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Alleviation of Cd Metal-Induced Toxicity in *Raphanus sativus* L. by Exogenous Application of Plant Steroid Hormone

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ABSTRACT

The response of radish (Raphanus sativus L.) was evaluated after soaking seeds in different concentrations of 24-epibrassinolide (EBL) and then grown under cadmium (Cd) metal. Seeds were soaked in three different concentrations of EBL (10^{-11}, 10^{-9} and 10^{-7} M) for 8 hours and treated with 0.25 mM concentration of Cd. Metal was found to affect the growth (root length, shoot length, fresh weight, dry weight and percent germination), proline content, total osmolytes, levels of sodium (Na^+), potassium (K^+) ions, Cd uptake, malondialdehyde content (MDA), photosynthetic pigments (total chlorophyll, chl a, chl b, carotenoids, anthocyanins and flavonoids) and antioxidative defence system (antioxidants (ascorbic acid, tocopherol, glutathione), antioxidative enzymes (SOD, POD, CAT, APOX, GR, DHAR, MDHAR, PPO, GST, GPOX) and protein content) of 7-days old seedlings. The deleterious effects induced by metal were ameliorated by EBL. Maximum improvement was observed by 10^{-9} M EBL.

Key words: Cadmium toxicity, 24-EBL, Osmoprotectants, Metal uptake, Photosynthetic pigments, Antioxidative defence system**.**

INTRODUCTION

Radish (*Raphanus sativus*) is an edible root vegetable belonging to Brassicaceae family. It is grown and consumed globally and is rich source of carbohydrates, energy, sugars and dietary fibres. It contains certain medicinal compounds like peroxidases and isothiocyanates. Radish plant extract has anti-oxidant, anti-diabetic, choleretic, anti-hepatotoxic and multi-potent chemo-preventive potential (1).

In many parts of the world, heavy metals like Cd, Cu, Pb, Co, Cr and As contaminate agricultural soils slightly to moderately. This may be because of enduring utilization of phosphatic fertilizers, application of sewage sludge, industrial wastes, dust from smelters and wrong watering practices in agricultural lands (2). Cadmium (Cd) is considered as most toxic metal for plants and animals including human beings due to its phytotoxicity and high water solubility. In non-polluted soil, Cd content is usually in the range of 0.1–2 ppm and mostly it is below 1 ppm (3). Cd is present as free hydrated ions in the soil solution or they may be complexed by organic or inorganic ligands. Inhibition of growth and leaf chlorosis are the visible symptoms which appear in plants due to high Cd doses. Stomatal opening is inhibited and water balance and photosynthetic apparatus of plants are also disturbed due to Cd stress. Various metabolic enzymes are also sensitive to this metal (4).

Generation of reactive oxygen species (ROS) is the primary response of plants due to high levels of heavy metals. ROS production either occurs directly through Haber-Weiss reactions or they can be produced indirectly due to occurrence of oxidative stress in plants (5). Lipid peroxidation is one of the most adverse effects caused by heavy metals exposure in plants, which leads to distortion of biomembrane. Malondialdehyde (MDA) is considered as reliable indicator of oxidative stress, which is one of the decomposition products of polyunsaturated fatty acids of membrane (6).

One of the elementary fields for biotechnological advances in the improvement of agriculture is stimulation of internal defence mechanism of plants, so that plants can survive in adverse environmental cues. Regarding this, external application of brassinosteroids (BRs), a plant protectant is a promising choice. BRs are plant polyhydroxy steroids, which play key role in growth and development of plants. They initiate intracellular signal transduction cascade by binding to leucine-rich repeat receptor kinases (BRI1) at the cell surface, resulting in the changed expression of genes, which are concerned for various functions involving enhanced adaptation to various stresses (7). Keeping this in account, the present work describes the exogenous application of 24-EBL in measuring the growth, levels of osmoprotectants, photosynthetic pigments, elemental uptake, MDA content and activities of antioxidative enzymes and antioxidants in radish var. Pusa Chetki exposed to Cd stress.

EXPERIMENTAL SECTION

Plant Material, Growth Conditions and Treatments

Seeds of *Raphanus sativus* L. var. Pusa chetaki were obtained from Punjab Agricultural University, Ludhiana, Punjab. Healthy seeds were surface sterilized with 0.01% mercuric chloride solution, followed by the repeated washing with double distilled water (DDW). Then the seeds were soaked in 0 (control), 10^{-11}, 10^{-9}, 10^{-7} M concentrations of 24-EBL (Sigma Aldrich, Ltd., New Delhi) for 8 hours. Stock solution of EBL was prepared by dissolving the hormone in methanol and final volume was made by DDW. The seeds were then germinated in *Whatman No.1* filter paper lined glass petriplates (10 cm diameter, 20 seeds per petriplate) containing the 0.25 mM Cd metal. Cd was given in the form of cadmium chloride ($CdCl_2$) dissolved in distilled water. 3ml of test solution was given to each petriplate on first day and 2 ml on alternate days, upto 7 days. In control, seedlings were supplied only with distilled water. Three replicates of each treatment were grown. Controlled conditions ($25^oC \pm 0.5^oC$, 16 h photoperiod) were given to this experiment. Seedlings were harvested on 7^{th} day to study the following parameters:

***Growth Parameters*:** Growth parameters (root length, shoot length, fresh weight, dry weight and percentage germination) were determined on 7 days old seedlings.

