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Mercury-induced changes in growth, metal & ions uptake, photosynthetic pigments, osmoprotectants and antioxidant defence system in *Raphanus sativus* L. seedlings and role of steroid hormone in stress amelioration

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Abstract
The present work was undertaken to analyze the effects of 24-epibrassinolide (10^{-11}M, 10^{-9}M and 10^{-7}M) on growth, metal uptake, Na^+ & K^+ ions concentration, photosynthetic pigments, level of osmoprotectants, MDA content, level of antioxidants, protein content and activities of antioxidant enzymes in 7-days old seedlings of *Raphanus sativus* L. subjected to Hg metal (0.25 mM) stress. Seedling growth was improved by 24-epiBL treatments under heavy metal stress. Application of 24-EBL enhanced the level of osmolytes, antioxidants and also stimulated the activities of antioxidative enzymes. MDA content was found to enhance under stress conditions, which was reduced by treatment of EBL. Level of photosynthetic pigments and Na^+ & K^+ ions were also increased by the supplementation of EBL. 10^{-9} and 10^{-11} M concentrations of 24-EBL were proved to be the effective concentrations. In conclusion, treatment of 24-EBL helped in combating the metal stress by activating the various defence strategies of *Raphanus sativus*.

Keywords: 24-epibrassinolide, Hg toxicity, osmolytes, metal uptake

1. Introduction
Plants often face different environmental challenges affecting their growth and development [1]. Among these stresses, heavy metal toxicity has become a major focus because of the increased environmental pollution. Heavy metals are non-degradable pollutants which pose major occupational and environmental hazards. Various natural as well as anthropogenic factors like mining, sewage, industrial activities and traffic are the major sources of heavy metals, which further cause pollution in land areas [2]. Among these, mercury (Hg) is most toxic to plants, animals and human beings. In plants, it damages the photosynthetic membrane, mainly the membrane of photosystem II (PSII), which further blocks the oxygen evolution from PSII, as it causes perturbances in the binding sites of chlorine (which acts as cofactor in oxygen evolution complex). Hg affects the cell growth, cell division and triggers the formation of ROS in the cells. Generally it alters the reactivity with sulphydryl group of proteins, cell permeability and competence of ATP binding. Metabolism of glutathione, which plays significant role in metal homeostasis, is altered by Hg due to phytochelatins synthesis [3].
Exogenous application of plant hormones counteracts the toxic effect of various abiotic stresses. Brassinosteroids (BRs) have been recognized as natural polyhydroxy steroids, which are ubiquitously occurring phytohormones [4]. They have significant effects on vegetative and reproductive development, seed germination and stress tolerance [4]. At cellular level, BRs activate the synthesis of proteins and nucleic acids, change permeability of cell membranes and the mechanical properties of cell wall, promote photosynthetic capacity, cell elongation and fission [5].
Raphanus sativus is considered to be a hyperaccumulator of heavy metals [6]. This plant has economic value and also rich source of medicinal compounds like peroxidases and isothiocyanates [7]. Hypocotyls are the edible part of the plant and these can be polluted with heavy metals when in direct contact with soil [8]. Anthropogenic pollution of soil due to heavy metals lead to entrance of these metals into food chain through plants grown in that polluted environment.

The present investigation was conducted with an objective to examine the protective role of 24-epibrassinolide on growth, level of photosynthetic pigments & osmolytes, metal & ions uptake and antioxidant defence system of mercury-treated plants of *Raphanus sativus* L. and to find out how BRs mediating the deleterious effects of heavy metal.

2. Materials and Methods

Certified and disease free seeds of *Raphanus sativus* L. var. Pusa chetaki were procured from Punjab Agricultural University, Ludhiana, Punjab and surface sterilized with 0.01% mercuric chloride solution, followed by the repeated washing of sterile double distilled water (DDW). These surface sterilized seeds were soaked in 0, 10^{-11}, 10^{-9}, 10^{-7} M concentrations of 24-EBL for 8 hours, which was purchased from Sigma Aldrich, Ltd., New Delhi and then germinated in *Whatman No.1* filter paper lined glass petriplates (10 cm diameter, 20 seeds per petriplate) containing the 0.25 mM (IC_{50} value) concentration of Hg metal. Hg was given in the form of mercury acetate ($C_4H_6HgO_4$) dissolved in distilled water. Each petriplate was supplied with 3ml of test solution on first day and 2 ml of test solution on alternate days, up to 7 days. Control seedlings were supplied with distilled water only. Each treatment was replicated 3 times. The experiment was conducted under controlled conditions (25 ºC ± 0.5 ºC, 16 h photoperiod). These seedlings were harvested on 7th day to study the following parameters:

2.1 Growth Parameters

Growth parameters (root length, shoot length, fresh weight, dry weight and percentage gemination) were determined on 7 days old seedlings of *R. sativus* L.

