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Effect of 24-Epibrassinolide on Protein Content and Activities of Glutathione-*S*-Transferase and Polyphenol Oxidase in *Raphanus sativus* L. Plants under Cadmium and Mercury Metal Stress

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ABSTRACT

Heavy metal toxicity results in oxidative stress in plants. Cadmium and mercury are non-essential elements for plants and are thus toxic even at low concentrations. Brassinosteroids, an important group of plant hormones have been reported to ameliorate abiotic stress in plants. The present study was undertaken to evaluate the role of 24-epibrassinolide in ameliorating the stress caused by Cd and Hg metals in raddish plants. The seeds of *Raphanus sativus* L. were soaked in 24-epibrassinolide (0, 10^{-7}, 10^{-9}, 10^{-11} M) and were sown in soil medium containing Cd and Hg (0, 0.5, 1.0, 1.5 mM). Oxidative stress caused by heavy metals was assessed by studying the protein content and activities of Glutathione-*S*-transferase (GST) and Polyphenol oxidase (PPO) enzyme activities in 60 and 90 days old raddish plants. Results revealed that presence of metals in the soil medium lead to decrease in protein content which was improved with the treatment of 24-epibrassinolide. The treatment of metals enhanced the antioxidative enzymes activities. These activities were further enhanced by 24-epibrassinolide treatments.

Keywords: *Raphanus sativus* L., cadmium, mercury, 24-epibrassinolide, glutathione-*S*-transferase, polyphenol oxidase

Abbreviations: ABA, abscisic acid, **ANOVA**, analysis of variance; **ATP**, adenosine triphosphate, **CDNB**, 1-chloro-2,4-dinitrobenzene, **EDTA**, ethylenediamine tetraacetic acid, **H_2SO_4**, sulphuric acid, **JA**, jasmonic acid, **NaCl**, sodium chloride, **PPB**, potassium phosphate buffer, **PS**, photosystem, **-SH**, sulphydryl group, **UA**, unit activity, **UV**, ultraviolet

INTRODUCTION

Heavy metals are major environmental contaminants and their release in biologically active forms, as a result of anthropogenic activities, can damage natural as well as man-made ecosystems (Tyler *et al.* 1989). Contamination of soils with heavy metals has become a problem in many countries all over the world. The effects of soil contamination with heavy metals viz., cadmium (Cd), copper (Cu) and zinc (Zn) on alfalfa, lettuce, radish and *Thlaspi caerulescens* were studied by a mathematical interaction model. The effects of heavy metals was moderate at low concentrations and the dynamics was observed to be linear. However, an increase in concentrations lead to nonlinear behaviours (Guala *et al.* 2010). Inactivation of enzymes, blocking of functional groups of metabolically important molecules, displacment or substitution of essential elements and disruption of membrane integrity may be attributed to heavy metal phytotoxicity (Rascio *et al.* 2011). The accumulation of these metals in the plants grown in contaminated soils allows their entry into the food chain with great risks for human health (Keltjens *et al.* 1998). Metals like Cd and mercury (Hg) are the non essential metals and cause toxicity when present in amounts more than endurable limits. These may disrupt enzyme functions, replace essential metals in pigments (Van Assche *et al.* 1990) and may produce reactive oxygen species resulting in oxidative stress (Dietz *et al.* 1999). Cd enters the environment through traffic, metal-working industries, as a byproduct of mineral fertilizers and mining activities. Cd ion brings biochemical and physiological changes and thus leads to phytotoxicity (Benavides *et al.* 2005; Gratão *et al.* 2005). The toxicity symptoms of Cd include leaf chlorosis, stunted growth and even death (Baryla *et al.* 2001; Mallick *et al.* 2003). Hg is emitted in the atmosphere from natural as well as anthropogenic sources. Natural sources of mercury are volcanoes, evasion from superficial soils, vegetation surface and wild fires while combustion of coal, by products of electrochemical industry, fungicides and sewage sediments are main anthropogenic causes (Li *et al.* 2009). General effects of Hg in cells include changes in cell permeability, reactivity with –SH groups of proteins and ATP binding capability and thus its activity. It shows its harmful effects on photosynthetic membranes but membrane of PS II is most sensitive to Hg contamination (de Filippis *et al.* 1981).