Proline content: Proline content was determined by Bates et al. (8) method.

***Total osmolyte content*:** Total osmolyte content was determined by using vapour pressure osmometer (Vapro 5600).

Photosynthetic Pigments

Preparation of extract

1g of fresh seedlings was homogenized in chilled pestle and mortar by using 4ml of 80 % acetone. Then the homogenized material was subjected to centrifugation using cooling centrifuge (Eltek MP 400 R) for 20 minutes at 13000 rpm at a temperature of 4°C.

Chlorophyll content was measured by Arnon (9) method, Carotenoid content by Maclachlan and Zalik (10) method, Anthocyanin content by Mancinelli (11) method, Total Flavonoid content by the method given by Kim et al. (12).

Elemental analysis

Estimation of sodium and potassium ion concentration was done by flame emission photometer (Systronics 128). NaCl and KCl salts were used for running the standards of sodium and potassium respectively calibration curve was prepared.

Estimation of Cadmium Uptake:

To 0.5 g of plant sample, nitric acid (HNO_3) and perchloric acid ($HClO_4$) in the ratio of 2:1 was added and heated until complete digestion. The digested sample was then diluted by DW and the extract was filtered. Filtered extract was used for determination of cadmium uptake by using (AAS) Atomic Absorption Spectrophotometer (Shimadzu 6200). For standardization different concentrations of $CdCl_2$ were run for making standard curve.

Malondialdehyde (MDA) content: MDA content was determined by following the method of Heath and Packer (13).

Antioxidants

1g of seedlings was homogenized in 3ml of tris buffer (50 mM, pH 10.0). Homogenized sample was then subjected to centrifugation using Eltek cooling centrifuge for 20 minutes at 13000 rpm at a temperature of 4°C. The supernatant was further used for analysis of antioxidants.

Ascorbic acid content was determined by following the method of Roe and Kuether (14), tocopherol content by Martinek (15) and glutathione content was estimated by the method given by Sedlak and Lindsay (16).

Antioxidative enzymes

1g of seedings was crushed in 3 mL of 100 mM potassium phosphate buffer at pH 7. Centrifugation of homogenates was done at 13,000 rpm for 20 min at 4°C. Supernatant was used for the estimation of protein content by Lowry et al. (17) method and activity of antioxidative enzymes.

Guaiacol peroxidase (POD) activity was estimated by Putter (18) method, catalase (CAT) activity by method of Aebi (19), superoxide dismutase (SOD) activity per the method of Kono (20), ascorbate peroxidase (APOX) activity by Nakano and Asada (21), glutathione reductase (GR) activity as per the method of Carlberg and Mannervik (22), dehydroascorbate reductase (DHAR) activity by the method of Dalton et al. (23), mono-dehydroascorbate reductase (MDHAR) activity by Hossain et al. (24) method, polyphenol oxidase (PPO) activity according to the method given by Kumar and Khan (25), glutathione-s-transferase activity (GST) by following the method of Habig et al. (26) and glutathione peroxidase (GPOX) activity was determined according to the method of Flohe and Gunzlar (27).

Statistical Analysis

The data obtained was statistically analyzed using one-way analysis of variance (ANOVA) and presented as means ± SE.

RESULTS AND DISCUSSION

Growth Analysis

In comparison to control seedlings, metal treatment showed distinct adverse effect on growth parameters of 7-days old seedlings of radish like root length, shoot length, fresh weight, dry weight and percent germination (Fig. 1). However treatment of EBL along with the metal showed improvement in growth over their respective control i.e., metal alone. Maximum enhancement of root length (Cm) was observed in the samples growing in 10^{-9} M EBL in conjunction with Cd (3.94 ± 0.2) as compared to seedlings grown in metal alone (3.02 ± 0.3). Similar trends were found in case of shoot length (Cm), where treatment of 10^{-9} M EBL + Cd resulted in maximum increase (3.28 ± 0.11) as compared to control seedlings (2.93 ± 0.09). Similarly, in case of fresh weight (g) and percent germination (%), maximum improvement was observed with 10^{-9} M EBL + Cd (1.32 ± 0.03, 90 ± 3.7 respectively) as compared to control (1.22 ± 0.07, 67.67 ± 3.26 respectively). Though in case of dry weight (g) 10^{-11} M EBL + Cd resulted in maximum increase (0.13 ± 0.002) with respect to its control (0.12± 0.003).

