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CADMIUM TOXICITY IS DIMINISHED BY 24-EPIBRASSINOLIDE IN SEEDLINGS OF *TRIGONELLA FOENUM-GRAECUM* L.

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Summary. The effects of 24-epibrassinolide (EBL) on seed germination, seedling growth, lipid peroxidation and antioxidative enzyme activities in *Trigonella foenum-graecum* L. seedlings exposed to Cd at a concentration of 10 mM were studied. The activities of superoxide dismutase (SOD, E.C. 1.15.1.1), guaicol peroxidase (GPOX, E.C. 1.11.1.7), catalase (CAT, E.C.1.11.1.6) and ascorbate peroxidase (APOX, E.C.1.11.1.11) were analyzed in 7-day-old seedlings. It was observed that EBL treatments reduced considerably the toxicity of Cd on seedling growth. Lipid peroxidation level was significantly increased under Cd stress condition, but lowered after EBL application revealing less oxidative damage. Further, an enhancement in the activities of proline, CAT and APOX was observed. Supplementation of EBL to seedlings exposed to Cd stress was associated also with an increase in glutathione reductase (GR) activity and total glutathione content (GSH+GSSG) in the seedlings. It can be concluded that EBL could play a positive role in alleviation of oxidative damage caused by reactive oxygen species (ROS) overproduction through enhancing the activity of the antioxidant defense system, resulting in improving the tolerance of *T. foenum-graecum* seedlings to Cd stress.

Keywords: Antioxidative enzymes; 24-epibrassinolide; lipid peroxidation; *Trigonella foenum-graecum* L. seeds.

INTRODUCTION

Increasing environmental pollution with heavy metals has sharpened focus to their impact on various organisms including plants. Among non-nutrient heavy metals, Cd is the most widespread that results from human economic activities like burning fuels, smelting and soil-applied chemicals including fertilizers. Cd is a particularly dangerous environmental pollutant due to its relatively high solubility and mobility in soil. Extremely toxic even at very low doses (Clemens 2006), it is easily taken up and accumulated by plants. When bound to the cell surface and also within the

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cell, Cd ions interact with the functional groups of proteins, nucleic acids and polysaccharides and substitute other metal ions already bound to these functional groups that can lead to various metabolic disorders and reduction in growth (Seregin and Ivanov, 2001). Additionally, heavy metals also cause oxidative damage due to the production of reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). In plant systems ROS are removed by the antioxidative defense system comprising important enzymes like superoxide dismutase (SOD), guaiacol peroxidase (GPOX), ascorbate peroxidase (APOX), catalase (CAT) (Sharma and Dietz, 2009).

Several plant hormones have been reported to modulate plant responses to oxidative stress generated by abiotic stresses (Cao et al., 2005). Brassinosteroids (BRs) are a new group of naturally occurring phytohormones with a significant growth promoting activity, which are essential for many processes in plant growth and development (Rao et al., 2002; Sasse, 2003; Ren et al., 2009; Tanaka et al., 2009; Swamy and Rao 2011). Brassinosteroids have demonstrated a wide spectrum of physiological roles in plants that include stem elongation, pollen tube growth, leaf bending, xylem differentiation and regulation of gene expression (Khripach et al., 2000). Apart from plant growth and development BRs are also reported to confer resistance to plants against various abiotic and biotic stresses (Vardhini et al. 2006) and regulate antioxidative enzyme activities in plants. With stress-protective properties, it becomes important to study the role of BRs in the oxidative defense system of plants.

T. foenum-graecum is a legume crop grown in temperate regions of India usually on marginal lands which are contaminated to varying extents with heavy metals due to industrial activities. As forage it lowers the lipoprotein cholesterol density in serum and possess anti-diabetic and proved hepatoprotective effects in experimental models (Basch et al., 2003; Thirunavukkarasu et al., 2003). Recently, *T. foenum-graecum* seeds have been experimentally shown to protect against breast cancer (Amin et al., 2005). *T. foenum-graecum* seeds are used as a remedy for obesity and proved in Zucker obese rats (Raju et al., 2006).