Plants have enzymatic and non-enzymatic antioxidant molecules to deal with the oxidative stress caused by the production of free radicals under stress conditions (Foyer *et al.* 2003). Plant hormones, such as ethylene, ABA, salicylic acid, JA, auxins and brassinosteroids (BRs), have been found to be involved in modulating the plant responses to oxidative stress (Cao *et al.* 2005; Tuna *et al.* 2008). BRs are the upcoming group of phytohormones which are regarded as sixth group of plant growth regulators (Clouse *et al.* 1998; Bhardwaj *et al.* 2006). The structure of BRs is similar to animal steroid hormones and these are widely distributed in the plant kingdom (Mandava 1988; Clouse *et al.* 1998; Bhardwaj *et al.* 2006).

The role of BRs during environmental stresses have gained much attention such as conferring stress protection to plants against various biotic stresses like fungal (Churikova *et al.* 1997), bacterial (Rodkin *et al.* 1997), viral (Romanutti *et al.* 2007), cancer (Malíková *et al.* 2008) and abiotic stresses like heat (Sasse 2006), chilling (Huang *et al.* 2006), drought (Kagale *et al.* 2007) and heavy metals stress (Janeczko *et al.* 2005). In an experiment on two tomato cultivars, grown under cadmium metal stress, BRs were supplied with in the form of a foliar spray. In the activity of

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both photosynthetic machinery and antioxidant defence system, an improvement was observed with the application of BRs in both cultivars (Hasan *et al.* 2011).

24-Epibrassinolide (24-epiBL) is one of the most active brassinosteroids. It induces a large range of cell responses which include plant growth, seed germination and nitrogen fixation. It also improves the resistance of plants towards cold, pathogens and salt stress (Kulaeva *et al.* 1991). Exogenous application of 24-epiBL has been reported to have varying effects as activity of superoxide dismutase (SOD) remained unaffected in hexaploid wheat (*Triticuma estivum* L.) cultivars, 'S-24' (salt tolerant) and 'MH-97' (moderately salt sensitive), grown under saline conditions (150 mM of NaCl) but that of peroxidase (POD) and catalase (CAT) was promoted in the salt stressed plants of cv. S-24 only (Shahbaz *et al*. 2008). In *Brassica juncea* seedlings, application of 24-epiBL at 10^{-9} and 10^{-11} M blocked heavy metal uptake and accumulation (Sharma *et al.* 2007). *B. juncea* plants grown under Ni metal stress and treated with a foliar spray of 24-epiBL, isolated from Ni-stressed *B. juncea* plants, there was a lower metal uptake and increase in the activity of antioxidative enzymes (Kanwar *et al.* 2012).

Glutathione-*S*-transferases (GSTs, EC.2.5.1.18) are multifunctional enzymes which detoxify endobiotic and xenobiotic compounds by conjugating glutathione (GSH) to a hydrophobic substrate. As a result, a water-soluble and less toxic glutathione S-conjugates are formed that are coupled to internal compartmentation due to the lack of effective excretion pathways (Sandermann 1992; Rea 1999). Polyphenol oxidase (PPO, *o*-diphenol: oxygen oxidoreductase, EC1.14.18.1), is a copper-containing enzyme. It catalyzes the oxidation of phenols to the respective quinones. PPO is found in the chloroplast in healthy plant cells, although it is synthesized in the cytoplasm under nuclear control (Lax *et al.* 1984).

Radish plant is an important medicinal plant. Radish roots stimulate the appetite and digestion because they have a tonic and laxative effect upon the intestine and indirectly stimulating the flow of bile (Chevallier 1996). Leaves, seeds and old roots of radish plants are very useful in the treatment of asthma and other chest complaints (Duke *et al.* 1985). Radish plants are also known as hyperaccumulators of heavy metals (Máthé-Gáspár *et al.* 2002). Also, BRs have also been found to be present in significant amounts. The BRs isolated from radish plants are teasterone, brassinolide, castasterone and 28-homoteasterone (Schmidt *et al.* 1991, 1993).