2.2 Estimation of Mercury Uptake

To 0.5 g of dried 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 mercury uptake by mercury analyzer (MA 5800E, Electronic Corperation of India Limited).

2.3 Estimation of Na and K ion concentration

Determination of sodium and potassium ion concentration was done by flame emission photometer (128 systronics, Electronics Division of ASE Limited, India). Standards of sodium and potassium were prepared by using salts of NaCl and KCl (Fischer Scientific India, Pvt. Ltd.) respectively. Standardization of flame photometer was done by running standards and calibration curve was prepared.

2.4 Photosynthetic Pigments

Photosynthetic pigments like Chlorophyll content (chl a, chl b and total chl), carotenoids, anthocyanins and flavonoids were determined by Arnon [9], Maclachlan and Zalik [10], Mancinelli [11] and Kim *et al* [12] methods respectively.

2.5 Proline and Total Osmolyte Content

Proline was estimated by Bates *et al.* [13]. Method and total osmolyte content was analyzed by using vapour pressure osmometer (Vapro 5600).

2.6 Malondialdehyde (MDA) content

MDA content was estimated by following the method of Heath and Packer [14].

2.7 Antioxidants and Antioxidative Enzymes

Ascorbic acid content was determined by following the method of Roe and Kuether [15], tocopherol content by Martinek [16] and glutathione content was determined by the method given by Sedlak and Lindsay [17].

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

Statistical Analysis: The data obtained was statistically analyzed using one-way ANOVA (Bailey 1995). Data were presented as means ± SE.

3. Results and Discussion

3.1 Growth Parameters

Root and shoot lengths were found to increase in *Raphanus sativus* seedlings treated with 24-EBL when compared to untreated seedlings (Table 1). 10^{-9} M EBL alone enhanced root length to the maximum (6.2 cm) as compared to 10^{-7} M and 10^{-11} M EBL. Decrease in root and shoot length (2.35 and 1.41 times respectively) was noticed in 0.25 mM Hg in comparison to control seedlings. Shoot length was observed to be maximum (4.48 cm) in untreated controls. However, EBL treatment restored the growth of shoots in the presence of metal. Reduction in fresh and dry weights (1.21 g and 1.11g) was noticed respectively in the seedlings treated with 0.25 mM Hg. Application of EBL along with metal increased fresh and dry weight and maximum stress ameliorating effect was observed with 10^{-7} M EBL and 0.25 mM Hg. Percent germination was enhanced in the seedlings treated with 24-EBL as compared to untreated seedlings. 10^{-9} M EBL proved most effective concentration which enhanced the percent germination to the maximum (95%).

In *Raphanus sativus* seedlings, fall in fresh weight and dry weight was observed when metal treatment was given to seedlings. EBL treatment aided in restoring the fresh weights, thus reducing the toxic effects of metal. Similar results were restored by Ali *et al*. [28] in *Vigna radiata* L. when treated with BRs under Al stress. 24-EBL application also increased the fresh weight of salinity stressed seedlings of rice, but no effect was observed in dry weight. Percentage germination of seeds was highest in 10^{-9} M EBL treatment in case of *Raphanus sativus* seedlings.