In view of the adverse effects of heavy metals on growth and development of plants, the present study was carried out to investigate the influence of EBL on seedling growth and antioxidant system in *T. foenum-graecum* seedlings exposed to Cd stress.

MATERIAL AND METHODS

Chemicals and plant material

24-epibrassinolide was procured from CID Technologies Inc., Brampton, Ontario, Canada. Seeds of *T. foenum-graecum* L. were obtained from the National Seeds Corporation, Hyderabad, India.

Seed germination

Seeds were surface sterilized with dilute sodium hypochlorite solution (0.5%, v/v) and washed thoroughly with sterile distilled water. They were soaked for 24 h in: (i) distilled water (control), (ii) 10 mM Cd^{+2} ($CdCl_2$), and (iii) 10 mM Cd^{+2} supplemented with 0.5, 1.0 and 2.0 µM EBL. For each treatment 5 replicates

(Petri dishes), each with 20 seeds were maintained. The seeds were allowed to germinate in the dark at 25±1°C, and the number of seeds germinated was recorded. After 48 h, five seedlings were retained in each plate, and 5 ml of treatment solution was added to the respective Petri dishes. On the 5th day 5 seedlings were selected randomly from each Petri dish and length (hypocotyl), fresh weight and dry weight were recorded. On the 7th day proline content, activities of antioxidant enzymes (CAT, POD, SOD, APOX, GPOX, GR), total glutathione content, AAO together with lipid peroxidation were assayed.

Free proline

Proline was extracted with 3% sulphosalicylic acid, and determined according to Bates et al. (1973). It was determined in the supernatant by measuring the absorbance of the proline ninhydrin product formed at 520 nm using toluene as a solvent.

Extraction and assay of antioxidant enzymes

The seedlings were ground in sodium phosphate buffer at pH 7.0 for CAT, POD, APOX, GPX and at pH 7.8 for SOD. The supernatant was used to measure the activity of the enzymes, and the protein content in the supernatant was determined according to Lowry et al. (1951).

Catalase (EC 1.11.1.6)

Catalase activity was assayed by the method of Barber (1980). The enzyme extract (0.5 ml) was added to 2.0 ml of hydrogen peroxide and 3.5 ml of phosphate buffer (pH 7.0). The reaction was stopped by adding 10 ml of 2% (v/v) concentrated sulphuric acid, and the residual hydrogen peroxide was titrated against 0.01 M $KMnO_4$ until a faint purple color persisted for at least 15 seconds. The activity of the enzyme was expressed as enzyme units.

Peroxidase (EC 1.11.1.7)

Peroxidase activity was assayed adopting the method of Kar and Mishra (1976). To 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7.0), 1.0 ml of 0.01 M pyrogallol and 1.0 ml of 0.005 M H_2O_2 were added. After incubation, the reaction was stopped by adding 1.0 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The enzyme activity was expressed in absorbance units.

Ascorbate peroxidase (EC 1.11.1.11)

The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 250 mM H_2O_2 and 50 µg of protein. The activity of APOX was determined spectrophotometrically by measuring the rate of ascorbate oxidation at 290 nm for 1 min. The amount of ascorbate was calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1} by the method of Nakano and Asada (1981).

Guaiacol peroxidase (E.C 1.11.1.7)

GPOX was measured by the method of Mazhoudi et al. (1997). The reaction mixture contained 50 mM phosphate buffer, 0.2 mM guaiacol, 10 mM H_2O_2 and distilled water in a total volume of 3.0 ml. The reaction was started by adding 50 µg of protein. The change in absorbance of one unit per min at 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) gave the activity of GPOX, and it was expressed in enzyme units.

Superoxide dismutase (EC 1.15. 1.1)

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium adopting the method of Beauchamp and Fridovich (1973). The reaction mixture (3.0 ml) contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA, 0.1 ml of enzyme extract and 2 µM riboflavin. After mixing the contents, test tubes were shaken and placed 30cm below light source consisting of two 20 W fluorescent lamps for 15 min. A tube with protein kept in the dark served as a blank, while the control tube was without the enzyme and kept in the light. The absorbance was measured at 540 nm. The activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction in light with protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

Glutathione reductase (GR, E.C. 1.6.4.2)

The assay was performed according to Jiang and Zhang (2001). 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 started by the addition of protein and NADPH oxidation was recorded as the decrease in absorbance at 340 nm for 1 min. The activity was calculated using the extinction coefficient for NADPH ε= 6.22 mM^{-1} cm^{-1}.

