) were grinded in liquid nitrogen to a fine powder and homogenized in 50 mM phosphate buffer (pH 7.0) and 100 mM triethanolamine buffer (pH 7.4). The homogenates were centrifuged at 16000 rpm for 20 min at 4 °C and the supernatants were collected for assay of APX, CAT, DHAR, GR and SOD activities. The protein content of the crude extract was quantified by the protein dye binding assay with bovine serum albumin as the standard (Bradford *et al*, 1976) [14]. APX activity was determined according to the method of Nakano and Asada (Nakano *et al*, 1981) [55]. and oxidation of AsA was determined by monitoring the decrease in absorbance at 300 nm (extinction coefficient 0.74 mM^{-1} cm^{-1}). One unit of APX was defined as the amount of enzyme oxidizing 1.0 nmol of AsA per min. Catalase activity was measured according to the method described by Aebi (Aebi *et al*, 1983). The CAT activity was estimated by the decrease in absorbance at 240 nm. One unit of CAT was defined as the amount of enzyme dismuting 1.0 nmol of H_2O_2 per min. DHAR activity was determined as described by Nakano and Asada (1981) [55]. DHAR activity was assayed at 25°C by following the increase in absorbance at 265 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) measuring the GSH-dependent synthesis of AsA. Glutathione reductase (GR) activity was determined according to the method of Foyer and Halliwell (Foyer *et al*, 1976) [28]. And oxidation of NADPH was determined by monitoring the decrease in absorbance at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). SOD activity was determined according to the protocol described by Paoletti *et al* (1986) [59]. based on the inhibition of superoxide-driven NADH oxidation. The oxidation of NADH was measured at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). One unit of SOD was defined as the amount of enzyme oxidizing 1 nmol of NADPH per min.

2.10. Extraction and estimation of endogenous plant hormones

Levels of plant hormones, indole-3-acetic acid (IAA) and abscisic acid (ABA) were measured using HPLC according to the method described by Kelen *et al* (2004) [45]. About 1.0 g fresh leafs were homogenized in 70% (v/v) methanol and stirred overnight at 4 ºC. The extracts were filtered and methanol was evaporated under vacuum. The pH of aqueous phase was adjusted to 8.5 with 0.1 M phosphate buffer. The aqueous phase was partitioned three times with ethyl acetate. After removal of ethyl acetate phase, the pH of the aqueous phase was adjusted to 2.5 with 1 N HCl. The aqueous phase was again partitioned

with three volumes of diethyl ether and passed through anhydrous sodium sulphate. The ether phase was evaporated and the pellet was dissolved in 2.0 ml of absolute methanol. The chromogenic analysis was performed on a Shimadzu Model HPLC. The mobile phase used was acetonitrile: water (26:74; v/v) at 25±1 ºC. The separation was carried by isocratic elution with a flow rate of 0.8 ml min^{-1}. The optimum wavelengths were set at 280 and 265 nm for IAA and IBA, respectively.

2.11. Statistical analysis

The experiment was conducted in a simple randomized block design (RBD). A total of three replicates for each treatment were taken. Treatment means were compared by analysis of variance (ANOVA) using SPSS.10 (SPSS, Chicago, IL, USA). Statistically significant differences between means were determined by Duncan's multiple range test at *P* (<0.01) level (Duncan *et al* 1955) [21].

3. Results

3.1. Effect of EBR on seeds germination and growth

Significant differences were observed in shoot length, average root length, seedling dry weight of tomato germinated under different abiotic stresses and treated with EBR. However, no significant differences were observed in germination percentages among the treatments (Table 2). Average shoot length of the seedlings was maximum (5 cm) when the seeds germinated in distilled water while the longest roots (5. 3 cm) were observed in seedlings grown under drought induced by mannitol (100 mM).

Table 2: Effect of 24- epibrassinolide (EBR) germinating and growing on filter paper under NaCl, drought and $ZnCl_2$ stress

Treatment	Root length (cm)	Soot length (cm)	Root dry weight (g)	Seedling dry weight (g)	RWC (%)	Total chlorophyll (mg/ 100 g FW)
Control	5.9 ± 0.4^{b}	10.6±0.2^{a}	0.0072±0.0005^{ab}	0.0867±0.0020^{a}	97.5^{a}	0.0093±0.0003^{a}
NaCl stress	2.0±0.2^{e}	4.0±0.1^{cde}	0.0030±0.0001^{c}	0.0126±0.0009^{cd}	87.0^{a}	0.0050±0.0003^{b}
NaCl + EBR (1 µm)	3.8±0.1^{c}	5.2±0.1^{b}	0.0036±0.0001^{b}	0.0173±0.0007^{ab}	92.1^{a}	0.0055±0.0003^{b}
NaCl + EBR (2 µm)	3.7±0.3^{c}	5.8±0.2^{b}	0.0048±0.0001^{b}	0.0237±0.0002^{ab}	97.1^{a}	0.0070±0.0001^{a}
Mannitol	4.8±0.1^{a}	4.2±0.1^{c}	0.0030±0.0003^{a}	0.0139±0.0002^{ab}	87.3^{d}	0.0045±0.0003^{c}
Drought + EBR (1 µm)	5.3± 0.1^{cd}	6.2±0.2^{c}	0.0049±0.0002^{bc}	0.0224±0.0003^{bc}	86.3^{d}	0.0049±0.0003^{c}
Drought + EBR(2µm)	4.7±0.1^{dc}	6.3±0.1^{bc}	0.0047±0.0003^{bc}	0.0247±0.0002^{ab}	86.7^{d}	0.0052±0.0001^{c}
$ZnCl_2$ stress	3.5±0.1^{f}	4.1±0.2^{e}	0.0036±0.0001^{d}	0.0173±0.0016^{de}	91.4^{b}	0.0038±0.0001^{de}
$ZnCl_2$ + EBR (1 µm)	5.6±0.1^{cd}	4.5±0.1^{cd}	0.0059±0.00005^{d}	0.0284±0.0020^{de}	93.4^{b}	0.0045±0.0003^{be}
$ZnCl_2$+EBR (2µm)	6.5±0.1^{e}	5.2±0.1^{e}	0.0057±0.0002^{d}	0.0363±0.0008^{e}	92.5^{c}	0.0059±0.0004^{e}