Entry of heavy metal occurs through root system in the plants. A decrease in root and shoot lengths were found in the Cd treated plants as compared to untreated control. Metal accumulated in the roots and its mobility is controlled by root cell wall. (28). Negative effects on nutrition and water supply is produced by root damage, so growth and physiology of aerial parts of plants are affected. Whereas 24-EBL was found to increase the growth of plant. BRs treatment showed the similar ameliorative effects (29) in barley seedlings, where growth was significantly enhanced. Similarly, exogenous applications of EBL have been studied on *Brassica juncea* and *Raphanus sativus* plants under copper stress. It has been found that EBL blocked the uptake and accumulation of metal in these plants. Treatment of EBL to the radish seeds also showed the reduced Cu toxicity by stimulating the root and shoot growth of seedlings (30).

Osmoprotectants

Metal treatment enhanced the level of osmoprotectants like proline (μ mol g^{-1} FW) and total osmolytes (m mol Kg^{-1}) as compared to their control seedlings (Fig. 2). Supplementation of EBL further significantly improves the proline and total osmolytes content as compared to seedlings grown in metal alone. In both cases 10^{-9} M EBL + Cd proved as effective treatments as they caused maximum increase (45.71±1.43, 206.67±1.85 respectively) with respect to control (20.81±2.3, 183.33±0.88 respectively). Osmolyte content was increased during stress. This might be due to stimulation of Δ^{1} pyrroline-5-carboxylate synthase which triggers the formation of osmolytes like proline under stress. osmolytes scavenge the free radicals and stabilize the membranes (31).

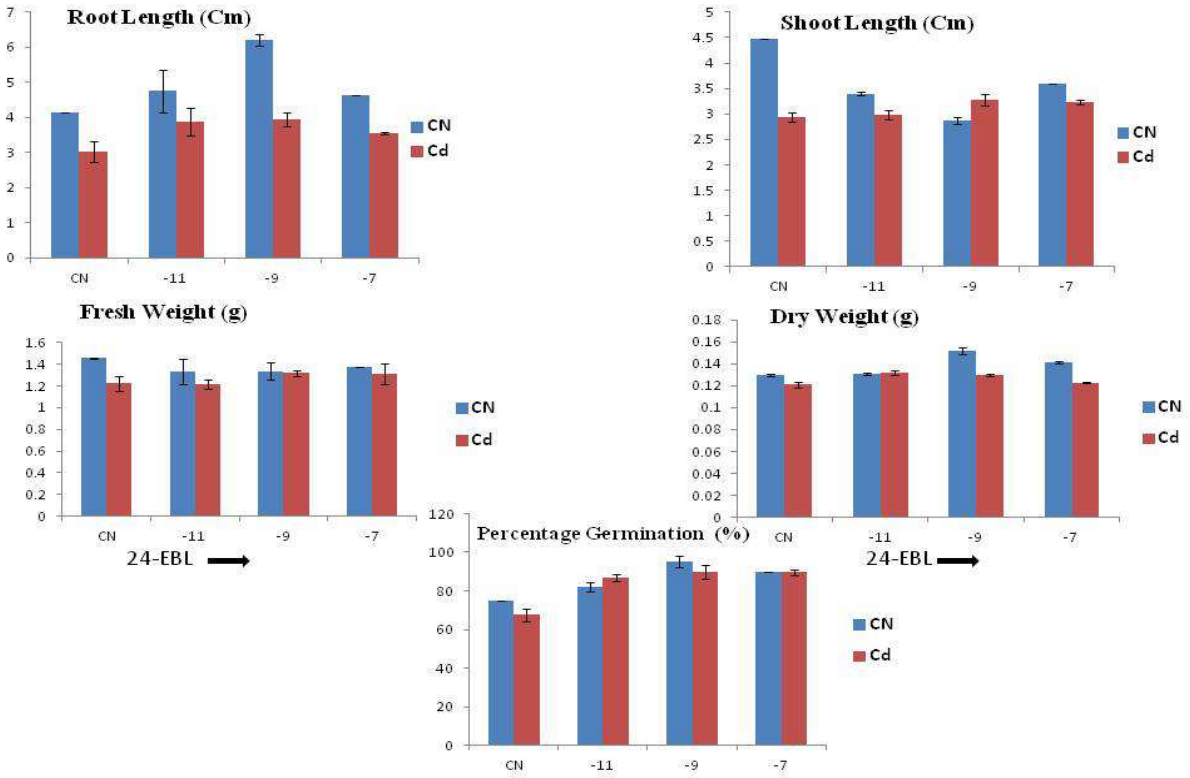

Fig.1 Effect of 24-EBL on Growth of 7-day old seedlings of *Raphanus sativus* under Cd stress.

