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A N I N T E R N A T I O N A L J O U R N A L

Brassinosteroids Reciprocates Heavy Metals Induced Oxidative Stress in Radish by Regulating the Expression of Key Antioxidant Enzyme Genes

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ABSTRACT

Heavy metal toxicity in plants lead to accumulation of reactive oxygen species (ROS). Antioxidant enzyme system is also not able to revert altered ROS homeostasis. The present study reports the heavy metal induced ROS toxicity by up-regulating the expression of key antioxidant enzyme genes through Brassinosteroids pre-soaking treatment in radish.

Keywords: Brassinosteroids, oxidative stress, antioxidant enzymes, gene expression

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INTRODUCTION

Anthropogenic activities have posed a serious threat of heavy metal pollution. These heavy metals further get incorporated in the food chain and thereby degrade the ecosystem[1]. Through metal contaminated soils, the heavy metals get infiltrated into the plants. Although plants have developed various mechanisms to overcome heavy metal induced phyto-toxicity, yet their accumulation beyond the threshold concentrations lead to severe oxidative stress[1, 2]. In plants, heavy metals affect the normal growth, development, reproduction, photosynthesis and accumulation of osmolytes, proteins, carbohydrates as well as polynucleotides[3, 4]. Our previous studies reported that cadmium (Cd) and chromium (Cr) stress leads to suppression of normal plant metabolism in radish[3, 5]. However, application of Brassinosteroids (BRs), the polyhydroxylated steroidal plant hormones, helps in overcoming the metal induced oxidative stress in plants[5, 6]. However, BRs-mediated stresses tolerances are highly depend on their specific concentration and mode of application[3, 4, 5]. The optimum concentration of BRs that can enhance plant adaptability varies with plant species, developmental stage and environmental conditions[7]. We have earlier reported that seed pre-soaking treatments of two commercially available (and biochemically active) BRs *viz*., 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) are most effective at 10^{-7} M concentration[3, 4, 5]. Furthermore, BRs mediate the process of stress amelioration by interacting with other hormones, regulating the activities of several antioxidant enzymes and expression of various genes though different metabolic pathways[6, 7]. Therefore, the present study was carried out to study the effect of 10^{-7} M EBL or HBL on the regulation of gene expression of key antioxidant enzymes in radish seedlings under Cd or Cr metal toxicity.

The certified and disease-free seeds of radish (*Raphanus sativus* L. var. Pusa Chetki) were surface sterilized with 0.4% sodium hypochlorite for 15 min followed by repeated washings by sterile distilled water. Further, the seeds were given 8-hour presoaking treatment with different concentrations (0 and 10^{-7} M) of EBL (24-epibrassinolide) or HBL (28-homobrassinolide). These pre-treated seeds were then germinated on Whatman No. 1 filter paper lined pre-sterilized glass petridishes (10 cm diameter, 20 seeds/dish) each containing different concentrations (0 and 0.5 mM) of Cd salt [i.e., Cd (II) from Cadmium chloride anhydrous ($CdCl_2$)] or different concentrations (0 and 1.0 mM) Cr salt [i.e., Cr (VI) from Potassium chromate (K_2CrO_4)]. The IC-50 value (*i.e*., value of that concentration of heavy metal at which 50% growth of the radish seedlings is inhibited) of both heavy metal was used to conduct the present study. Therefore, to calculate IC-50 value a range of various concentrations from 0 mM to 5.0 mM of either Cd or Cr was prepared. The IC-50 value for Cd (II) and Cr (VI) was obtained as 0.5 mM Cd and 1.0 mM Cr respectivelty. The experiment was conducted under controlled conditions of light (16 h photoperiod under fluorescent white light with 175 μmol m^{-2} s^{-1} intensity), temperature (25 ± 5˚C) and relative humidity (80-90%).

The shoots of 7-days old radish seedlings treated with 10^{-7} M EBL alone, 10^{-7} M HBL alone, control, 0.5 mM Cd alone, 1.0 mM Cr alone and 0.5 mM Cd or 1.0 mM Cr in combination with HBL and EBL were harvested and immediately kept in liquid nitrogen. They were stored at -80˚C until subsequent isolation of RNA was done.