Table 1: Effect of different concentrations of 24-EBL on Growth Parameters of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Root Length (Cm)	Shoot Length (Cm)	Fresh Weight (g)	Dry Weight (g)	% Germination
1.	0 (Control)	4.14 ± 0.001	4.48 ± 0.001	1.45 ± 0.002	0.13 ± 0.001	75 ± 0.001
2.	10^{-11} M EBL	4.75 ± 0.61	3.4 ± 0.03	1.33 ± 0.221	0.13 ± 0.001	82 ± 2.5
3.	10^{-9} M EBL	6.2 ± 0.16	2.87 ± 0.07	1.33 ± 0.18	0.15 ± 0.003	95 ± 3
4.	10^{-7} M EBL	3.64 ± 0.001	3.6 ± 0.001	1.37 ± 0.001	0.141 ± 0.001	90 ± 0.001
		F-Ratio $_{(df\ 3,8)}$ = 29.61*, HSD = 0.93	F-Ratio $_{(df\ 3,8)}$ = 29.61*, HSD = 0.93	F-Ratio $_{(df\ 3,8)}$ = 0.62, HSD = 0.29	F-Ratio $_{(df\ 3,8)}$ = 46.58*, HSD =0.007	F-Ratio $_{(df\ 3,8)}$ = 5.12*, HSD = 17.64
5.	0.25 mM Hg	1.76 ± 0.1	3.18 ± 0.15	1.21 ± 0.02	0.114 ± 0.006	88.33 ± 2.41
6.	10^{-11} M EBL + 0.25 mM Hg	3.26 ± 0.3	3.17 ± 0.2	1.22 ± 0.17	0.13 ± 0.01	83.33 ± 3.009
7.	10^{-9} M EBL + 0.25 mM Hg	2.82 ± 0.13	3.09 ± 0.3	1.29 ± 0.02	0.13 ± 0.008	83.33 ± 1.67
8.	10^{-7} M EBL + 0.25 mM Hg	1.77 ± 0.05	2.94 ± 0.1	1.14 ± 0.042	0.15 ± 0.004	88.33 ± 3.66
		F-Ratio $_{(df\ 3,8)}$ = 2.67, HSD = 2.09	F-Ratio $_{(df\ 3,8)}$ = 2.68, HSD = 2.09	F-Ratio $_{(df\ 3,8)}$ = 0.14, HSD = 0.44	F-Ratio $_{(df\ 3,8)}$ = 2.10, HSD = 0.05	F-Ratio $_{(df\ 3,8)}$ = 0.32, HSD = 22.96

3.2 Hg Uptake

Hg concentration was found highest in the plants with metal stress (81.0µg/L) in comparison to the seedlings exposed to EBL and decreases with the treatment of EBL (Table 2). 10^{-9} M EBL was proved as most effective concentration, which caused maximum reduction (62.33 µg/L) in metal uptake.

Table 2: Effect of different concentrations of 24-EBL on the concentration of Hg metal in 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Metal Concentration (µg/L)
1.	0.25 mM Hg	81 ± 4.36
2.	10^{-11} M EBL + 0.25 mM Hg	70.92 ± 5.26
3.	10^{-9} M EBL + 0.25 mM Hg	62.33 ± 1.99
4.	10^{-7} M EBL + 0.25 mM Hg	73.1 ± 2.8
		F-Ratio $_{(df\ 3,8)}$ = 4.40, HSD = 17.45

3.3 Na^+ and K^+ ion concentration

In present work Na^+ and K^+ ion concentrations were found to reduce (1.34 and 1.46 times respectively) due to Hg stress as compared to control (Table 3). EBL application enhanced the level of elements. EBL supplementation when given in combination with Hg, concentration of both ions increased as compared to treatment of Hg given alone. Maximum Na^+ and K^+ concentration (5.88 ppm, 3.49 ppm respectively) were found with application of 10^{-9} M EBL. Potassium and sodium ion content was found to reduce with Hg treatment as compared to control in 7-d old radish plants. At the cellular level, plants have a range of potential mechanisms which are involved in the detoxification of heavy metal. Thus heavy metal stress tolerance of plants is found to increase. It might be due to the involvement of plasma membrane in decreasing the heavy metal uptake or by stimulation of efflux pumping of metals which entered the cytosol. The other mechanism is the chelation of the metal ion by ligands, like organic acids, amino acids, peptides and polypeptides [29]. BRs have ability to regulate cell membrane permeability and ions transport, and it can be considered a good agricultural application in the heavy metal polluted areas. 24-epiBL when applied in combination with heavy metals in algal cells, blocked metal accumulation [30]. Similarly, in the present work 24-EBL lowered the uptake of heavy metal, thus decreasing their toxic effects in plants.

Table 3: Effect of different concentrations of 24-EBL on the concentration of Sodium ion and Potassium ion in 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Sodium ion (ppm)	Potassium ion (ppm)
1.	0 (Control)	5.22 ± 0.07	2.71 ± 0.095
2.	10^{-11} M EBL	5.15 ± 0.03	3.03 ± 0.04
3.	10^{-9} M EBL	5.88 ± 0.05	3.49 ± 0.02
4.	10^{-7} M EBL	5.35 ± 0.04	3.05 ± 0.06
		F-Ratio $_{(df\ 3,8)}$ = 41.64*, HSD = 0.25	F-Ratio $_{(df\ 3,8)}$ =27.48*,HSD = 0.29
5.	0.25 mM Hg	3.90 ± 0.05	1.85 ± 0.08
6.	10^{-11} M EBL + 0.25 mM Hg	4.87 ± 0.50	2.41 ± 0.12
7.	10^{-9} M EBL + 0.25 mM Hg	5.53 ± 0.06	2.74 ± 0.03
8.	10^{-7} M EBL + 0.25 mM Hg	4.85 ± 0.04	2.68 ± 0.03
		F-Ratio $_{(df\ 3,8)}$ =121.07*,HSD = 0.25	F-Ratio $_{(df\ 3,8)}$ =30.41*,HSD = 0.35