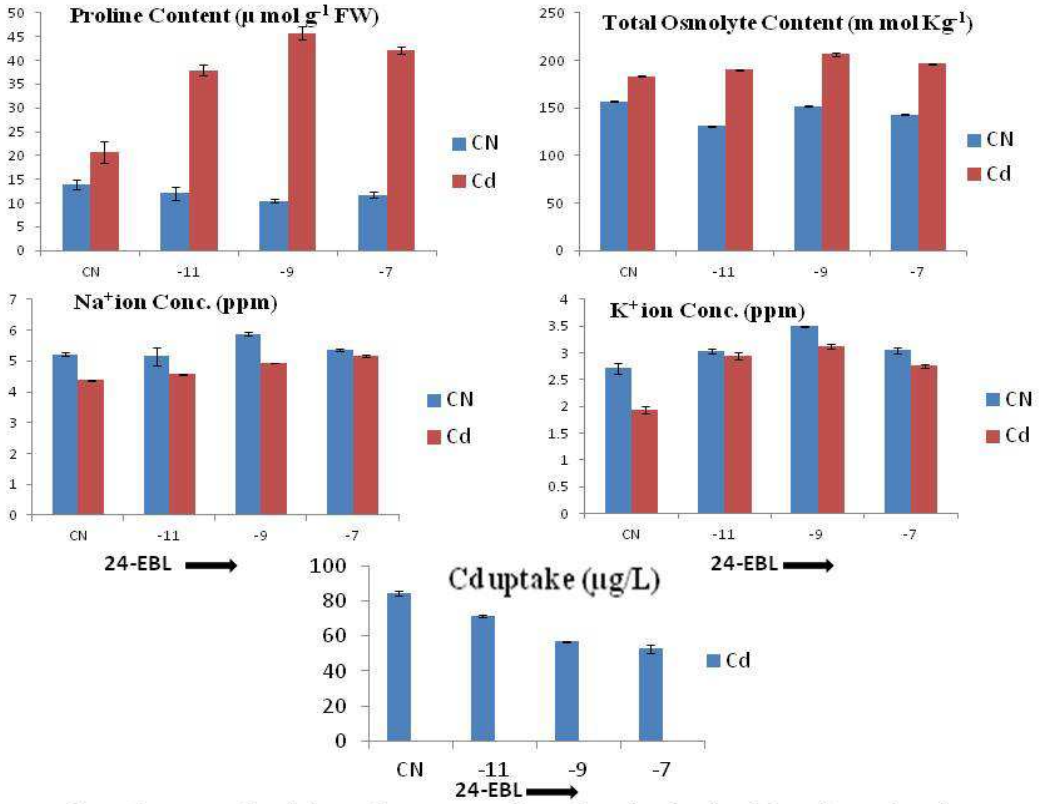

Fig. 2 Effect of 24-EBL on level of osmolytes, ions and metal uptake of 7-day old seedlings of *Raphanus sativus* under Cd stress.

Photosynthetic pigments

It was observed that chlorophyll content (chl a, b, total chl) declined with metal stress (Fig. 3). Treatment of EBL however led to enhancement of chlorophyll levels (mg/ml) in the seedlings exposed to metal. Maximum increase in

total chl content (30.2± 2.8) was observed with 10^{-7} M EBL + Cd treatment as compared to its respective control i.e. metal alone (24.65±3.1). Similar trends were found with chl a and chl b, where 10^{-7} M EBL + Cd treatment showed the most effective results (15.0±1.5, 18.37±0.2) as compared to control (9.1±0.9, 11.15±1). Similarly, total flavonoid content (µg/ml) also reduced with metal and further stimulated with EBL application. In this case 10^{-11}M EBL + Cd sharply enhanced the flavonoid content (189.2±11.2) (Fig. 3). However, increase in total carotenoid and anthocyanin content was observed with metal stress and they further showed enhancement with EBL supplementation with respect to control (Fig. 3). Most effective concentration of EBL in both cases was observed as 10^{-11}M in conjunction with metal. Maximum carotenoid content (mg g^{-1} FW) was observed as 12.32±0.34 and that of anthocyanin (mg g^{-1} FW) was 0.38±0.03.