Total RNA was isolated through TRIzol method for RNA isolation. In plant tissue (100 mg) homogenized in liquid nitrogen, 1 mL TRIzol (RNAzol®RT) was added followed by vortex for 60 s. Then, reaction mixture was incubated for 10 min with slow-agitation followed by centrifugation at 12,000g for 10 min at 4˚C. Later, 200 μL chloroform was added to the supernatant and mixed vigorously by vortexing it for 15 s. After incubating for 7 min at room temperature**,** the reaction mixture was centrifuged at 12,000g for 15 min at 4˚C and transferred the clear aqueous phase into a new tube. To this, 650 μL of solution [that contained iso-propanol and sodium citrate (0.8 M)/sodium chloride (1.2 M) mixed in equal ratio] was added and mixed gently.

After incubating at 4˚C for 15 min, reaction mixture was centrifuged at 12,000g for 10 min at 4˚C. The pellet was subsequently washed with 1 mL of 70 % ethanol followed by centrifugation at 12,000 g for 2 min at 4˚C. Then, the pellet was air-dried in laminar flow for 10 min and further dissolved in 20 µL pre-chilled RNase free-water.

Isolated RNA was quantified spectrophotometricaly (Perkin Elmer Lambda 25, UV-Visible spectrophotometer), and 4 µg RNA was used to prepare cDNA. AuPrep Gold cDNA Synthesis Kit (Life Technologies, India) was used to prepare cDNA following the methodology and reagents provided by Kit manufacturers. The cDNA synthesized from seedling mRNA was used as template for PCR or amplification with specific primers. Degenerate oligonucleotide primers (Table 1) corresponding to conserved regions in the plant *SOD*[8] and *CAT* genes[9] were used as primers for PCR reactions. Tubulin (*Tub2*) was used as a housekeeping control. The program included denaturation at 94˚C for 5 min; followed by 40 PCR cycles of annealing at 45˚C for 1 min, and polymerization at 72˚C for 2 min. The results of amplified PCR product were analyzed on ethidium bromide stained 1% agarose gel under ultra-violet light using Gel documentation system (BIORAD-GEL DOC, Milan, Italy). Analysis of relative band intensity (*i.e.* integrated density values or IDVs) was performed using AlphaEase®FC software, Version 6.0.0, Alpha Innotech Corporation.

Table 1. List of Primers Used

Primer	Nucleotide sequence of the Primers
1) **CAT1**	**F:** CTGAACGTGAGACCAAGCATC
2) CAT1	**R:** TAACAGCATGAGACAAAACCA
3) CAT2	**F:** CGTCTCAATGTAAGGCCAAGCA
4) CAT2	**R:** TCAAATAAAATAATAGTCGTCGAA
5) CAT3	**F:** CTGAACGTGAGGCCAAGCATC
6) CAT3	**R:** TAGTACTGCGTTTATTTTCATTGA
7) Cu/Zn-SOD	**F:** CCTGG(AC)CT(CT)CATGG(GC)TT(CT)CAT
8) Cu/Zn-SOD	**R:** CTGAG(ATG)TC(AG)TGTCC(AT)CCCTT
9) Fe-SOD	**F:** CT(CT)CC(AT)GC(AT)TTCAACAA(TC)GC
10) Fe-SOD	**R:** GTA(AT)GCATG(TC)TCCCA(AG)AC(AG)TC
11) Mn-SOD	**F:** GA(AG)GG(AT)GGTGG(GTC)GA(GA)CC(GAT)CC
12) Mn-SOD	**R:** GTA(AT)GCATG(TC)TCCCA(AG)AC(AG)TC
13) TUB2	**F:** 5'-ATCCGTGAAGAGTACCCAGAT-3'
14) TUB2	**R:** 5'-AAGAACCATGCACTCATCAGC-3'

Both EBL and HBL were observed to increase the expression of *Cat1*, *Cat2*, *Cat3*, *Cu/ZnSod*, *FeSod* and *MnSod* (Fig.1, 2) when compared with only heavy metals (Cd or Cr) stressed radish seedlings. However, seed pre-soaking treatments of BRs alone could not change the expression of these antioxidant enzymes in comparison to untreated radish seedlings. Besides, applications of BRs or metal treatments had no significant effect on constitutively expressed *Tub2* genes in treated or untreated radish seedlings. The detailed analysis of influence of BRs (EBL or HBL) on regulation of expression of antioxidant genes with respect to untreated seedlings in heavy metals (Cr or Cd) stressed radish seedlings were described in terms of fold change in band intensity in terms of relative IDVs.