In this study, we investigated the protein content of plants treated with Cd and Hg and variations in the activities of GST and PPO that have been associated with the possible role of 24-epiBL against Cd and Hg stress in radish plants.

MATERIALS AND METHODS

Field experiment

To study the effects of 24-epiBL on the biochemical parameters of radish plants grown under Cd and Hg metal stress, a field experiment was conducted in Botanical Garden of Guru Nanak Dev University, Amritsar. The certified and disease free seeds of *Raphanus sativus* L. var. 'Pusa chetaki' were procured from Punjab Agricultural University, Ludhiana, Punjab. The 24-Epibrassinolide used for the study was purchased from Sigma-Aldrich Ltd., New Delhi. The seeds were surface sterilized with 0.01% sodium hypochlorite for 2 min followed by rinsing 5 times with distilled water. The seeds were then soaked in different concentrations (0, 10^{-7}, 10^{-9}, 10^{-11} M) of 24-epiBL for 8 h prior to sowing. A piece of land (10 × 11 feet) was used to raise the plants. The soil was arranged in form of crests and troughs and was supplied with Cd and Hg metals at the concentrations 0, 0.5, 1.0, 1.5 mM. The plants were regularly irrigated with the respective metal solutions. The shoots of 60-and 90-days-old plants were then subjected to biochemical analysis.

Biochemical analysis

1. Preparation of extracts

The apical leaves of both 60- and 90-days-old plants were harvested at respective time period and (5 g) were homogenized in 50 mM phosphate buffer [pH 7.0, EDTA (1 mM), Triton X-100 (0.5%)] in a pre-chilled pestle and mortar. The homogenate was centrifuged at 13,000 × *g* for 20 min at 4°C. The supernatant was then used for assessing the protein content and activities of GST and PPO enzymes using UV-Visible PC-based Double Beam Spectrophotometer (Systronics 2202).

2. Protein content

The total protein content in the shoots was estimated by following the method of Lowry *et al.* (1951) using bovine serum albumin as standard. A graph of absorbance vs concentration for standard solutions of protein was plotted and the amount of protein in the sample was calculated from the graph. The amount of protein is expressed as mg/g tissue.

3. GST assay

GST (EC.2.5.1.18) activity was estimated using the method proposed by Habig *et al.* (1974). The method is based on the reaction of the GSTs in a mixture of CDNB (20 mM) and GSH (100 mM). The change in optical density due to the emergence of complex CDNB-GSH is measured spectrophotometrically every 15 sec for 2 min at 340 nm. The assay mixture (2.25 ml) contained 2 ml PPB (0.2 M, pH 7.4), 100 µl GSH (20 mM), 100 µl CDNB (20 mM) and 50 µl enzyme sample. The concentration of GST was expressed in µmole UA mg^{-1} protein. Unit activity (UA) is defined as the change in absorbance by 0.1 min^{-1} mg^{-1} protein.

4. PPO assay

PPO (EC 1.10.3.1) activity was assayed by the method of Kumar *et al.* (1982). The assay mixture for PPO contained 2 mL of 0.1 M phosphate buffer (pH 7.0), 1 mL of 0.1 M catechol and 0.5 mL of enzyme extract. This was incubated for 5 min at 25°C, after which the reaction was stopped by adding 1 mL of 2.5 N H_2SO_4. The absorbance of the benzoquinone formed was read at 495 nm. To the blank 2.5 N H_2SO_4 was added of the zero time of the same assay mixture. PPO activity is expressed in µmole UA mg^{-1} protein. (UA = change in absorbance by 0.1 min^{-1} mg^{-1} protein).

Statistical analysis

All the experiments were performed in triplicates and the values presented here are the mean of three values ± standard error. The statistical differences between means were assessed with one-way ANOVA according to the methodology proposed by Bailey (1995) using Microsoft excel. A significant difference was evaluated at a level of $P < 0.05$.