Tomato seeds were soaked in distilled water (control) or in NaCl (150 mM) or $ZnCl_2$ (25 mM) or Mannitol (100 mM) with or without EBR (1-2 µM) Mannitol was used to induce drought stress to seeds and seedlings. Values represent means represented by ± SE (n=3) and means represented by similar alphabets are statistically not different at $P<0.01$.

The exposure of 7-day old tomato seedlings to NaCl (150 µM), drought and $ZnCl_2$ (25 µM) stresses under *ex vitro* conditions significantly affected their growth characteristics (plant height, root length, dry weight and total leaf area) (Table 3). The average height of the tomato plants was decreased by 26, 17 and 23%, respectively in salt, drought and heavy metal stresses compared to control plants. Similarly, the reduction in total dry weight was 38, 33 and 53%, respectively in salt, drought and heavy metal stresses over control.

However, spraying of leaves with EBR in stressed plants significantly enhanced the values of these parameters over their respective controls. Among the two concentrations, spraying of EBR at 2 µM was found to be more effective in neutralizing the toxicity and growth inhibition induced by these stresses. Relative water content (RWC) of tomato seedlings germinated under mannitol induced drought stress showed significant reduction when compared to salt and heavy metal stresses. Addition of EBR (1 or 2 µM) could alleviate the effect of these stresses (Table 2).

As expected tomato plants grown under drought conditions showed significantly lower values for RWC under *ex vitro* conditions also (Table 3). Over all the plants under stress Conditions showed a reduction in RWC in the range of 10 to 15% and the RWC values increased by moderate to normal levels with the application of EBR with no significant variation between the EBR levels.

3.2. Effect of EBR on leaf chlorophyll content

Leaf total chlorophyll content showed significant variation among different stresses and EBR treatments (Table 2 and 3). Leaf chlorophyll content showed a drastic reduction (58.2% of control) in tomato seedlings germinated on ZnCl2 (25 mM) while salt and drought stresses showed slight to moderate effect (Table 2). In *ex vitro* studies, tomato seedlings grown under drought and ZnCl2 (25 mM) stress showed a reduction of 23 and 37%, respectively in leaf chlorophyll content and the application of EBR recovered up to 70 - 80% of their normal levels (Table 3). Salt stress had no significant effect on leaf chlorophyll content during the experimental period under *ex vitro* conditions.

Table 3: Effect of 24- epibrassinolide (EBR) spray on seeds germinating and growing in pots under salt, drought and $ZnCl_2$ stresses

Treatment	Root length (cm)	Soot length (cm)	Root dry weight (g)	Total dry matter(g)	RWC (%)	Total leaf area (sq.cm)	Total chlorophyll (mg/ 100 g FW)
Control	9.6 ± 0.3^{AB}	17.5 ± 0.6^{a}	0.106 ± 0.017^{bc}	0.886 ± 0.009^{b}	91.4^{a}	58.0 ± 4.0^{a}	0.0103 ± 0.0004^{a}
NaCl stress	6.3 ± 0.4^{d}	10.5 ± 0.9^{abc}	0.054 ± 0.003^{f}	0.414 ± 0.047^{f}	82.6^{c}	33.3 ± 2.5^{b}	0.0103 ± 0.0001^{a}
NaCl + EBR (1 µm)	7.5 ± 0.5^{bcd}	12.0 ± 0.6^{ab}	0.071 ± 0.002^{ef}	0.511 ± 0.008^{d}	88.8^{ab}	38.7 ± 1.9^{ab}	0.0101 ± 0.0004^{ab}
NaCl + EBR (2 µm)	7.0 ± 0.1^{d}	14.0 ± 0.9^{bc}	0.078 ± 0.001^{de}	0.560 ± 0.006^{e}	86.4^{b}	41.2 ± 3.3^{bc}	0.0096 ± 0.0001^{b}
Drought	7.7 ± 0.3^{bc}	10.0 ± 0.3^{c}	0.036 ± 0.002^{f}	0.424 ± 0.025^{e}	78.8^{d}	23.0 ± 1.3^{d}	0.0080 ± 0.0003^{d}
Drought + EBR (1 µm)	8.5 ± 0.3^{ab}	12.5 ± 0.9^{abc}	0.087 ± 0.002^{cd}	0.619 ± 0.030^{c}	88.5^{b}	32.0 ± 2.6^{bc}	0.0103 ± 0.0002^{a}
Drought + EBR(2µm)	9.5 ± 0.3^{a}	13.5 ± 0.6^{abc}	0.100 ± 0.020^{a}	0.710 ± 0.023^{a}	87.7^{b}	44.7 ± 1.9^{d}	0.0103 ± 0.0002^{a}
$ZnCl_2$ stress	6.5 ± 0.1^{cd}	9.0 ± 0.6^{c}	0.051 ± 0.002^{ef}	0.314 ± 0.011^{f}	80.2^{d}	18.3 ± 1.0^{d}	0.0065 ± 0.0004^{e}
$ZnCl_2$ + EBR (1 µm)	7.5 ± 0.3^{bcd}	13.0 ± 0.9^{abc}	0.092 ± 0.003^{cd}	0.630 ± 0.012^{d}	87.3^{b}	36.3 ± 3.7^{bc}	0.0083 ± 0.0001^{d}
$ZnCl_2$+EBR (2µm)	9.0 ± 0.3^{a}	15.5 ± 0.9^{bc}	0.123 ± 0.008^{b}	0.755 ± 0.233^{e}	88.5^{b}	53 ± 1.4^{cd}	0.0090 ± 0.0001^{cd}