3.4 Photosynthetic Pigments

Hg treatment caused reduction in total chlorophyll content (as in Table 4). Least value (12.31mg/ml) for total chlorophyll content was observed with 0.25 mM Hg. However, EBL treatment further enhanced the level of total chlorophyll when EBL was given in combination with metals. 10^{-7} M EBL was found to be most effective (1.48 times) concentration which helped in overcoming the effect of 0.25 mM Hg. Chlorophyll a

content was reduced with 24-EBL treatment as compared to untreated control. Metal treatments, whether given alone or in combination, caused decrease in chl a content and its least value was found to be 7.26 mg/ml at 0.25 mM Hg. Chl b was reduced by the metal treatment. But supplementation of different concentration of EBL did not affect much significantly. Carotenoid content was decreased during Hg treatment but further enhanced when plants were given EBL treatment. 10^{-11} M EBL was found to be the most effective concentration that increased the carotenoid level 1.15 times more as compared to Hg treatment. Similarly, anthocyanin content was found to increase by the Hg stress. Application of EBL further enhanced anthocyanin content. 10^{-7} M EBL was found to be the most effective concentration, which enhanced the anthocyanin level 3.4 times with respect to metal stress (Table 4). Metal treatment decreased the total flavonoid content (112.6 µg/ml) as compared to untreated plants (176.1 µg/ml), while EBL supplementation further helped in enhancement of flavonoid level. 10^{-7} M EBL was found to be 1.32 times more effective as compared to metal treatment (Table 4).

In the seedlings of *Raphanus sativus*, total chlorophyll, chl aand chl b contents were decreased in Hg treated seedlings and EBL application increased the chlorophyll content and 10^{-7} M EBL was found to be most effective concentration. It was reported that Fe and Mg supply to the plants was inhibited by the metal stress, which is required for the synthesis of chlorophyll. Present work showed that Hg stress reduced the total carotenoid content in radish plants. These findings are in coherence with results of Singh and Malik [31], where carotenoid content decreased in *Brassica juncea* under Hg stress. Hg stress enhanced the anthocyanin content as compared to untreated plants. This is due to the reason that metal stress can stimulate the synthesis of glutathione-S-transferase (GST) which stimulates the synthesis of anthocyanin [32].

Table 4: Effect of different concentrations of 24-EBL on Photosynthetic Pigments of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Total Chl Content (mg/ml)	Chl a Content (mg/ml)	Chl b Content (mg/ml)	Carotenoid Content (mg/g)	Anthocyanin Content (mg/g)	Flavonoid Content (µg/ml)
1.	0 (Control)	46.3 ± 1.4	23.9 ± 1.19	14.97 ± 1.0	3.4 ± 0.03	0.12 ± 0.01	176.1 ± 9.9
2.	10^{-11} M EBL	17.31 ± 3.3	4.93 ± 0.2	9.13 ± 0.68	3.39 ± 0.03	0.13 ± 0.006	134 ± 5.8
3.	10^{-9} M EBL	13.75 ± 1.06	4.9 ± 0.8	6.37 ± 0.3	3.99 ± 0.02	0.24 ± 0.06	131.2 ± 3.5
4.	10^{-7} M EBL	27.47 ± 4.8	8.73 ± 0.4	13.1 ± 2.8	4.64 ± 1.11	0.21 ± 0.01	141.4 ± 2.7
		F-Ratio $_{(df\ 3,8)}$ = 22.14*, HSD = 14.05	F-Ratio $_{(df\ 3,8)}$ = 137.88*, HSD = 3.49	F-Ratio $_{(df\ 3,8)}$ = 3.98, HSD = 8.9	F-Ratio $_{(df\ 3,8)}$ = 33.39*, HSD =0.48	F-Ratio $_{(df\ 3,8)}$ = 3.69, HSD = 0.15	F-Ratio $_{(df\ 3,8)}$ = 11.24*, HSD = 27.93
5.	0.25 mM Hg	12.31 ± 0.47	7.27 ± 0.8	4.8 ± 0.5	9.72 ± 1.03	0.18 ± 0.01	112.6 ± 4.2
6.	10^{-11} M EBL + 0.25 mM Hg	16.4 ± 0.37	6.85 ± 0.9	4.15 ± 0.11	11.19 ± 1.11	0.1 ± 0.02	119.9 ± 0.96
7.	10^{-9} M EBL + 0.25 mM Hg	15.75 ± 1.4	7.48 ± 0.2	5.38 ± 0.3	10.36 ± 0.24	0.47 ± 0.03	106.8 ± 5.8
8.	10^{-7} M EBL + 0.25 mM Hg	18.3 ± 0.5	7.15 ± 0.05	4.05 ± 0.1	10.41 ± 0.14	0.612 ± 0.0003	148.3 ± 5.3
		F-Ratio $_{(df\ 3,8)}$ = 0.96, HSD = 12.1	F-Ratio $_{(df\ 3,8)}$ = 0.87, HSD = 3.68	F-Ratio $_{(df\ 3,8)}$ = 0.69, HSD = 3.58	F-Ratio $_{(df\ 3,8)}$ = 0.61, HSD = 3.68	F-Ratio $_{(df\ 3,8)}$ = 106.47*, HSD = 0.09	F-Ratio $_{(df\ 3,8)}$ = 16.63*, HSD = 20.47