RESULTS

Metal-induced oxidative stress in plants is generally studied through their biochemical responses and interpreted in terms of their variation trend and patterns (Schutzendubel and Polle 2002). Both the heavy metals *viz.*, Cd and Hg significantly affected the protein content as well as the activities of GST and PPO in the shoots of 60 and 90 days old radish plants. Seed presoaking treatment with 24-epiBL considerably enhanced the protein content and activities of enzymes under the heavy metals stress.

Effects of cadmium

1. Protein content

The presence of Cd metal in the soil medium resulted in a decrease in the protein content of shoots of both 60- and 90-days-old plants. Among 60-days-old shoots, maximum de-

Table 1 Effect of 24-epiBL on protein content (mg/g f.w.) in 60-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	616.7 ± 2.96	296.6 ± 3.18	305.9 ± 1.0	332.6 ± 0.88
10^{-11} EBL	542.6 ± 0.88	374.6 ± 0.33	370.2 ± 0.33	406.2 ± 0.88
10^{-9} EBL	622.9 ± 1.15	384.2 ± 11.17	512.2 ± 4.05	400.9 ± 7.21
10^{-7} EBL	531.6 ± 3.93	421.0 ± 4.16	533.9 ± 9.53	386.9 ± 1.0
F value (HSD)	353.99* (12.2)	0.581 (158)	234.54* (24.9)	328.70* (17.8)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 2 Effect of 24-epiBL on protein content (mg/g f.w.) in 90-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	298.2 ± 0.86	163.6 ± 2.72	164.9 ± 2.0	122.6 ± 1.52
10^{-11} EBL	246.6 ± 1.20	229.2 ± 3.66	264.2 ± 1.85	376.9 ± 2.0
10^{-9} EBL	328.9 ± 0.55	351.6 ± 3.28	462.6 ± 2.66	367.6 ± 1.20
10^{-7} EBL	345.4 ± 2.51	324.6 ± 2.18	307.5 ± 2.51	296.6 ± 1.85
F value (HSD)	855.63* (7.1)	826.46* (14.4)	4180.5* (9.01)	4934.24* (8.0)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 3 Effect of 24-epiBL on GST activity (unit activity/mg protein) in 60-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.058 ± 0.02	0.074 ± 0.02	0.076 ± 0.02	0.113 ± 0.02
10^{-11} EBL	0.103 ± 0.011	0.064 ± 0.017	0.139 ± 0.007	0.154 ± 0.004
10^{-9} EBL	0.062 ± 0.015	0.110 ± 0.035	0.136 ± 0.005	0.198 ± 0.031
10^{-7} EBL	0.086 ± 0.018	0.059 ± 0.023	0.146 ± 0.011	0.242 ± 0.064
F value (HSD)	0.83 (0.66)	0.98 (0.12)	0.92 (0.09)	2.27 (0.17)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 4 Effect of 24-epiBL on GST activity (unit activity/mg protein) in 90-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	**GST activity** (unit activity/mg protein) **in 90 days old shoots**			
	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.050 ± 0.013	0.200 ± 0.05	0.280 ± 0.082	0.110 ± 0.24
10^{-11} EBL	0.190 ± 0.04	0.177 ± 0.025	0.142 ± 0.01	0.133 ± 0.027
10^{-9} EBL	0.066 ± 0.013	0.049 ± 0.004	0.105 ± 0.039	0.076 ± 0.007
10^{-7} EBL	0.155 ± 0.004	0.201 ± 0.116	0.116 ± 0.032	0.174 ± 0.009
F value (HSD)	4.99* (0.11)	7.02* (0.13)	2.40 (0.24)	11.82* (0.07)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 5 Effect of 24-epiBL on PPO activity (unit activity/mg protein) in 60-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	1.562 ± 0.06	2.397 ± 0.06	2.573 ± 0.06	2.152 ± 0.17
10^{-11} EBL	1.660 ± 0.03	1.619 ± 0.06	1.186 ± 0.03	2.322 ± 0.05
10^{-9} EBL	1.119 ± 0.03	1.491 ± 0.08	1.236 ± 0.05	1.631 ± 0.03
10^{-7} EBL	1.233 ± 0.12	1.596 ± 0.07	1.651 ± 0.11	1.524 ± 0.10
F value (HSD)	24.13* (0.35)	51.62* (0.32)	81.36*(0.34)	13.25* (0.51)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 6 Effect of 24-epiBL on PPO activity (unit activity/mg protein) in 90-days-old shoots of *Raphanus sativus* L. plants grown under cadmium metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	1.304 ± 0.02	1.956 ± 0.03	2.455 ± 0.04	1.779 ± 0.04
10^{-11} EBL	1.763 ± 0.03	1.182 ± 0.01	2.932 ± 0.03	2.078 ± 0.05
10^{-9} EBL	1.774 ± 0.01	1.968 ± 0.02	0.864 ± 0.02	2.157 ± 0.03
10^{-7} EBL	1.140 ± 0.05	2.278 ± 0.04	1.788 ± 0.06	1.797 ± 0.05
F value (HSD)	199.53* (0.11)	417.70* (0.13)	794.48* (0.15)	18.07* (0.21)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