Tomato plants at four leaf stage were irrigated with 150 mM NaCl, 25 mM $ZnCl_2$ and with held water for 5 days to impart salt, heavy metal and drought stresses, respectively. Simultaneously seedlings were sprayed with EBR (1-2 µM). Values represent mean ± SE (n=3) and means represented by similar alphabets are statistically not different at $P<0.05$

3.3. Effect of EBR on lipid peroxidation

The MDA content significantly reduced with the application of EBR on tomato plants grown under different abiotic stresses (Table 4). Under *in vitro* conditions, MDA content increased significantly in salt, drought and $ZnCl_2$ stresses. However, addition of EBR in the germinating condition completely recovered the seedlings from membrane peroxidation as reflected in lowered MDA levels. Similarly under *ex vitro* conditions, the increased MDA content observed in leaves of tomato plants grown under the three stresses had reverted back to almost normal with spraying of EBR at 2 µM.

Table 4: Effect of 24- epibrassinolide (EBR) on lipid peroxidation and leaf proline content of tomato seeds Germinated and grown under salt, drought and $ZnCl_2$ stresses

Treatment	***in* vitro conditions**		***Ex vitro conditions***	
	MDA content (nmol /100 g FW)	Proline content (mg/100 g FW)	MDA content (nmol /100 g FW)	Proline content (mg/100 g FW)
Control	22.5 ± 0.99^{de}	0.19 ± 0.01^{e}	22.13^{d}	1.34 ± 0.052^{e}
NaCl stress	33.55 ± 0.90^{a}	2.23 ± 0.16^{cd}	42.26^{b}	45.00 ± 2.51^{b}
NaCl + EBR (1 µm)	23.23 ± 1.18^{d}	4.50 ± 0.33^{a}	27.68^{cd}	98.90 ± 0.75^{a}
NaCl + EBR (2 µm)	20.00 ± 0.87^{de}	3.28 ± 0.32^{ab}	23.39^{c}	96.65 ± 0.76^{a}
Drought	31.39 ± 1.12^{ab}	2.48 ± 0.13^{cd}	47.10^{ab}	25.20 ± 2.68^{d}
Drought + EBR (1 µm)	27.10 ± 0.60^{c}	3.83 ± 0.15^{ab}	31.90^{c}	45.65 ± 2.80^{b}
Drought + EBR(2µm)	21.94 ± 0.74^{de}	2.70 ± 0.02^{c}	29.03^{cd}	33.13 ± 2.14^{c}
$ZnCl_2$ stress	28.39 ± 1.30^{bc}	1.80 ± 0.14^{d}	49.03^{a}	23.03 ± 1.43^{d}
$ZnCl_2$ + EBR (1 µm)	27.68 ± 1.17^{c}	3.60 ± 0.16^{b}	23.23^{d}	29.03 ± 0.63^{d}
$ZnCl_2$+EBR (2µm)	19.35 ± 0.57^{e}	1.80 ± 0.09^{d}	25.81^{cd}	36.40 ± 2.20^{c}

Values represent mean ± SE (n=3) and means represented by similar alphabets are statistically not different at $P<0.01$.

3.4. Effect on leaf proline content

The change in leaf proline content is an indication of plant tolerance to various stresses. In our study, we found a significant increase in leaf proline content under all the abiotic stresses as compared to plants grown under control conditions (Table 4). The leaf proline content in tomato seedlings germinated under stress treatments further increased with the addition of EBR, where EBR at 2 µM concentration showed greater influence than 2 µM concentration. Similar results were observed under *ex vitro* conditions also suggesting that EBR (2 µM) spray is more effective for inducing stress tolerance in tomato plants grown under salt, drought and heavy metal stresses.

3.5. Effect on nitrate reductase (NR) activity

Nitrate reductase activity showed an abrupt reduction in plants grown under salt, drought or heavy metal stresses. The percentage decrease in NR activity ranged between 50 – 25% under these stresses (Fig. 1). Further inhibitory effect was observed under drought and $ZnCl_2$ stresses. However, EBR Application to germinating condition or foliar application to tomato plants reverted back the inhibited NR activity under stress conditions and among the EBR levels EBR (2 µM) was Found more effective in elevating NR activity.