Cellular glutathione content

Total glutalthione [GSH (reduced) + GSSG (oxidized)] content was estimated according to the method of Hissin and Hilf (1976). One gram of seedlings was homogenized with 10 ml Tris-EDTA (pH 8.2) and centrifuged at 25,000 rpm for 30 min at 4°C. From the homogenate about 0.3 ml (300 µl) was pipetted into 1 ml of tube to which 60 µl of 25% phosphoric acid was added and kept in ice for 5 min, then centrifuged at 25,000 rpm for 30 min at 4°C. The supernatant was collected for the estimation of GSH and GSSG. For reduced glutathione (GSH) estimation, to 50 µl of supernatant, 0.45 ml of cold phosphate EDTA buffer (pH 8) was added and mixed thoroughly. Aliquots of 25 and 50 µl were taken into 5 ml test tubes and made up to 100 µl with cold glass distilled water. 1.8 ml of phosphate EDTA buffer was added to the tubes and mixed. 100 µl of OPT (ortho-phthalaldehyde) solution was then added and after thorough mixing, incubated at room temperature (25°C) for 15 min. Fluorescence was measured using a JASCO, FP-750 spectrofluorometer at wavelengths of 350 nm and 420 nm. For oxidized glutathione (GSSG) estimation, an aliquot of 50 µl was incubated at room temperature (25°C) with 20 µl of NEM reagent for 30 min. To the above mixture 0.43 ml of 0.1 N NaOH was added and mixed thoroughly. From this 50 and 100 µl aliquots were taken into 5 ml test tubes and made up to 100 µl with glass distilled water. 100 µl of OPT was added to this mixture and 1.8 ml of 0.1 N NAOH was added and incubated at room temperature for 15 min. After incubation, fluorescence was measured with a JASCO, FP-750 spectrofluorometer at 350 nm and 420 nm.

Ascorbic acid oxidase (EC 1.10.3.3)

Ascorbic acid oxidase was extracted and assayed following the method of Povolotskaya and Sedenka (1956)

as modified by Gopalachari (1963). Seedlings (200 mg) were homogenized with cold phosphate citrate buffer (pH 5.0) and filtered through glass wool. The filtrate was taken as the enzyme extract for the assay of the ascorbic acid oxidase. 2.5 ml of phosphate citrate buffer (pH 5.0) and 0.2% ascorbic acid were added to 4 ml of the enzyme extract, and they were kept for 30 min with occasional shaking. 1.0 ml of 10% (v/v) trichloroacetic acid was added to stop the enzyme activity after 30 min.1.0 ml of the above solution diluted with 5.0 ml of distilled water was titrated against 0.001 N 2,6-dichlorophenol indophenol till a permanent pink color was obtained. The ascorbic acid oxidase activity was calculated using the formula:

$$A=V/W \times 0.088 \times 10 \times 25/4 = 5.5V/W$$

Where: A – ascorbic acid oxidase activity; V – difference in titre values between the control and treatment in 1.0 ml; W – fresh weight [g] of plant material.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde content following the method of Heath and Packer (1968). Seedlings (1.0 g) were homogenized with 3 ml of 0.5% TBA in 20% TCA (v/v). The homogenate was incubated at 95°C for 30 min and the reaction was stopped in ice. The samples were centrifuged at 10,000×*g* for 5 min and the absorbance of the resulting supernatant was recorded at 532 nm and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of malondialdehyde was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

The data represent the mean values of 5 replicates. The data were analyzed by one-way ANOVA, followed by Post Hoc Test (Multiple Comparisons). The differences were considered significant when $P \leq 0.05$. The mean values were compared and lower case letters were used in the tables and figures to show the significant differences between the treatments.