3.5 Proline and Total Osmolyte Content

It was observed that proline content was enhanced by the metal treatment (23.09 µ mol g^{-1} FW) as compared to control (13.90 µ mol g^{-1} FW) (Table 5). Supplementation of EBL further enhanced the content, 1.20 times more in 10^{-9} M in comparison to Hg stressed seedlings. Total osmolytes content were found to increase in plants due to Hg (167.20 m mol/Kg) than control (156 m mol/Kg). Content was further increased with EBL treatment and maximum level of osmolytes was analyzed 1.09 times higher in seedlings treated with 10^{-9} M EBL (183 m mol/Kg). Proline content was increased during Hg stress. Increase in proline content is due to the stimulation of Δ^{1} pyrroline-5-carboxylate synthase which helps in production of proline during stress conditions. Proline acts as a protectant osmolyte as it scavenge the free radicals and stabilize the membranes [33].

3.6 MDA content

MDA content was found to enhance when plants treated with metal (10.97 µ mol g^{-1} FW) and it reduced with EBL supplementation. 10^{-9} M EBL was found as most effective concentration as it decreased MDA content to7.74 µ mol g^{-1} FW (Table 5). MDA content was found to increase due to Hg stress in present study. As heavy metal triggers the lipid peroxidation and these results are supported by De Britto *et al* [34] in *Capsicum annum*.

Table 5: Effect of different concentrations of 24-EBL on the level of Osmoprotectants and MDA content of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Proline Content (µ mol g^{-1} FW)	Total Osmolyte Content (m mol/Kg)	MDA Content (µ mol g^{-1} FW)
1.	0 (Control)	13.90 ± 1.06	156 ± 1.00	5.81 ± 0.008
2.	10^{-11} M EBL	12.05 ± 1.4	131 ± 0.66	8.72 ± 0.01
3.	10^{-9} M EBL	10.43 ± 0.45	152.5 ± 0.33	10.80 ± 0.009
4.	10^{-7} M EBL	11.76 ± 0.79	143.5 ± 0.33	9.79 ± 0.008
		F-Ratio $_{(df\ 3,8)}$ = 2.08, HSD =	F-Ratio $_{(df\ 3,8)}$ = 291.98*, HSD =	F-Ratio $_{(df\ 3,8)}$ = 4001.46*, HSD

		4.74	3.08	= 0.162
5.	0.25 mM Hg	23.09 ± 2.94	167.2 ± 0.88	10.97 ± 0.008
6.	10^{-11} M EBL + 0.25 mM Hg	25.57 ± 2.71	154 ± 1.66	12.15 ± 0.01
7.	10^{-9} M EBL + 0.25 mM Hg	27.76 ± 2.34	183 ± 1.33	7.74 ± 0.01
8.	10^{-7} M EBL + 0.25 mM Hg	26.86 ± 0.35	168.5 ± 0.58	10.73 ± 0.008
		F-Ratio $_{(df\ 3,8)}$ = 0.76, HSD = 19.13	F-Ratio $_{(df\ 3,8)}$ = 99.52*, HSD = 5.69	F-Ratio $_{(df\ 3,8)}$ = 235.17*, HSD = 0.58