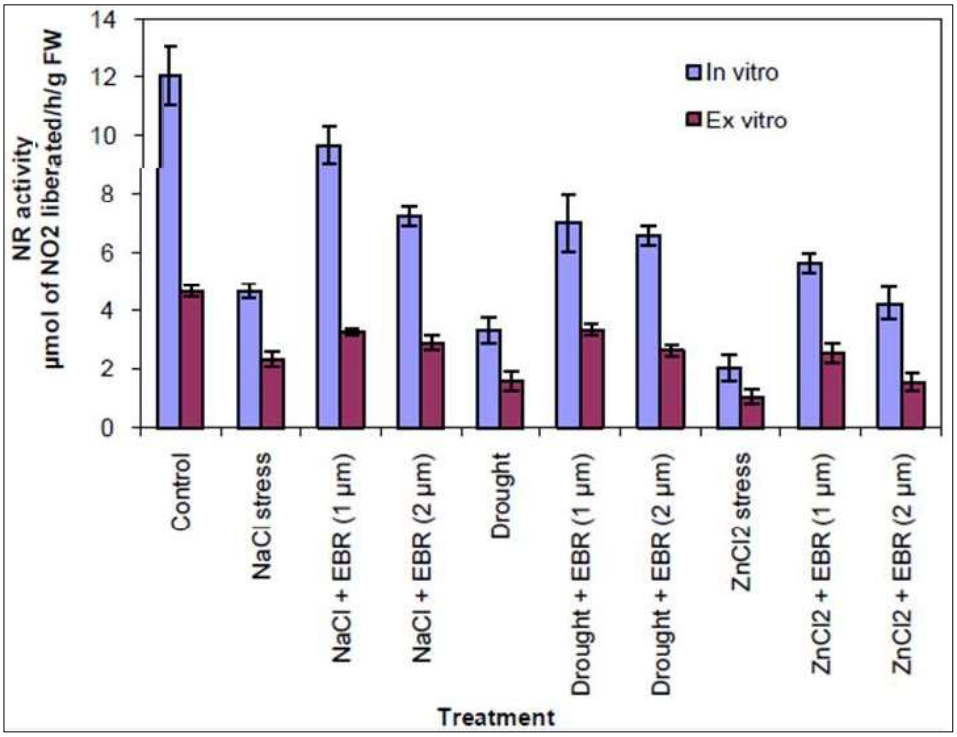

Fig. 1: Effects of EBR on nitrate reductase (NR) activity in tomato seedling and plant grown under NaCl, Drought and ZnCl2 stress. Bars represent mean ± SE (n=3) and are statistically different at $P<0.05$.

3.6. Estimation of antioxidants

Application of EBR substantially enhanced the ascorbic acid (AsA) contents in plants germinated under various abiotic stresses. The endogenous levels of AsA were significantly higher under heavy metal stress as compared to salt and drought stresses (Table 5). Their levels further increased with the addition of EBR to the seed germination medium. Similar results were observed in *ex vitro* studies also. Leaf ascorbic acid content increased by 3 folds (from 8.17 in control to 24.11 mg/ 100g FW) in tomato seedlings grown under drought stress and sprayed with EBR (2 µM). EBR at 2 µM showed greater influence than 1 µM concentration.

Table 5: Effect of 24- epibrassinolide (EBR) on antioxidants level of tomato plants germinated and Grown under salt, drought and $ZnCl_2$ stresses.

	In vitro conditions		Ex vitro conditions	
Treatments	**AsA content (mg/100 g FW)**	**Proline content (µmol/ g FW)**	**AsA content (mg /100 g FW)**	**Proline content (µmol/ g FW)**
Control	6.34± 0.18 ^{e}	0.52 ± 0.029^{f}	8.17^{f}	0.39 ± 0.06^{f}
NaCl stress	7.35 ± 0.18^{de}	0.60 ± 0.29^{de}	11.36^{e}	0.42.00 ± 0.8^{e}
NaCl + EBR (1 µm)	8.42 ± 0.017^{c}	0.60 ± 0.01^{ab}	14..68^{d}	0.85 ± 0.03^{bc}
NaCl + EBR (2 µm)	10.56 ± 0.13^{b}	0.53 ± 0.01^{c}	12.75^{dc}	0.56 ± 0.06^{de}
Drought	6.65 ± 0.09^{e}	0.50± 0.01^{c}	11.37^{e}	0.40 ± 0.02^{e}
Drought + EBR (1 µm)	8.67 ± 0.087^{de}	0.80 ± 0.8^{a}	21.79^{a}	0.43 ± 0.12^{a}
Drought + EBR(2µm)	8.65 ± 0.23^{de}	0.85 ± 0.06^{b}	19.59^{c}	0.65 ± 0.07^{d}
$ZnCl_2$ stress	9.43 ±0.07^{bc}	0.42 ± 0.006^{e}	13.10^{de}	0.67 ±0.01^{c}
$ZnCl_2$ + EBR (1 µm)	11.87 ± 0.22^{a}	0.45 ± 0.03^{cd}	24.11^{b}	0.95 ± 0.09^{b}
$ZnCl_2$+EBR (2µm)	13.98 ± 0.98^{a}	0.44 ± 0.0^{cd}	23.46^{c}	0.65±0.11^{d}

Values represent mean ± SE (n=3) and means represented by similar alphabets are statistically not different at $P<0.01$

3.7. Effect of EBR on total protein content

Total protein isolated from leaves of tomato plants grown under the abiotic stresses showed varied results. As a general trend, the protein contents showed slight to moderate decrease under salt and drought stress conditions and a substantial reduction under $ZnCl_2$ stress. Their levels reverted back to control or elevated over control with the application of 0.2 µM EBR. Under *in vitro* conditions, drought stress caused a 30% reduction in leaf protein content and with the application of EBR (2 µM), the protein content increased by 1.5 folds over drought stress plants (Fig. 2A). Similar trend was observed under *ex vitro* conditions also. Leaf protein content was the maximum (190.28 mg/100 g FW) in drought stressed tomato plants sprayed with EBR (2 µM).