RESULTS AND DISCUSSION

There was a considerable reduction in seed germination in response to Cd stress when compared to unstressed control. The supplementation with EBL reduced the inhibitory effect of metal stress on seed germination and enhanced seed germination. The inhibitory effect of metal stress on seed germination was reduced with increasing the concentrations of EBL (Table 1). Similarly, stimulation of seed germination by BRs was reported in response to water stress (Vardhini and Rao, 2005) and salinity stress (Anuradha and Rao, 2002). However, application of EBL resulted in stimulation of seed germination under metal stress.

In the present study, there was a considerable decrease in seedling growth when subjected to Cd stress (Table 2). Earlier, Li et al. (2005) reported that seedling growth of *Arabidopsis thaliana* was more sensitive to heavy metal. Similarly, heavy metal toxicity was reduced by the application of 24-epibrassinolide in 7-day-old seedlings of *Brassica juncea* (Sharma and Bharadwaj, 2007). Thus, BRs help in alleviating the suppression of growth caused by the heavy metal stress by increasing seedling length,

Table 1. Effect of 24-epibrassinolide on the percentage of germination of *T. foenum-graecum* seeds exposed to Cd stress.

Treatments	12 h	24 h
Control	53.2±3.1[a]	83.6±2.9[a]
Cd 10 mM	24.7±4.3[e]	39.1±3.6[e]
Cd 10 mM + EBL 0.5 µM	38.1±2.7[d]	52.8±6.2[d]
Cd 10 mM + EBL 1.0 µM	45.2±3.3[c]	65.3±4.1[c]
Cd 10 mM + EBL 2.0 µM	50.8±5.4[ab]	74.2±2.3[b]

Table 2. Effect of 24-epibrassinolide on the growth of *T. foenum-graecum* seedlings exposed to Cd stress.

Treatments	Seedling growth [cm]	Seedling fresh weight [mg]	Seedling dry weight [mg]
Control	5.8±0.6[a]	0.082±0.004[a]	0.013±0.001[a]
Cd 10 mM	2.6±0.8[e]	0.049±0.001[d]	0.008±0.002[d]
Cd 10 mM + EBL 0.5 µM	4.3±0.2[d]	0.054±0.005[d]	0.008±0.001[d]
Cd 10 mM + EBL 1.0 µM	4.8±0.5[c]	0.063±0.002[c]	0.009±0.001[c]
Cd 10 mM + EBL 2.0 µM	5.5±0.3[b]	0.076±0.008[b]	0.011±0.002[b]

seedling fresh and dry weight. The above findings clearly demonstrated the ability of EBL to alleviate Cd stress both on seed germination and seedling growth.

Cd stress increased free proline content in *T. foenum-graecum* seedlings when compared to control seeds. The seedlings exposed to heavy metal stress further supplemented with EBL had higher proline content as compared to the control (Fig. 1). Proline is a proteinogenic amino acid and functions as an osmolyte, free radical scavenger, electron sink stabilizer of macro molecules and cell wall components (Matysik et al., 2002). Proline is involved in plant heavy metal stress by different mechanisms, in osmo and redox regulation, metal chelation and scavenging of free radicals. Free proline content correlated positively with Cu tolerance of the lichen photobiont *Trebouxia eriei* (Backor et al.2004). Similarly, proline was suggested to act as an antioxidant in Cd treated cells; consequently increased GSH levels and favored enhanced phytochelatin synthesis and sequestration of Cd (Siripornadulsi et al., 2002). In the present study, the elevated levels of free proline might have protected the seedlings from the injurious impact of Cd stress.