crease was at a concentration of 0.5 mM Cd. The concentration of 1.5 mM Cd decreased protein content to the maximum in 90-days-old shoots. In case of plants treated with metal and 24-epiBL both, the presence of 24-epiBL increased the protein content and 10^{-7} and 10^{-9} M were found to be the most effective in 60- and 90-days-old shoots, respectively (**Tables 1, 2**).

2. GST activity

In case of 60-days-old shoots, specific activity of GST (µmole UA mg^{-1} protein) increased with increasing metal concentrations with a maximum in 1.5 mM Cd compared to the control. 24-epiBL further enhanced the GST activity with all concentrations of Cd metal. Maximum effect was shown by 10^{-7} M in 1.5 mM Cd and 1 mM Cd. In case of plants treated with 0.5 mM Cd, results were variable, GST activity decreased with application of 24-epiBL, except for 10^{-9} M 24-epiBL (**Table 3**). However, among 90-days-old plants, the GST specific activity raised to the maximum in 1.0 mM Cd-treated shoots compared to the control. 10^{-11} M of 24-epiBL was most effective with different metal treatments and maximum effect was found with 0.5 mM Cd (**Table 4**).

Table 7 Effect of 24-epiBL on protein content (mg/g f.w.) in 60-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.434 ± 0.007	0.299 ± 0.008	0.378 ± 0.012	0.356 ± 0.02
10^{-11} EBL	0.293 ± 0.009	0.348 ± 0.01	0.290 ± 0.011	0.301 ± 0.016
10^{-9} EBL	0.303 ± 0.01	0.410 ± 0.009	0.264 ± 0.007	0.377 ± 0.01
10^{-7} EBL	0.488 ± 0.009	0.396 ± 0.02	0.422 ± 0.01	0.315 ± 0.007
F value (HSD)	125.7* (0.04)	18.1* (0.06)	52.32* (0.05)	6.79* (0.06)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 8 Effect of 24-epiBL on protein content (mg/g f.w.) in 90-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.239 ± 0.001	0.230 ± 0.02	0.307 ± 0.01	0.365 ± 0.007
10^{-11} EBL	0.266 ± 0.009	0.249 ± 0.015	0.207 ± 0.01	0.277 ± 0.01
10^{-9} EBL	0.263 ± 0.009	0.256 ± 0.012	0.230 ± 0.004	0.338 ± 0.008
10^{-7} EBL	0.226 ± 0.008	0.221 ± 0.012	0.230 ± 0.004	0.338 ± 0.008
F value (HSD)	6.44* (0.036)	1.82 (0.068)	19.98* (0.05)	9.22* (0.051)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 9 Effect of 24-epiBL on GST activity (unit activity/mg protein) in 60-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

reatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.012 ± 0.003	0.144 ± 0.016	0.0139 ± 0.02	0.094 ± 0.023
10^{-11} EBL	0.012 ± 0.003	0.044 ± 0.018	0.115 ± 0.011	0.152 ± 0.013
10^{-9} EBL	0.199 ± 0.011	0.108 ± 0.022	0.068 ± 0.011	0.141 ± 0.018
10^{-7} EBL	0.041 ± 0.003	0.219 ± 0.01	0.073 ± 0.007	0.144 ± 0.007
F value (HSD)	22.14* (0.029)	6.932* (0.13)	7.99* (0.05)	3.334 (0.06)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 10 Effect of 24-epiBL on GST activity (unit activity/mg protein) in 90-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.090 ± 0.01	0.211 ± 0.014	0.113 ± 0.01	0.088 ± 0.006
10^{-11} EBL	0.128 ± 0.002	0.070 ± 0.01	0.394 ± 0.04	0.016 ± 0.006
10^{-9} EBL	0.099 ± 0.015	0.129 ± 0.009	0.313 ± 0.05	0.046 ± 0.016
10^{-7} EBL	0.107 ± 0.01	0.090 ± 0.026	0.024 ± 0.01	0.129 ± 0.011
F value (HSD)	1.210 (0.06)	16.25* (0.07)	25.03* (0.16)	8.99* (0.07)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

3. PPO activity

Specific activity of PPO was observed to increase considerably under Cd metal stress with a maximum value observed with 1.0 mM Cd in 60-days-old plants. Treatment with 24-epiBL alone, as well as in combinations with Cd metal, increased the values to the maximum in 1.5 mM Cd treatment supplemented with 10^{-11} 24-epiBL. 10^{-9} M 24-epiBL showed a negative effect with various concentrations studied (**Table 5**). Different was the case with 90-days-old shoots. In response to various concentrations of 24-epiBL, 10^{-9} M 24-epiBL was effective with 1.0 mM Cd while 0.5 mM Cd showed maximum increase with 10^{-7} M 24-epiBL and with 1.5 mM Cd, 10^{-9}M 24-epiBL enhanced the activity to the maximum (**Table 6**).

Effects of mercury

1. Protein content

In 60-days-old shoots of *R. sativus* plants, Hg metal stress resulted in decreased protein content as compared control plants. Seed presoaking treatments with 10^{-7} M and 10^{-9} M 24-epiBL significantly increased the protein content in plants treated with 0.5 mM Hg (**Table 7**). In 90-days-old radish shoots, except for 10^{-7} M 24-epiBL, for which protein content was lower than water-treated control plants, seed presoaking treatment with 24-epiBL increased protein content in Hg-stressed plants (**Table 8**).

2. GST activity

GST activity increased considerably in the presence of Hg with a maximum increase with 0.5 mM Hg treatment in both 60- and 90-days-old shoots. Treatment with 24-epiBL was able to increase GST activity to a considerable amount and maximum activity was observed in the presence of 1.5 mM Hg in 60-days-old shoots and in the presence of 1.0 mM Hg among 90-days-old shoots. 10^{-11} M 24-epiBL was the most effective concentration (**Tables 9, 10**).

3. PPO activity

In 60-days-old shoots, Hg treatment showed an increase in the specific activity of PPO with a maximum increase at 1.0 mM. The presence of 24-epiBL alone did not show any increase but when combined with Hg treatments, 24-epiBL significantly increased the activity; maximum increase was observed in plants supplemented with 10^{-7} M 24-epiBL and 1.0 mM Hg (**Table 11**). In 90-days-old shoots, 24-epiBL treatments alone significantly enhanced PPO activity except for 10^{-11} M 24-epiBL. Treatment of seeds with 10^{-7} M 24-epiBL before sowing them effectively increased the enzyme activity at all metal concentrations. However, treatment of 10^{-11} M and 10^{-9} M 24-epiBL along with metal only enhanced the enzyme activity when the metal concentration was 0.5 mM and 1 mM Hg, respectively compared to only metal-treated plants (**Table 12**).