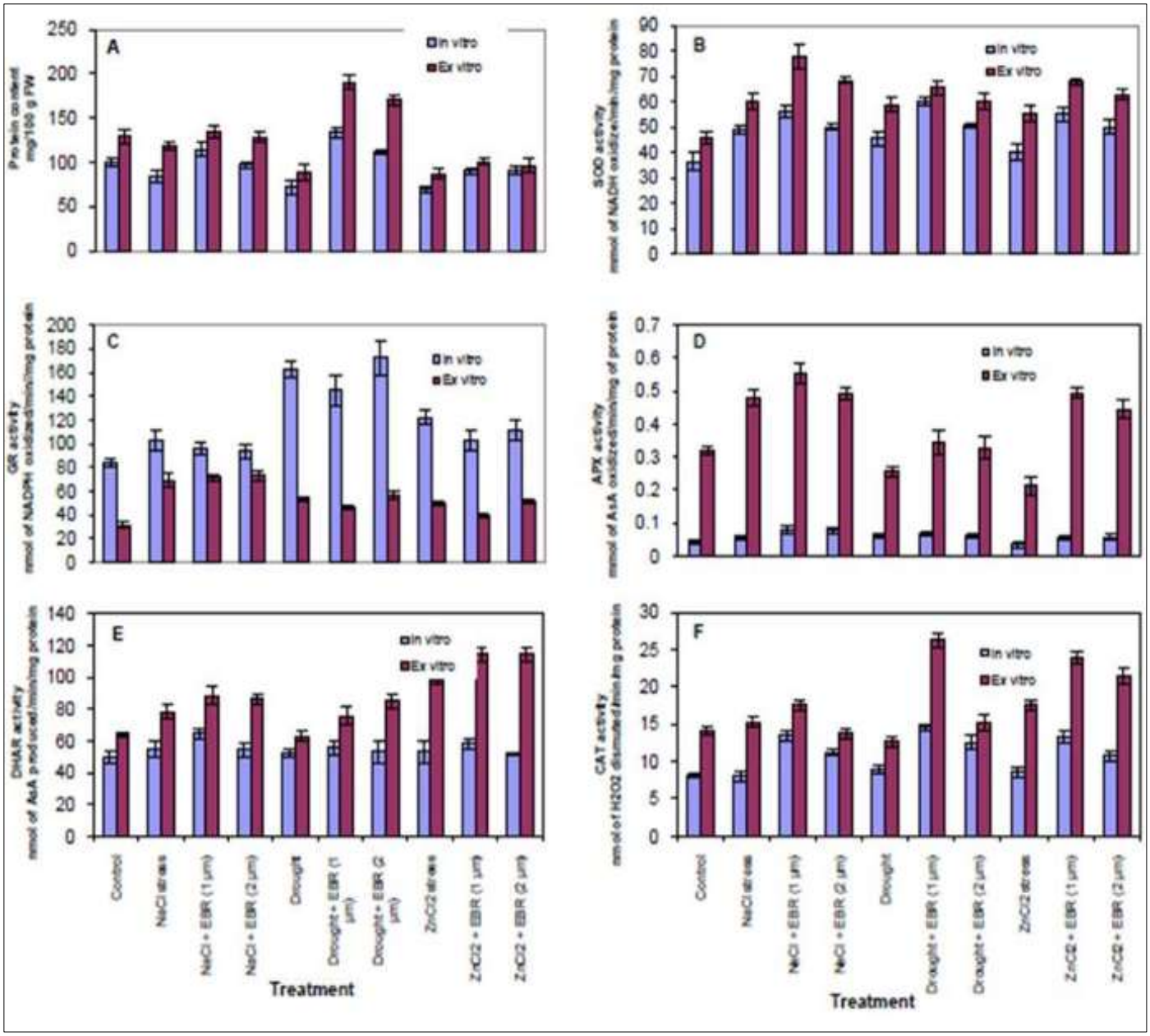

Fig 2: Effects of EBR on protein content (A), SOD (B), GR (C), APX (D), DHAR (E) and CAT (F) activities in leaves of tomato seedlings and plants grown under NaCl, drought and ZnCl2 stresses. Bars represent mean ± SE (n=3) and are statistically different at $P<0.01$.