3.7 Antioxidants and Antioxidative Enzymes

With the application of 24-EBL to the seedlings, content of ascorbic acid was observed to increase (Table 6). At 10^{-9} M EBL concentration, ascorbic acid content was found to be maximum (4.63 mg/g fw). Treatment of metal enhanced the level of ascorbic acid in plants and maximum content (4.72 mg/g fw) was observed with 0.25 mM Hg. Tocopherol content was increased in seedlings when 24-EBL treatment was given. Maximum value (6.33 mg/g fw) for tocopherol content was observed with 10^{-11} M EBL and this value was 1.11 folds more as compared to the seedlings subjected to Hg stress. Tocopherol content decreased when metal treatment was given to the seedlings. Minimum content was found to be 4.51 mg/g fw in seedlings treated with 0.25 mM of Hg (Table 6). Glutathione content was slightly reduced in Hg treated plants (9.113 mg/g FW) as compared to control (9.187 mg/g FW). Plants supplemented with EBL further showed decrease in the content. Maximum reduction (3.36 mg/g FW) was observed with 10^{-9} M EBL. Ascorbic acid content was increased with the application of 24-EBL as compared to control. 10^{-9} M EBL (alone) treatment was found more effective for enhancing ascorbic acid content in control plants. During Hg stress, ascorbic acid and tocopherol content enhanced. Glutathione content enhanced only with 10^{-11} M EBL application as compared to control and it also enhanced with Hg treatment. Similar findings were found in *Phragmites australis* under Cd stress (Pietrini *et al*., 2003). Total flavonoid content was stimulated by EBL supplementation in present work as they act as antioxidants and help in scavenging the free radicals.

Increase in protein content was observed in seedlings treated with metal (10.26 mg/g FW) as compared to control (7.97 mg/g FW) (Table 7). With EBL supplementation, protein content was found to reduce and maximum reduction (6.54 mg/g FW) was observed in the seedlings treated with 10^{-11} M EBL. It was observed that specific activity of guaiacol peroxidase was reduced by the metal treatment (0.44 µmole UA mg protein^{-1}) as compared to control (1.09 µmole UA mg protein^{-1}) (Table 7). Treatments of EBL further caused reduction in POD activity upto 0.49 µmole UA mg protein^{-1}. 10^{-9} M EBL enhanced the activity of POD 1.5 folds more as compared to metal treated seedlings. Treatment of metal enhanced the specific activity of catalase (12.8 µmole UA mg protein^{-1}) as compared to control (6.705 µmole UA mg protein^{-1}). However, supplementation of EBL further increased the enzyme activity and most effective concentration was found as 10^{-11} M EBL, which enhanced the activity upto 24.23 µmole UA mg protein^{-1} i.e. 1.89 times higher than that of metal stressed seedlings (Table 7). A very little decrease was observed in the activity of superoxide dismutase (2.46 µmole UA mg protein^{-1}) in seedlings when treated with metal as compared to control (2.64 µmole UA mg protein^{-1}). Application of 24-EBL enhanced the enzyme activity upto 3.87 µmole UA mg protein^{-1}, where 10^{-11} M EBL was recorded to rise the enzyme activity upto 1.57 folds than the seedlings subjected to Hg (Table 7). Activity of ascorbate peroxidase was found to decrease (14.4 µmole UA mg protein^{-1}) in plants treated with Hg as compared to untreated plants (14.95 µmole UA mg protein^{-1}) (Table 7). Application of EBL enhanced the enzyme activity, where maximum increase (1.61 folds) was observed (23.2 µmole UA mg protein^{-1}) with 10^{-11} M EBL as compared to Hg treatment. Specific activity of GR increased (5.38 µmole UA mg protein^{-1}) during metal stress. Increase in enzyme activity was observed with EBL treatments (Table 8). Maximum activity (12.33 µmole UA mg protein^{-1}) was observed with 10^{-11} EBL, i.e., 2.28 times more than metal stressed seedlings. Activity of DHAR was reduced in Hg stressed plants (22.75 µmole UA mg protein^{-1}) as compared to control (23.02 µmole UA mg protein^{-1}) (Table 8). Enzyme activity was found to enhance by EBL treatment. Maximum activity (34.812 µmole UA mg protein^{-1}) was observed in seedlings supplemented with 10^{-11} EBL, which increased enzyme activity 1.53 folds with respect to metal treatment.