Levels of photosynthetic pigments like chlorophyll and flavonoids were found to inhibit during metal stress, whereas carotenoids and anthocyanin contents were enhanced during stress. But further the treatment of EBL stimulated the pigments level. For proper synthesis of chlorophyll pigment, the adequate supply of Fe and Mg ions is necessary which is reported to be affected by heavy metal stress (32). On the other hand, EBL supplementation stimulates the antioxidant system, thus protect the photosynthetic machinery (33). Carotenoid and flavonoid level was increased during stress as plants own defence strategies help in overcoming the oxidative stress induced by metal (34). Similar findings were observed by Singh et al. (35), in *Hydrilla verticillata* when exposed to Pb and Cd metals. Cd^{2+} stimulates the synthesis of glutathione-S-transferase (GST) enzyme which further increases the synthesis of anthocyanin. Similarly, in the leaves of *Echium amoenum*, anthocyanin content increased under Cd^{2+} stress (36).

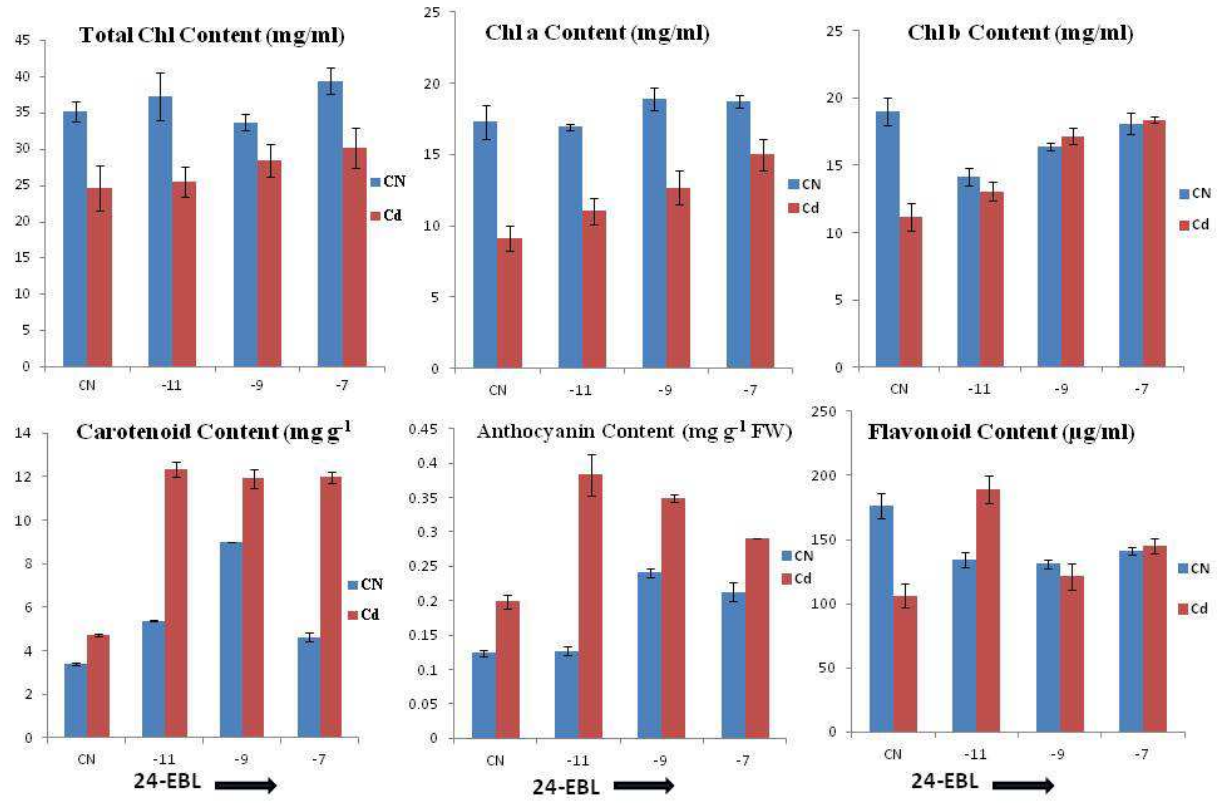

Fig.3 Effect of 24-EBL on Photosynthetic Pigments of 7-day old seedlings of *Raphanus sativus* under Cd stress.

Elemental Analysis

A decrease in sodium and potassium ions were found to reduce with the metal stress and further increased in the seedlings supplemented with EBL (Fig. 2). Sodium ion concentration (ppm) was enhanced maximum with 10^{-7}M EBL + Cd (5.11±0.04) as compared to treatment of metal alone (4.37±0.03). Similar trend was observed in potassium ion concentration (ppm). The most effective treatment in this case was 10^{-11}M + Cd, which contained the maximum potassium ions (2.95±0.07) as compared to seedlings growing in metal alone (1.94±0.06). Concentrations of ions were reduced with metal treatment and further enhanced with BRs application in present study. Similar results were reported by Alaoui-Sosse et al. (37) in cucumber plant. Whereas, the concentrations of Cd metal was more in the seedlings exposed in metal stress, which further decreased with EBL application. This might be because BRs reduce the metal uptake and block the accumulation of metal. Thus overcome the toxic effects of heavy metals in plants. These results are similar with the observations of Bajguz (38).