Cd decreased CAT activity and increased POD activity in *T. foenum-graecum* seedlings (Fig. 2, A, B). Both CAT and POD are important oxidative enzymes. CAT ensures the removal of H_2O_2, thus providing free O_2 and detoxifying harmful metabolic products

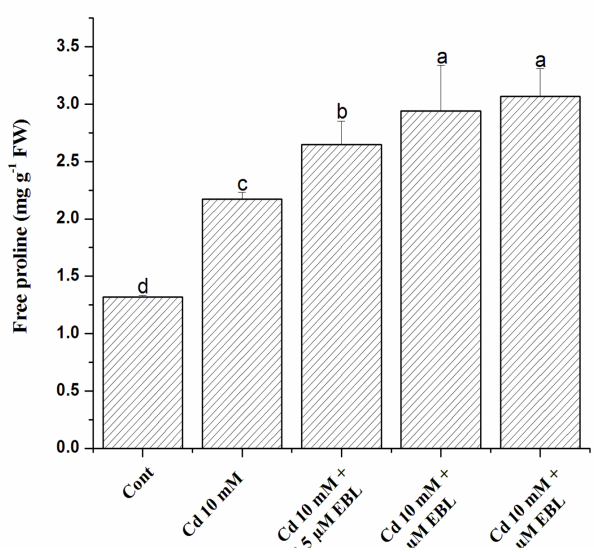

Fig. 1. Effect of 24-epibrassinolide on free proline levels in *T. foenum-graecum* seedlings under Cd stress. Different letters on the top of bars denote significant differences at $p \leq 0.05$ according to Post Hoc Test.

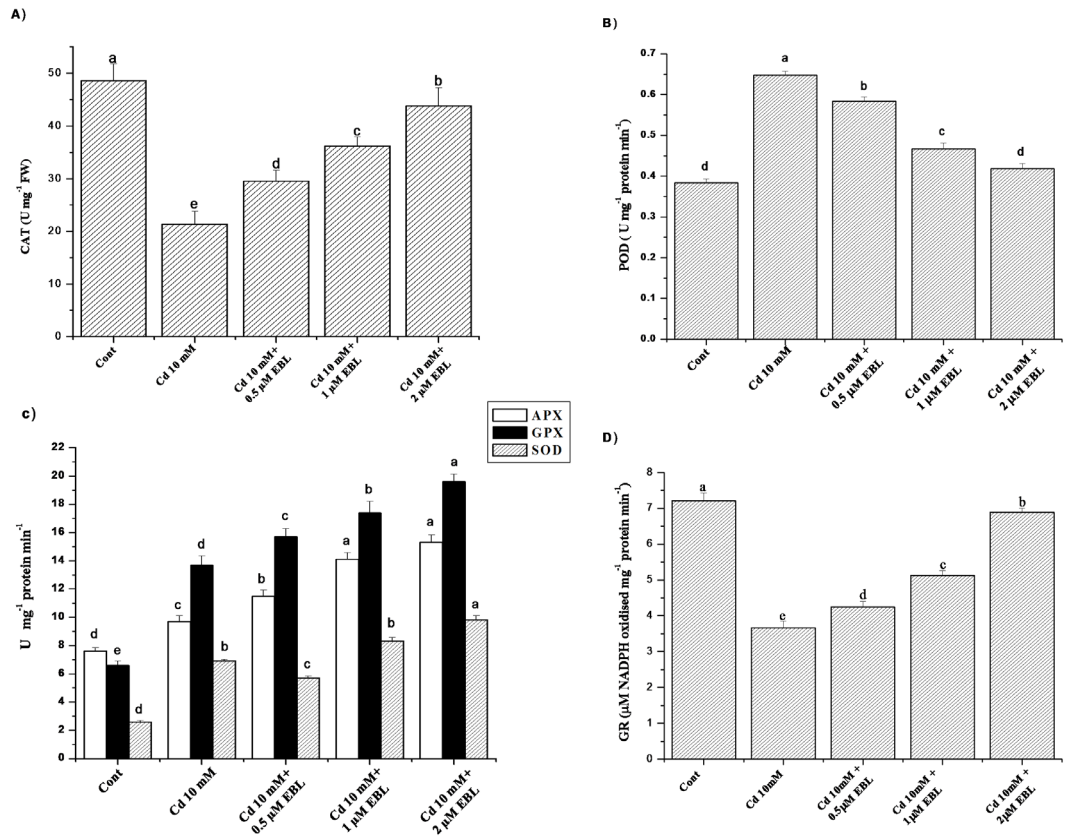

Fig. 2. Effect of 24-epibrassinolide on the activity of catalase (A), peroxidase (B), ascorbate peroxidase, guaiacol peroxidase, superoxidase dismutase (C) and glutathione reductase (D) in *T. foenum-graecum* seedlings under Cd stress. Different letters on the top of bars denote significant differences at $p \leq 0.05$ according to Post Hoc Test.