Decrease in MDHAR activity was observed when treated with Hg metal (2.95 µmole UA mg protein^{-1}) as compared to untreated plants (4.83 µmole UA mg protein^{-1}). No significant changes in enzyme activity were observed in plants which were supplemented with EBL. (Table 8). Hg treatment caused increased PPO activity (22.45 µmole UA mg protein^{-1}) as compared to control (20.1 µmole UA mg protein^{-1}) (Table 8). Enzyme activity was further enhanced in plants due to EBL treatment, where 10^{-9} M EBL was noticed as most effective concentration as it caused maximum increase (38.1 µmole UA mg protein^{-1}, i.e., 2.24 folds higher than Hg treatment) in activity of PPO. Activity of glutathione-S-transferase was decreased in plants when treated with Hg (0.485 µmole UA mg protein^{-1}), while in control plants, it was 1.43 µmole UA mg protein^{-1}. Whereas EBL application further stimulated the enzyme activity (Table 8). 10^{-11} M EBL caused 1.27 folds increase in the activity of enzyme with respect to metal treated seedlings. Activity of glutathione peroxidase was inhibited with metal stress (0.40 µmole UA mg protein^{-1}) as compared to control (0.53 µmole UA mg protein^{-1}). Enzyme activity was enhanced by 24-EBL and it was increased maximum (0.78 µmole UA mg protein^{-1}) by 10^{-11} M EBL, i.e. 1.95 folds higher in comparison to the seedlings subjected to metal stress (Table 8). 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 growth regulators [35]. Alteration in activities of antioxidative enzymes in the present study was in coherence with the reports of Behnamia *et al* [36].

Table 6: Effect of different concentrations of 24-EBL on Antioxidants of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Ascorbic acid Content (mg/g FW)	Tocopherol Content (mg/g FW)	Glutathione Content (mg/g FW)
1.	0 (Control)	1.60 ± 0.005	5.87 ± 0.01	9.19 ± 0.2
2.	10^{-11} M EBL	2.77 ± 0.01	6.21 ± 0.02	9.59 ± 0.1
3.	10^{-9} M EBL	4.63 ± 0.01	5.39 ± 0.08	7.77 ± 0.3
4.	10^{-7} M EBL	1.78 ± 0.2	6.34 ± 0.3	8.94 ± 0.3
		F-Ratio $_{(df\ 3,8)}$ = 183.28*, HSD = 0.46	F-Ratio $_{(df\ 3,8)}$ = 5.34*, HSD = 0.83	F-Ratio $_{(df\ 3,8)}$ = 8.65*, HSD = 1.2
5.	0.25 mM Hg	4.72 ± 0.01	4.81 ± 0.16	9.11 ± 0.3
6.	10^{-11} M EBL + 0.25 mM Hg	0.87 ± 0.03	3.24 ± 0.01	5.68 ± 0.1
7.	10^{-9} M EBL + 0.25 mM Hg	1.61 ± 0.02	2.72 ± 0.07	3.36 ± 0.02
8.	10^{-7} M EBL + 0.25 mM Hg	0.82 ± 0.01	4.02 ± 0.03	5.81 ± 0.5
		F-Ratio $_{(df\ 3,8)}$ = 5923.99*, HSD = 0.11	F-Ratio $_{(df\ 3,8)}$ = 93.32*, HSD = 0.43	F-Ratio $_{(df\ 3,8)}$ = 47.29*, HSD = 1.56

Table 7: Effect of different concentrations of 24-EBL on Protein Content and Antioxidative Enzyme activities of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	Protein Content (mg/g FW)	POD (µmole UA mg protein^{-1})	CAT (µmole UA mg protein^{-1})	SOD (µmole UA mg protein^{-1})	APOX (µmole UA mg protein^{-1})
1.	0 (Control)	7.97 ± 0.48	1.09 ± 0.06	6.70 ± 0.8	2.64 ± 0.3	14.95 ± 0.25
2.	10^{-11} M EBL	7.43 ± 0.46	0.16 ± 0.03	9.23 ± 0.41	3.23 ± 0.2	17.75 ± 0.05
3.	10^{-9} M EBL	9.06 ± 0.28	0.2 ± 0.009	6.82 ± 0.6	2.13 ± 0.13	11.13 ± 0.3
4.	10^{-7} M EBL	8.22 ± 0.33	0.38 ± 0.002	4.95 ± 0.3	3.15 ± 0.04	13.7 ± 0.8
		F-Ratio $_{(df\ 3,8)}$ = 2.92, HSD = 1.80	F-Ratio $_{(df\ 3,8)}$ = 88.07*, HSD = 0.21	F-Ratio $_{(df\ 3,8)}$ = 15.14*, HSD = 2.05	F-Ratio $_{(df\ 3,8)}$ = 5.117*, HSD = 1.02	F-Ratio $_{(df\ 3,8)}$ = 37.53*, HSD = 2.03
5.	0.25 mM Hg	10.26 ± 0.49	0.44 ± 0.02	12.8 ± 1.12	2.46 ± 0.2	14.4 ± 0.8
6.	10^{-11} M EBL + 0.25 mM Hg	6.54 ± 0.43	0.55 ± 0.008	24.23 ± 4.7	3.87 ± 0.2	23.2 ± 0.7
7.	10^{-9} M EBL + 0.25 mM Hg	7.53 ± 0.47	0.66 ± 0.007	8.66 ± 0.32	3.19 ± 0.2	19.65 ± 0.85
8.	10^{-7} M EBL + 0.25 mM Hg	7.55 ± 0.05	0.49 ± 0.001	3.96 ± 0.04	3.33 ± 0.3	17.5 ± 0.65
		F-Ratio $_{(df\ 3,8)}$ = 15.41*, HSD = 1.84	F-Ratio $_{(df\ 3,8)}$ = 0.76, HSD = 0.50	F-Ratio $_{(df\ 3,8)}$ = 4.92*, HSD = 17.69	F-Ratio $_{(df\ 3,8)}$ = 4.01, HSD = 1.31	F-Ratio $_{(df\ 3,8)}$ = 52.21*, HSD = 2.32