3.8. Effect on activities of antioxidative enzymes

Activities of antioxidative enzymes (SOD, CAT, GR, APX and DHAR) enhanced in the leaves of tomato plants grown under various abiotic stresses and their activities further increased with application of EBR (1 or 2 µM) (Fig. 2B). Under *in vitro* conditions, the activity of SOD, involved in detoxification of superoxide (O_2^-) radical, enhanced from 36.4 mmol of NADH oxidized/min/mg protein in untreated control to 48.9, 45.5 and 40.5 mmol NADH of oxidized/min/mg protein in salt, drought and ZnCl2 stresses, respectively. The SOD activity further increased with the addition of EBR to the liquid medium used for seed germination. Similar results were observed in case of *ex vitro* studies also. The highest activity of SOD (78.2 mmol of NADH oxidized/min/mg protein) was observed in the leaves grown under salt stress and sprayed with 2 µM EBR. The concentration of EBR at 2 µM showed a greater influence on SOD activity than 1 or 3 µM concentrations. The activity of catalase, involved in detoxification of hydrogen peroxide radials, showed a slight to moderate increase under salt, drought and ZnCl2 stresses under *in vitro* and *ex vitro* conditions (Fig. 2C). CAT activity increased further with the application of EBR to the growth medium used for seed germination or spraying of EBR to the plants growing under these stresses. Highest CAT activity was produced by EBR (1 µM) both under *in vitro* and *ex vitro* conditions. 2 µM EBR could not show further increase in the CAT activity.

The activities of the two enzymes involved in ascorbate-glutathione cycle, APX and GR showed an increasing trend with the imposition of stress and their activities further increased with the application of EBR both under *ex vitro* and *in vitro* conditions (Fig. 2D). In the present study, there was an increase in the activity of APX activity from 0.042 mmol of AsA oxidized/min/mg protein (untreated control) to 0.061 mmol of AsA oxidized/min/mg protein (drought stress) under

in vitro conditions. The APX activity further increased to 0.070 mmol of AsA oxidized/min/mg protein with application of EBR (1 µM), whereas highest activity of APX (0.078 mmol of AsA oxidized/min/mg protein) was displayed by leaves of tomato seedlings germinated on salt stress and supplemented with 2 µM EBR. Similarly the highest activity of APX (0.552 mmol of AsA oxidized/min/mg protein) was displayed by leaves of tomato plants grown under salt stress and sprayed with 2 µM EBR. Glutathione reductase activity increased from 83.09 nmol of NADPH oxidized/min/mg protein in untreated control seedlings to 102.84,162.54 and 122.07 nmol of NADPH oxidized/min/mg protein under salt, drought and $ZnCl_2$ stresses, respectively, under *in vitro* conditions (Fig. 2E) while the supplementation of EBR did not show any significant effect on GR activity. Similar results were observed under *ex vitro* studies. The corresponding GR activities were increased by 1.2, 2.0 and 1.5 fold in leaves of tomato plants grown under salt, drought and $ZnCl_2$ stresses, respectively, over control.

The other enzyme involved in the ascorbate- glutathione cycle in detoxification of toxic oxygen species is DHAR. The activity of DHAR was enhanced considerably with the imposition of stress. DHAR activity showed an increasing trend from 49.82 nmol of AsA produced/min/mg protein in leaves of control seedlings to 55.07 nmol of AsA produced/min/mg protein in salt stress (Fig. 2F) and with the addition of EBR to seed germination medium further increased the DHAR activity in the leaves of tomato seedlings. Under *ex vitro* conditions, DHAR activity in leaves of tomato plants showed slight to moderate increase under salt and drought stresses while $ZnCl_2$ stress caused a significant increase in DHAR activity. Further, with the spraying of leaves with EBR significantly increased the DHAR activity in leaves of tomato with no significant difference between the two EBR concentrations. The highest activity of DHAR (114.43 nmol of AsA produced/min/mg protein) was observed in leaves of tomato plants grown under $ZnCl_2$ stress and leaves sprayed with EBR (2 µM). The results on quantitative real-time PCR of genes encoding these antioxidative enzymes showed similar trends showing the uniformity of results between transcript expression levels and enzyme activities (data not shown).

3.9. Effect on Endogenous Levels of IAA and ABA

The endogenous plant hormones, IAA and ABA were estimated in leaves of tomato grown under various abiotic stresses and EBR treatments. Under *in vitro* conditions, the free IAA in leaves of tomato were drastically reduced under all the stresses (Fig. 3A), and their level restored nearly to the control values with the supplementation of EBR with no significant differences between EBR concentrations (1 or 2 µM). Under *ex vitro* conditions, free IAA level decreased from 5.4 mg/100 g FW in control to 1.9, 2.3 and 1.4 mg/100 g FW under salt, drought and $ZnCl_2$ stresses, respectively, and restored up to 65% of control values with the application of EBR. Free ABA level increased under all stress conditions and their level further increased with the application of EBR (Fig. 3B). Among the treatments, soluble ABA was found to be maximum (21.7 mg/100 g FW) in leaves of tomato grown under drought stress and sprayed with EBR (2 µM). Under all the stress conditions, EBR at 2 µM induced higher ABA levels than 1 or 3 µM concentration suggesting its more significant effect on stress Tolerance to these stresses.