DISCUSSION

The observations in the present study revealed that 24-epiBL application to *R. sativus* plants grown under Cd and Hg metal stress resulted in an increased protein content as well as increased activity of antioxidative enzymes viz, GST and PPO, which is consistent with Nunez *et al.* (2003),

Table 11 Effect of 24-epiBL on PPO activity (unit activity/mg protein) in 60-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	0.583 ± 0.037	0.854 ± 0.089	0.967 ± 0.024	0.937 ± 0.09
10^{-11} EBL	0.380 ± 0.025	1.456 ± 0.065	1.057 ± 0.035	1.240 ± 0.119
10^{-9} EBL	0.306 ± 0.024	1.248 ± 0.037	1.213 ± 0.051	0.410 ± 0.054
10^{-7} EBL	1.171 ± 0.046	1.103 ± 0.029	2.352 ± 0.024	1.611 ± 0.089
F value (HSD)	142.8* (0.15)	17.66* (0.28)	7.50* (1.30)	15.37* (0.63)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

Table 12 Effect of 24-epiBL on PPO activity (unit activity/mg protein) in 90-days-old shoots of *Raphanus sativus* L. plants grown under mercury metal stress (mean ± S.E.).

Treatments	Control (DW)	0.5 mM Cd	1.0 mM Cd	1.5 mM Cd
Control (DW)	1.898 ± 0.171	2.135 ± 0.108	2.183 ± 0.19	2.051 ± 0.056
10^{-11} EBL	1.531 ± 0.076	3.276 ± 0.071	1.219 ± 0.09	1.406 ± 0.101
10^{-9} EBL	2.579 ± 0.289	1.730 ± 0.076	2.922 ± 0.13	1.410 ± 0.04
10^{-7} EBL	2.968 ± 0.256	2.160 ± 0.10	2.496 ± 0.12	3.042 ± 0.18
F value (HSD)	10.1* (1.02)	58.71* (0.41)	26.16* (0.67)	48.54* (0.53)

*statistically significant values at $P \leq 0.05$. DW: distilled water.

who revealed that the application of BRs caused the activation of antioxidative enzymes under water and salt stresses and increased SOD and proline contents under NaCl stress. Zhu *et al.* (2010) investigated the effect of BRs against blue mould rot caused by *Penicillium expansum* and on the senescence of harvested jujube fruit. It was observed that BRs not only inhibited development of blue mould rot effectively, but also enhanced the activities of defense-related enzymes. Similarly, in a study conducted by Anuradha *et al.* (2007) *R. sativus* plants were grown under cadmium stress. The effect of exogenous application of 24-epiBL and 28-homobrassinolide (28-homoBL) on seed germination and seedling growth was studied. It was observed that BRs treated seedlings were able to overcome the toxic effects of heavy metal and increased the percentage of seed germination and seedling growth. The treatment of 24-epiBL resulted in increase content of chl-*a*, chl-*b* and carotenoids in the rape leaves under cold treatment at 2°C (Janeczko *et al*. 2007) and enhanced activities of POD and SOD and glutathione content as compared to control in suspension cultured cells of *Chorispora bungeana* (Liu *et al.* 2009). Rady (2011) reported that the exposure of *Phaseolus vulgaris* L. plants to NaCl and/or $CdCl_2$ resulted in a significant decline in growth, level of pigment parameters, green pod yield and pod protein. However, treatment with 24-epiBL ameriolated the stress caused by NaCl and/or $CdCl_2$ and significantly improved all the respective parameters under study.