Cadmium metal uptake

A continuous decrease in the concentration of cadmium was observed with increasing the concentration of EBL (Fig. 2). 10^{-7}M EBL was proved as the most effect concentration of hormone, which reduced the uptake of metal (µg/L) up to 52.87±2.3 as compared to seedlings exposed to metal stress alone (84.53±1.3).

MDA content

MDA content (µ mol g^{-1} FW) significantly increased in metal stress (14.4±0.01) in comparison to control (5.81±0.01). No significant results were observed with the supplementation of EBL (Fig. 4). MDA content was enhanced in the seedlings exposed to heavy metal. As heavy metal stimulates the lipid peroxidation and these results are in coherence with the observations of De Brotto et al. (39) in *Capsicum annum*.

Antioxidants

An increase in the content of ascorbic acid, glutathione and tocopherol (mg/g FW) was observed in the seedlings exposed to metal stress (Fig. 4). Further EBL supplementation enhanced the glutathione and tocopherol level (9.2±0.4, 6.7±0.04) as compared to their respective control (7.8±0.2). Ascorbic acid content was increased under stressed conditions (2.5±0.005) in comparison to untreated control (1.6±0.005), but no significant results were observed during EBL treatment. In the present study, levels of antioxidants enhanced with EBL treatment, as this hormone acts as stress protectant and scavenge the free radicals. These findings are similar with the reports of Pietrini et al. (40), in *Phragmites australis* under Cd metal stress.

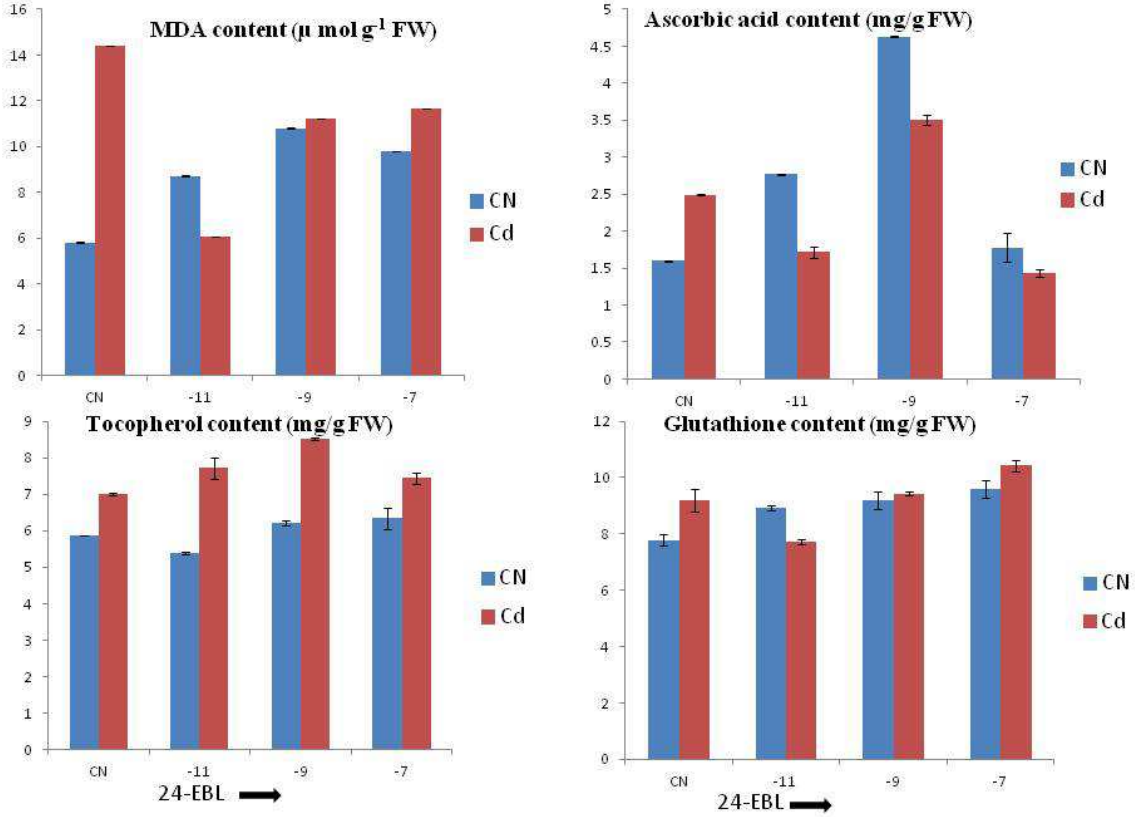

Fig.4 Effect of 24-EBL on MDA and Antioxidant level of 7-day old seedlings of *Raphanus sativus* under Cd stress.