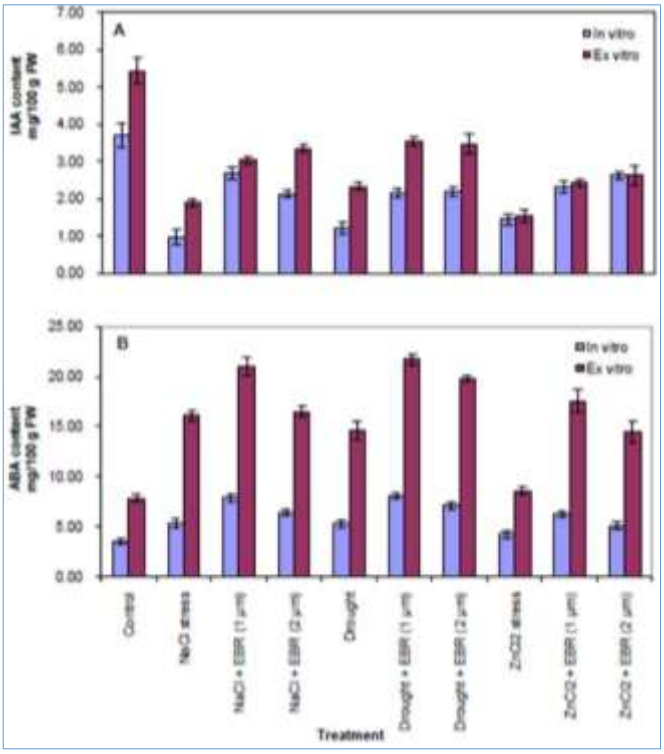

Fig 3: Effect of EBR on endogenous levels of free IAA (A) and ABA (B) contents in leaves of tomato seedlings and plants grown under NaCl, drought and ZnCl2 stresses. Bars represent mean ± SE (n=3) and are statistically different at $P<0.01$.

4. Discussion

Environmental stresses such as salinity, drought and heavy metal toxicity are the major abiotic factors limiting the crop production. Endogenous plant hormones such as BR, IAA, ABA and other hormones play important role in conferring tolerance to these stresses. In this study, it was observed that the growth pattern, plant length, total leaf area, fresh and dry weight, relative water content of the tomato decreased gradually under different abiotic stresses (Table 2 and 3). Decrease in dry weight could be attributed to reduction in photosynthesis, rate of leaf expansion and total leaf area. Reduction in relative water content of the plants growing under heavy metal stress is possibly an impact of the metal ions on the plasma membrane electrical potential which affect the absorption of ions (Harnandez *et al*, 1996, Zhang *et al*, 2005) [31, 75]. the reby creating physiological drought (Barcelo *et al*, 1990) [11]. Leaf chlorophyll content also showed a decreasing pattern with the presence of stress and it reverted back to almost normal with the application of EBR (Table 2 and 3). Increase in total leaf chlorophyll content with the application of EBR in the present study could be attributed to the removal of inhibitory effect caused by stress condition. Further, the growth promotion by EBR treatment is associated with increase in leaf chlorophyll content (Yu *et al*, 2004) [72]. as observed in beans (Krizek *et al*, 1983) [49]. and triticale (Kalituho *et al*, 1996) [41]. In addition, the increase in chlorophyll content could also be achieved via protection of thylakoid membranes by presence of proline which reduces negative effect of reactive oxygen species (ROS) (Kavi Kishor *et al*, 2005, Fariduddin *et al* 2012, 2014) [43, 73, 24]. Level of proline in tomato exposed to different stress was higher than those of control plants and their level further enhanced with the application of EBR, which could be

attributed to activation of proline biosynthesis enzymes with EBR treatment. Our findings are in agreement with earlier observations in *Brassica juncea* L. (Fariduddin *et al*, 2009, 2011, 2014) [22, 23, 24, 25]. Proline is reported to act as a protective osmolyte (Hartzendorf *et al*, 2001) [33], membrane stabilizer (Bandurska *et al*, 2001) [10]. And ROS scavenger in plants under stress (Matysik *et al*, 2002, Yusuf *et al*, 2012) [53, 73]. In general, ROS accumulation in plants caused by abiotic stress can result in considerable damage to the membrane lipids and proteins (Shalata *et al* 2001; Apel *et al*, 2004) [64, 7]. A reduction in membrane permeability during abiotic stress is partially due to the peroxidation of polyunsaturated fatty acids in the membranes resulting in the formation of MDA; therefore, its content was also measured as an indicator of lipid peroxidation. In our study, the MDA content in leaves showed enhanced pattern under stress conditions, indicating increased lipid peroxidation and its level decreased to almost normal with the application of EBR indicating the favorable role of EBR in reducing lipid peroxidation. Similar to our results EBR treatment could decrease the lipid peroxidation induced by cadmium toxicity in radish (Anuradha *et al*, 2007) [5], drought stress in soybean and spinach (Robinson *et al*, 2000) [61].

The activity of nitrate reductase (NR) is a measure of the habitat-dependent nitrate utilization of plants (Larcher *et al*, 1995) [51]. The reduced NR activity in the plants growing under salt and heavy metal stress is attributed to inhibition of nitrate transport to the shoot, interference with nitrate uptake and xylem loading (Cramer *et al*, 1995) [18]. NR enzyme partially represents a proportion of leaf protein (Calza *et al*, 1987) [15]. hence, its activity plays a pivotal role in N_2 supply, growth and productivity of plants (Srivastava *et al*, 1995) [66]. Our study demonstrated the ability of EBR, given as a foliar spray, to reduce the negative impact of salt and heavy metal stress on NR enzyme activity in tomato. Similar to our studies, application of BRs enhanced the NR activity and growth of the rice plants under salt stress (Anuradha *et al*, 2003; Fariduddin *et al*, 2009; Yusuf *et al*, 2012) [4, 22, 23, 73].