The protein content in both 60- and 90-days-old radish plants decreased due to Cd and Hg metal treatment. Decrease in the protein content was also observed under Cr metal stress in *Ocimum tenuiflorum* L. (Rai *et al*. 2004), *Citrullus vulgaris* (Tiwari *et al.* 2008), and *Vigna radiata* L. cv. 'Wilczek' (Karuppanapandian *et al*. 2007). In contrast, application of BRs led to an increase in protein levels. Similar results were obtained in our previous study conducted on *Brassica juncea* seedlings grown under Zn metal stress (Sharma *et al.* 2007). The study revealed that that 28-homoBL seed pre-sowing treatments enhanced protein content in 7-days old *B. juncea* seedlings under Zn metal stress conditions as compared to control. The application of epibrassinolide on winter rape plants under Cd stress have also shown stress-protective effects (Janeczko *et al.* 2005). 24-epiBL and 28-homoBL enhanced the protein content in *Oryza sativa* (Anuradha and Rao 2003) and in wheat (Kulaeva *et al.* 1991). It has been reported that BRs induced increase in protein is related to stress protective mechanism of plants (Khripach *et al.* 1999). In a study conducted by Müssig *et al.* (2002), the oxidative stress-related genes were identified. These genes were responsible for encoding monodehydroascorbate reductase and thioredoxin-h, the cold and drought reponse genes and genes related to heat stress (HSP-83, HSP-70). The study was conducted through microarray analysis of either BR deficient or BR treated plants. Yuan *et al.* (2010) studied the effect of 24-epiBL on relative water content (RWC), stomatal conductance (gs), net photosynthetic rate (PN), intercellular CO_2 concentration (Ci), lipid peroxidation level, activities of antioxidant enzymes and abscisic acid concentration (ABA) in tomato (*Lycopersicon esculentum*) seedlings under water stress. The RWC, gs, Ci and PN were found to decrease under water stress. However, treatment with 24-epiBL considerably increased the RWC and PN as well as the activities of antioxidant enzymes (catalase, ascorbate peroxidase and superoxide dismutase) while it decreased gs, Ci and content of H_2O_2 and malondialdehyde (MDA).

GST and PPO play a key role in plant defence systems (Shi *et al*. 2001; Öztetik 2008). Increase in the activities of these enzymes in response to stress helps the plant to withstand the effects of stress. Several workers have reported an increase in their activity in the presence of various types of stresses.

The increase in GST activity in the present study is in accordance with the findings of Marrs and Walbot (1997). It was observed that Cd strongly induced maize GST Bronze2 (*Bz2*) gene and GSTIII gene in maize plants. As a result of cadmium treatment spliced *Bz2* increased 20-fold and unspliced *Bz2* increased more than 50-fold than the levels of unspliced *Bz2* RNA in control protoplasts. B37, which is a maize inbred line, was the only one to show a Cd-responsive GST activity. Mauch *et al.* (1993) also observed that presence of Cd increased wheat GST25 and GST26 gene activities. An increase in GST activity was also reported by Cataneo *et al.* (2002). An experiment was conducted to evaluate the acetochlor, atrazine and oxyfluorfen herbicides plant selectivity, in relation to GST in plants of the Poaceae family *viz.*, maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) and wheat (*Triticum aestivum* L.). GST activity was detected after 24, 48 and 72 h of treatment applications. The activity of GST was found to increase. The highest GST activity was observed in presence of acetochlor, at 48 h after treatment. Qi *et al.* (2010) introduced the *Suaeda salsa* GST gene into *Arabidopsis* under the control of the cauliflower mosaic virus 35S promoter and noticed higher GST and Glutathione peroxidise (GPX) activities in transgenic plants (GT) than in wild type plants (WT). Further, it was reported that the expression of the GST gene was the reason for a higher level of salt tolerance in transgenic *Arabidopsis* plants.

An increase in the activity of PPO was observed by Jayakumar *et al*. (2007), in *Raphanus sativus* plants with the treatment of Cobalt (Co) metal. In another experiment conducted on *Vigna radiata* (L.) by Jayakumar *et al*. (2009) similar increase in activity of PPO was observed under Co stress. The seedlings of *Phaseolus trilobus*, commonly used as green manure and fodder, were subjected to UV-B radiation and an increase in the activity of PPO was observed (Ravindran *et al.* 2008). A similar trend where activity was increased with increase in the metal concentration was observed in *Arachis hypogaea* L by Jaleel *et al*. (2008) and in *Vigna radiata* (L.) by Azooz *et al*. (2008). Further, in response to drought stress in leaves of *Dolichos lablab*, an increase in PPO activity has been reported (D'Souza *et al.* 2011).

CONCLUSION

In the present study, the decrease in protein content and increase in activities of GST and PPO enzymes under Cd and Hg metal stress reveals their toxicity to *R. sativus* plants. The enhancement in content of proteins and activity of enzymes with 24-epiBL application to the metal stressed plants in the form of seed pre-soaking treatment strengthens its role in the heavy metal stress management in plants.

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