Antioxidative Enzymes

Protein content was found to increase in the seedlings exposed to Cd stress (Fig. 5). Further it has been observed that EBL supplementation enhanced the protein content as compared to their respective control. For increasing protein content (mg/g fw) maximum under stress conditions, 10^{-7} M EBL was proved as most effective concentration, which enhanced the content (11.98±0.57) as compared to control (10.16±0.78).

Protein content and specific activities of enzymes, POD, SOD, CAT, GR, DHAR, MDHAR, PPO and GST were found to enhance in Cd treated seedlings in comparison to untreated control (Fig.5 and Fig. 6). Further EBL treatment also increased the activities of enzymes as compared to their respective control. Protein content (mg g^{-1}FW) increased (10.16±0.78) in Cd exposed seedlings as compared to untreated control (7.97±0.48). In case of POD and GR, 10^{-11} M EBL was found as most effective concentration, which increased the enzyme activity (µmole UA

mg protein^{-1}) maximum (0.45±0.01 and 7.55±0.43 respectively) in comparison to its control (0.21±0.02 and 2.75±0.56). Whereas in case of SOD activity, 10^{-9} M EBL was proved as most effective concentration. It enhanced the enzyme activity upto 3.95±0.4 as compared to its control (3.43±0.2). 10^{-9} M EBL enhanced the CAT, DHAR, MDHAR and PPO activity maximum (11.96±0.25, 41.70±5.7, 6.09±0.9 and 34.25±1.05 respectively) in comparison to its control (9.95±1.5, 25.74±2.7, 4.93±0.6 and 23.65±2.05). Maximum increase in GST activity was observed with 10^{-7} M EBL (0.94±0.03) in comparison to control (0.86±0.01). However in case of GPOX, enzyme activity was enhanced in the seedlings exposed to metal (1.03±0.05) as compared to untreated seedlings (0.53±0.05). But EBL supplementation did not show any significant effect. Whereas, activity of APOX decreased with the metal treatment in comparison to control. Further EBL treatment helps in stimulating the enzyme activity. 10^{-9} M EBL enhanced the enzyme activity maximum (14.6±0.5) as compared to its control (12.1±0.4). Protein content and activities of antioxidative enzymes were found to alter during metal stress and also with the treatment of EBL with respect to their respective control. This may be due to the reason that plants defence system is induced against stress conditions and certain stress proteins also released in plants exposed to metals (41). Alteration in activities of antioxidative enzymes in the present study was in coherence with the reports of Behnamia et al. (42).