The ROS such as O_2^-, H_2O_2 and ^-OH produced in the plants under various stresses, oxidizes proteins, lipids and nucleic acids leading to mutation at the cellular level (Asada *et al*, 1987; Halliwell *et al*, 1999; Millar *et al*, 2010) [9, 30, 54]. However, in order to neutralize the toxicity of ROS, plants have evolved endogenous system of antioxidative enzymes system (e.g. catalase, peroxidases, superoxide dismutases and glutathione reductase) and metabolites (e.g. ascorbate, glutathione and tocopherols). The physiological stress caused by salt, drought and heavy metal in the present study, resulted in the increase in the level of AsA and GSH apart from proline (Table 5) and that of all the enzymes in antioxidant system (Fig. 2) to boost the tolerance capacity of the tomato to overcome the deleterious effects of these stresses. Neutralization of ROS by glutathione (Schafer *et al*, 2001) [63]. AsA and proline is the primary method of metal detoxification (Shao *et al*, 2008; Andre et al, 2010) [65, 3]. The increase in the level of the antioxidative enzymes as antioxidants with the application of EBR in present study corroborates earlier reports on cucumber seedlings subjected to hypoxia stress (Kang *et al*, 2007) [42]. chickpea grown under cadmium stress (Hasan *et al*, 2008) [34, 2] and *Vigna radiate* plants grown under salt stress (Fariduddin *et al*, 2009; Hayat *et al*, 2010,) [22, 23, 35]. These results suggested that EBR could play a positive role in the protection of plant cells against abiotic stress induced oxidative damage partially through scavenging the ROS and via increasing the activities of antioxidative enzymes. The enhanced activities of antioxidative enzymes seem to be the result of *denovo* synthesis and/or activation of the enzymes, mediated through transcription and/or translation of specific genes (Ali *et al*, 2008) [34, 2]. In the present study, EBR treatment enhanced the ABA content in tomato plants grown under salt, drought or heavy metal stresses and its content increased further with the application of EBR, both, under *in vitro* and *ex vitro* conditions (Fig. 3). Same results with similar findings in *Brassica napus* plants grown under high temperature stress (Kurepin *et al*, 2008) [50]. radish seedlings subjected to copper stress (Choudhary *et al*, 2010) [17]. And tomato seedlings grown under drought (Yuan *et al*, 2010) [71]. It is well established that drought stress induces the accumulation of ABA in the leaves (Davies *et al*1991) [19]. Which acts as a biochemical adaptation to reduce the water loss during scarcity. ABA is a key factor in controlling downstream responses essential for adaptation to the stresses and these responses are controlled by a complex web of signaling events such as proline accumulation and level of ROS (Pastori *et al*, 2002) [58]. Enhanced levels of ABA have been shown to increase metalothionenins, and thereby stress management ability against Cu and Zn stress as observed in *P. juliflora* (Usha *et al*, 2009) [68]. However, a significant decrease in free IAA was also recorded in salt, drought or heavy metal stressed plants when compared with controls. Further, EBR treatment restored the contents of free IAA content up to 65% of their control value. The improved growth of EBR treated plants under various abiotic stresses in the present investigation may be due to enhanced biosynthesis of IAA similar to earlier reports in radish seedlings grown under copper stress (Choudhary *et al*, 2010) [17]. Auxins have also been reported to ameliorate the inhibitory effects of salinity, drought (Farooq *et al*, 2009) [26]. Heat and heavy metal stress (Dimpka *et al*, 2008) [20]. In plants. The stress protective role of auxins in the restoration of cambial root growth under Hg^{2+} stress has also been observed by Khan and Chaudhry (2006) [46]. in *Luffa cylindrica*. The interactions of BRs with auxins have been shown to improve the stress management efficacy of plants (Hasan *et al*, 2008) [34, 2] and their co-application has shown a synergistic effects in the promotion of hypocotyls elongation in *Arabidopsis thaliana* (Tanaka *et al*, 2003) [67]. Recently, it was found that interactions between ABA and BR signals in *Arabidopsis* occur at a platform called ARF2 (auxin response factor - an integration point for auxin and BR promoters), a transcription factor that responds to auxin, could be regulated by BR insensitive (BIN2), a negative regulator of BR signaling (Zhang *et al*, 2009) [74]. These earlier reports and the findings from the present study could advance our understanding about the stress mitigation role of EBRs via auxins.

In conclusion, the data represent study gives a clear impression that the presence of salt, drought or heavy metal stress adversely affect growth and metabolism of tomato plant under *in vitro* and *ex vitro* conditions. On the other hand, EBR application has an added effect on the general metabolism and the tolerance capacity of the plants to overcome the toxic effect of these stresses. The enhanced tolerance in tomato plants to a variety of abiotic stresses upon EBR treatment involves changes in the expression of genes encoding regulatory proteins and proteins in antioxidative enzymes. Findings in our study also revealed the interaction of EBR with IAA, ABA and other stress-related parameters for ameliorating various abiotic stresses. On one

hand, EBR could increase the endogenous levels of IAA to restore plant growth, whereas on the other hand it enhances the biosynthesis of ABA to improve the plant tolerance to different abiotic stresses. Among the two concentrations of EBR, the 2 µM concentration was found to be superior in alleviating the inhibitory effects of abiotic stress in tomato plants. As per our knowledge, this is the first comprehensive report on the influence of EBR on alleviating abiotic stress tolerance in tomato plants under both *in vitro* and *ex vitro* conditions. Studies aiming at characterization of global gene expression changes will extend our understanding of how EBR improves tolerance in plants to a wide range of environmental stresses.

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