Table 8: Effect of different concentrations of 24-EBL on Antioxidative Enzyme activities of 7 days old seedlings of *Raphanus sativus* L. under Hg stress.

S. No.	Treatments	GR (µmole UA mg protein^{-1})	DHAR (µmole UA mg protein^{-1})	MDHAR (µmole UA mg protein^{-1})	PPO (µmole UA mg protein^{-1})	GST (µmole UA mg protein^{-1})	GPOX (µmole UA mg protein^{-1})
1.	0 (Control)	2.75 ± 0.56	23.02 ± 4.09	4.83 ± 0.5	20.1 ± 1.3	1.43 ± 0.05	0.53 ± 0.05
2.	10^{-11} M EBL	10.51 ± 1.58	20.95 ± 3.38	7.54 ± 0.88	38.1 ± 4	0.74 ± 0.09	0.64 ± 0.01
3.	10^{-9} M EBL	5.72 ± 0.81	17.97 ± 1.06	9.76 ± 1.28	23.8 ± 0.2	0.33 ± 0.03	0.56 ± 0.05
4.	10^{-7} M EBL	10.09 ± 1.31	23.15 ± 1.35	6.85 ± 0.42	34.7 ± 2.3	0.85 ± 0.08	0.79 ± 0.12
		F-Ratio $_{(df\ 3,8)}$ = 10.57*, HSD = 5.45	F-Ratio $_{(df\ 3,8)}$ = 0.75, HSD = 13.33	F-Ratio $_{(df\ 3,8)}$ = 1.72, HSD = 7.41	F-Ratio $_{(df\ 3,8)}$ = 26.07*, HSD = 7.62	F-Ratio $_{(df\ 3,8)}$ = 12.70*, HSD = 0.59	F-Ratio $_{(df\ 3,8)}$ = 2.66, HSD = 0.34
5.	0.25 mM Hg	5.38 ± 0.22	22.76 ± 2.72	2.95 ± 0.24	22.45 ± 3.7	0.48 ± 0.01	0.40 ± 0.01
6.	10^{-11} M EBL + 0.25 mM Hg	12.33 ± 1.73	34.81 ± 1.60	2.46 ± 0.48	26.33 ± 3.9	0.61 ± 0.01	0.78 ± 0.06
7.	10^{-9} M EBL + 0.25 mM Hg	5.65 ± 0.35	28.93 ± 1.56	2.78 ± 0.50	50.3 ± 4.1	0.62 ± 0.07	0.54 ± 0.03
8.	10^{-7} M EBL + 0.25 mM Hg	12.09 ± 2.36	33.92 ± 3.23	6.37 ± 0.77	43.43 ± 2.7	0.40 ± 0.05	2.22 ± 0.2
		F-Ratio $_{(df\ 3,8)}$ = 6.85*, HSD = 7.07	F-Ratio $_{(df\ 3,8)}$ = 5.36*, HSD = 11.43	F-Ratio $_{(df\ 3,8)}$ = 11.77*, HSD =2.55	F-Ratio $_{(df\ 3,8)}$ = 16.36*, HSD = 14.98	F-Ratio $_{(df\ 3,8)}$ = 1.65, HSD = 0.37	F-Ratio $_{(df\ 3,8)}$ = 5.73*, HSD = 1.58

Data shown are Mean ± SE. Each treatment consisted of three replicates.
* Statistically significant differences from control at $P \leq 0.05$.

4. Conclusion

Results of present study revealed that Hg metal toxicity caused deleterious effects on *Raphanus sativus* seedlings by retarding their growth, altering the level of ions and pigments. On the same way exposure of seedlings to 24-EBL helped in overcoming the Hg stress by enhancing the activities of antioxidative enzymes, contents of antioxidants and osmolytes that led to removal of free radicals generated during oxidative stress.

5. References

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