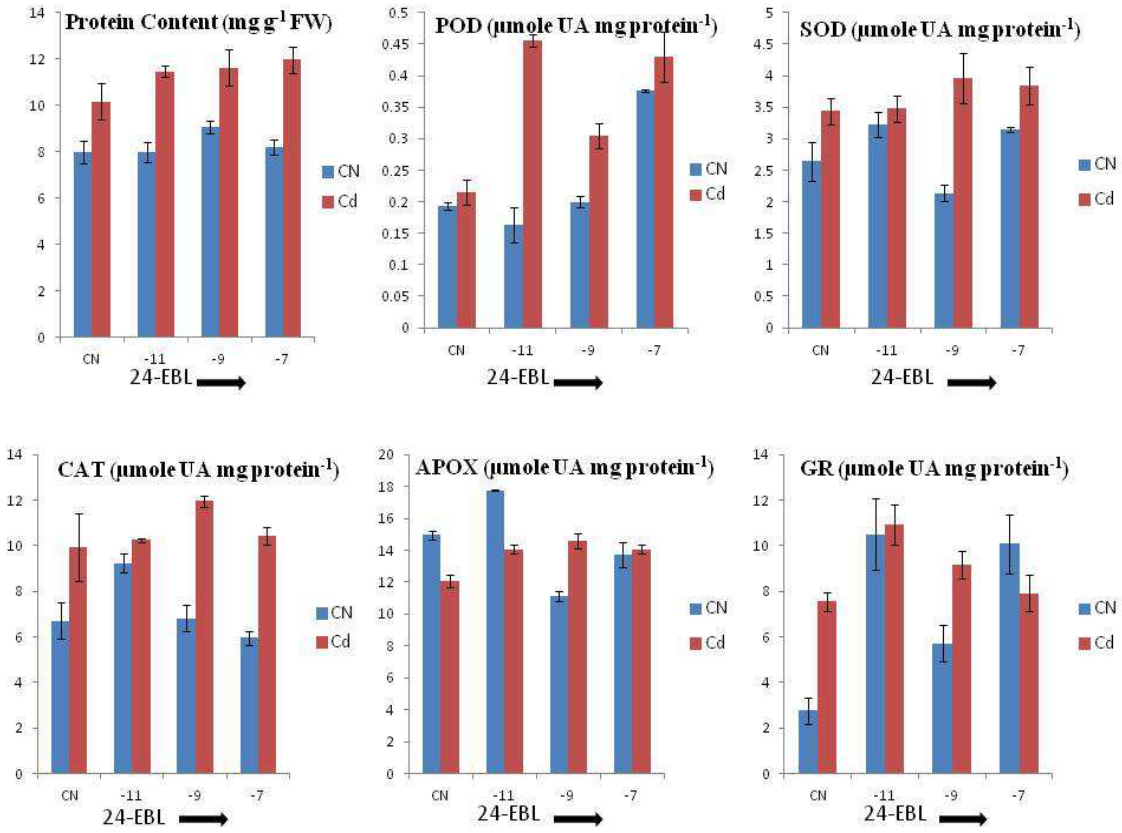

Fig. 5 Effect of 24-EBL on Activities of Antioxidative Enzymes of 7-day old seedlings of *Raphanus sativus* under Cd stress.

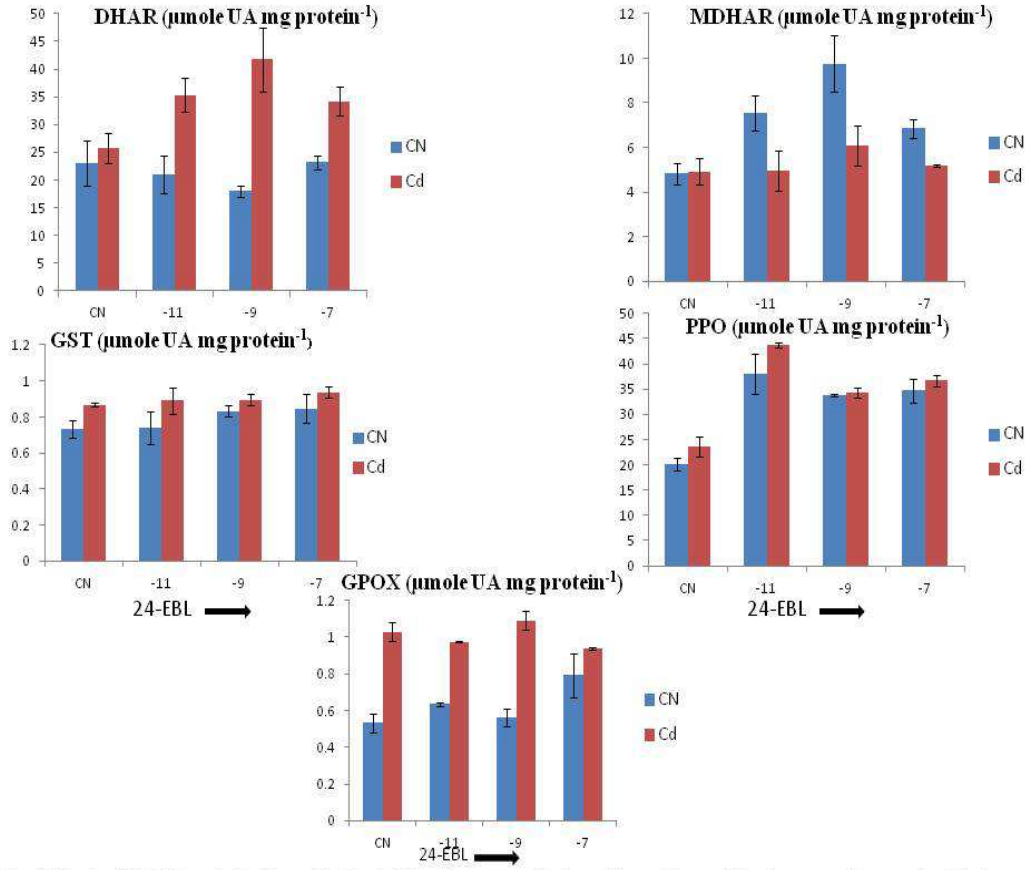

Fig.6 Effect of 24-EBL on Activities of Antioxidative Enzymes of 7-day old seedlings of *Raphanus sativus* under Cd stress.

CONCLUSION

EBL treatment activates the antioxidative defence system of plants. Elevated level of antioxidants, osmo-protectants and antioxidative enzymes under cadmium stress indicates the enhanced tolerance of radish plants to Cd stress so that photosynthetic machinery and growth of plants are protected.

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