(Burris, 1960). POD is also known to play a significant role in oxidative stress conditions. The mode of action of POD on H_2O_2 substrate differs from CAT in that POD liberates free radicals rather than oxygen. CAT may scavenge H_2O_2 formed by POD. Treatments with Cd decreased CAT activity and increased POD activity, thus increasing superoxide radicals and hydrogen peroxide levels. A similar decrease in CAT activity and increase in POD activity was reported in wheat leaves under heavy metal stress (Panda et al., 2003). Mishra and Choudhuri (1999) reported that exogenous application of salicylic acid increased CAT activity and decreased POD activity in rice plants under Hg stress. However, seed treatment with EBL significantly increased CAT activity and decreased POD activity in *T. foenum-graecum* under Cd stress.

The activities of APOX and GPOX increased under Cd stress in *T. foenum-graecum* seedlings. The activities of APOX and GPOX increased in response to H_2O_2 in the cell (Morita et al.1999). Supplementation of BRs further enhanced the APOX and GPOX activity in *T. foenum-graecum* seedlings (Fig. 2, C). A similar increase in APOX activity was induced by salt stress in cotton callus tissue (Bellaire et al., 2000). Similarly, polyamines were found to be associated with increased APOX activity in *Virginia* pine in reducing the salt induced oxidative damage (Tang and Newton, 2005). Further, spraying with spermidine increased the activity of APOX and GPOX in *Typha latifolia* L. under Cd stress (Tang et al., 2005).

Total glutathione content of radish seedlings was affected by Cd stress. A significant decrease in the GSH/GSSG ratio was observed in the stressed seedlings (Fig. 3, A). A similar variation was the consequence of the decrease in the GSH content and the increase in GSSG caused by Cd. GSH is an important non-enzymatic antioxidant involved in quenching ROS via ascorbate-glutathione cycle. Under heavy metal stress GSH is converted to its oxidized form GSSG. The cellular GSH pool is maintained by GR which is

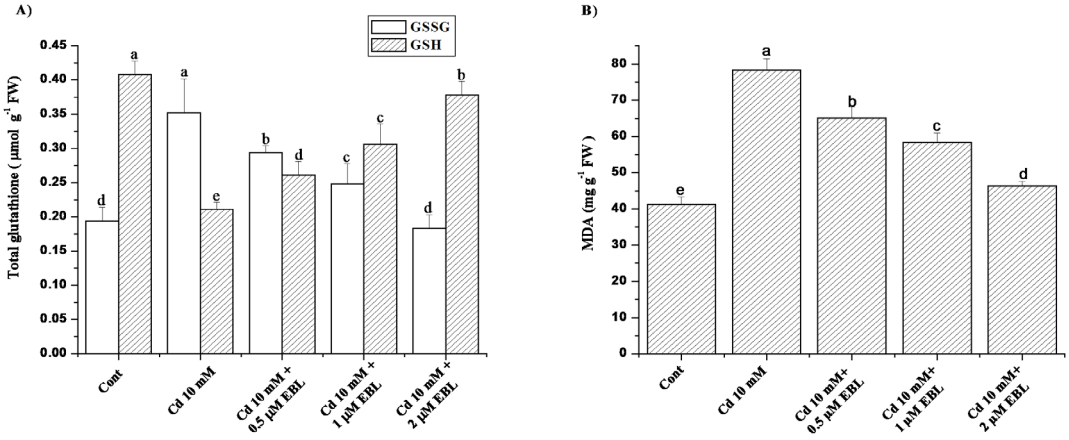

Fig. 3. Effect of 24-epibrassinolide on total glutathione content (A) and malondialdehyde (MDA) content (B) in *T. foenum-graecum* seedlings under Cd stress. Different letters on the top of bars denote significant differences at $p \leq 0.05$ according to Post Hoc Test.

the key enzyme in limiting glutathione biosynthesis rate. In the present study, Cd reduced the GR activity (Fig. 2, D), which further decreased the rate at which GSSG was reduced to GSH resulting in the induction of oxidative stress. Application of BRs to Cd treated seeds resulted in maintaining the redox state of GSH by reducing GSSG to GSH. EBL at all concentrations applied reduced the toxic effect of Cd and enhanced GR activity. Similarly, Kang et al. (2007) observed that application of EBL alleviated the oxidative stress in the roots of cucumber seedlings under hypoxia stress by elevating the activities of GR, APOX and the contents of GSH.

The activity of SOD in *T. foenum-graecum* seedlings under Cd stress increased as compared to control treatment. EBL further enhanced the activity of SOD (Fig. 2, C). SOD catalyzes the breakdown of O_2^- to O_2 and H_2O_2, and removes singlet oxygen as well as O_2^- and prevents formation of OH^- (Fridovich, 1973). Increased SOD activity in EBL treated seeds revealed that SOD must have catalyzed the disproportionate of super oxide O_2^- to molecular oxygen and H_2O_2, thereby reducing the risk of hydroxyl radical formation. Nunez et al. (2003) revealed that the application of BRs caused the activation of antioxidative enzymes under water and salt stresses and increased SOD activity in rice. Similarly, increased SOD activity in *Arabidopsis* in response to heavy metal toxicity caused by BRs application has been also reported by Cao et al. (2005).

Ascorbic acid plays a very important role in developing resistance against various environmental stresses.The activity of AAO was found elevated under Cd stress in *T. foenum-graecum* seedlings. Application of EBL resulted in a reduction of AAO activity (Fig. 4), which was described as an adaptive feature found in tolerant cultivars of rice (Kumar and Das 2000). The tolerant cultivars showed higher quantities of ascorbic acid.

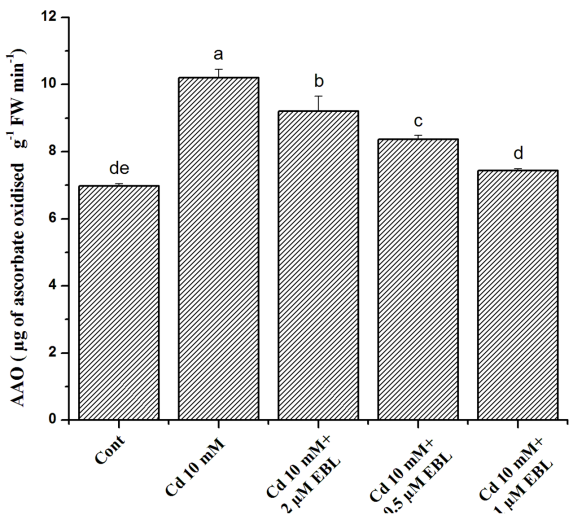

Fig. 4. Effect of 24-epibrassinolide on the activity of ascorbate oxidase in *T. foenum-graecum* seedlings under Cd stress. Different letters on the top of bars denote significant differences at $p \leq 0.05$ according to Post Hoc Test.

Cd stress induced an enhancement of MDA content in *T. foenum-graecum* seedlings reflecting the lipid peroxidation which increases oxidative stress and it decreased after the application of EBL (Fig. 3, B). Heavy metals cause severe lipid radical formation which leads to formation of alkenes and lipids which destroy the lipid structure. The amelioration of stress by plant growth regulators was found to be associated with the prevention of lipid peroxidation. Tang et al. (2005) showed that MDA content in leaves of *Typha* decreased after spraying with spermidine. Similarly, Panda and Choudhury (2004) observed that MDA content was lower in roots pretreated with salicylic acid. The present study clearly demonstrated the protective role of EBL against heavy metal induced lipid peroxidation.

In conclusion, supplementation of EBL enhanced the tolerance of *T. foenum-graecum* seedlings to oxidative stress generated by Cd by enhancing the activities of antioxidative enzymes. The higher activity of these enzymes suggests a possible role of EBL in amelioration of oxidative stress generated by Cd and in boosting the resistance capacity of the seedlings.

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