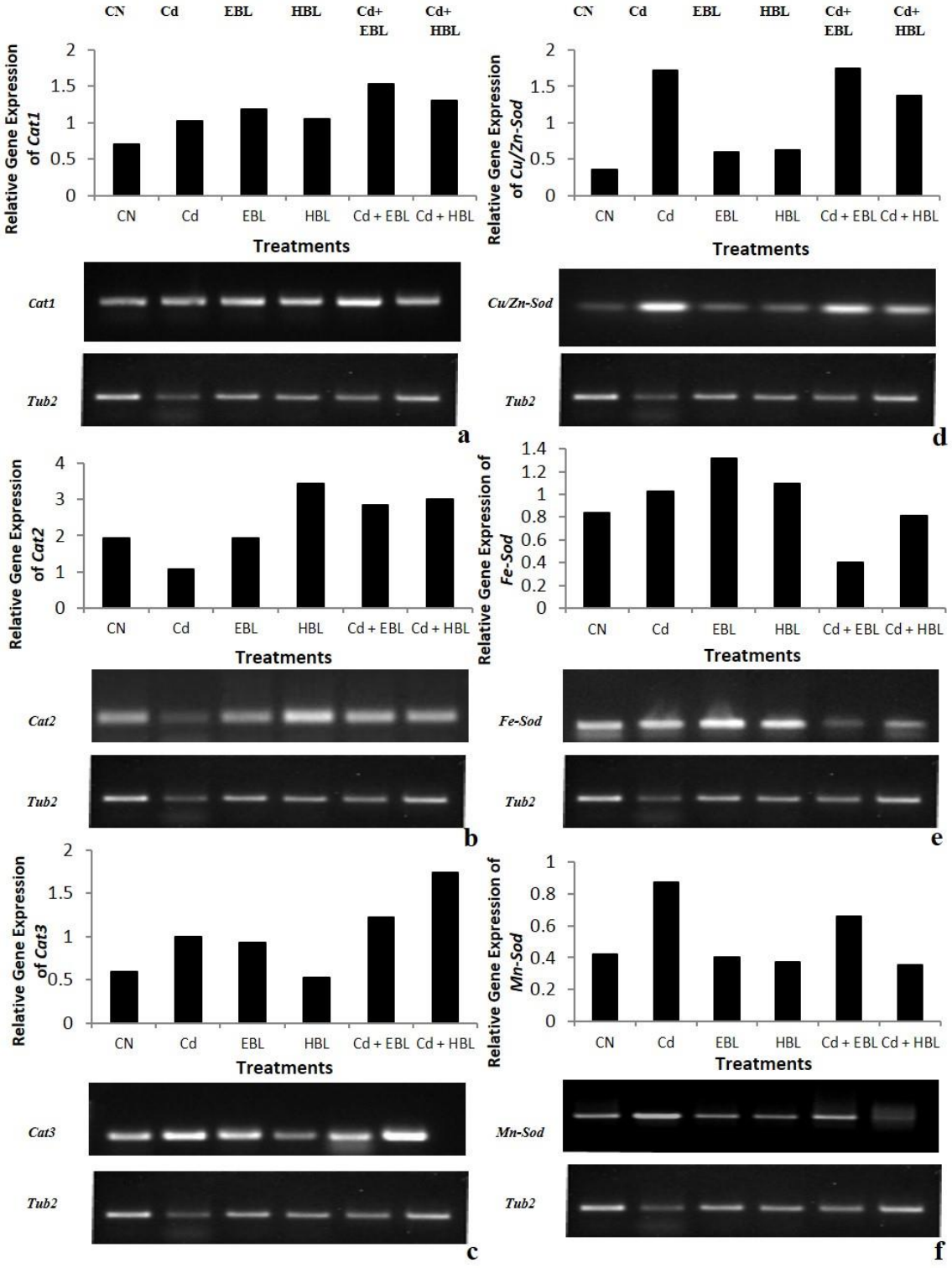

Figure 1. An ethidium bromide stained agarose gel electrophoresis products from reverse transcriptase PCR of 7 days old radish seedlings exposed to distilled water (Control, CN), 0.5 mM Cd, 10^{-7} M EBL (EBL), 10^{-7} HBL (HBL), both 0.5 mM Cd and 10^{-7} M EBL (Cd+EBL), both 0.5 mM Cd and 10^{-7} M HBL (Cd+HBL) for various key antioxidant genes: **(a)** *Cat1*, **(b)** *Cat2*, **(c)** *Cat3*, **(d)** *Cu/Zn-Sod*, **(e)** *Fe-Sod* and **(f)** *Mn-Sod*. Results were first normalized to the housekeeping gene *Tub2*, and then the relative expression of genes under various treatments was determined.

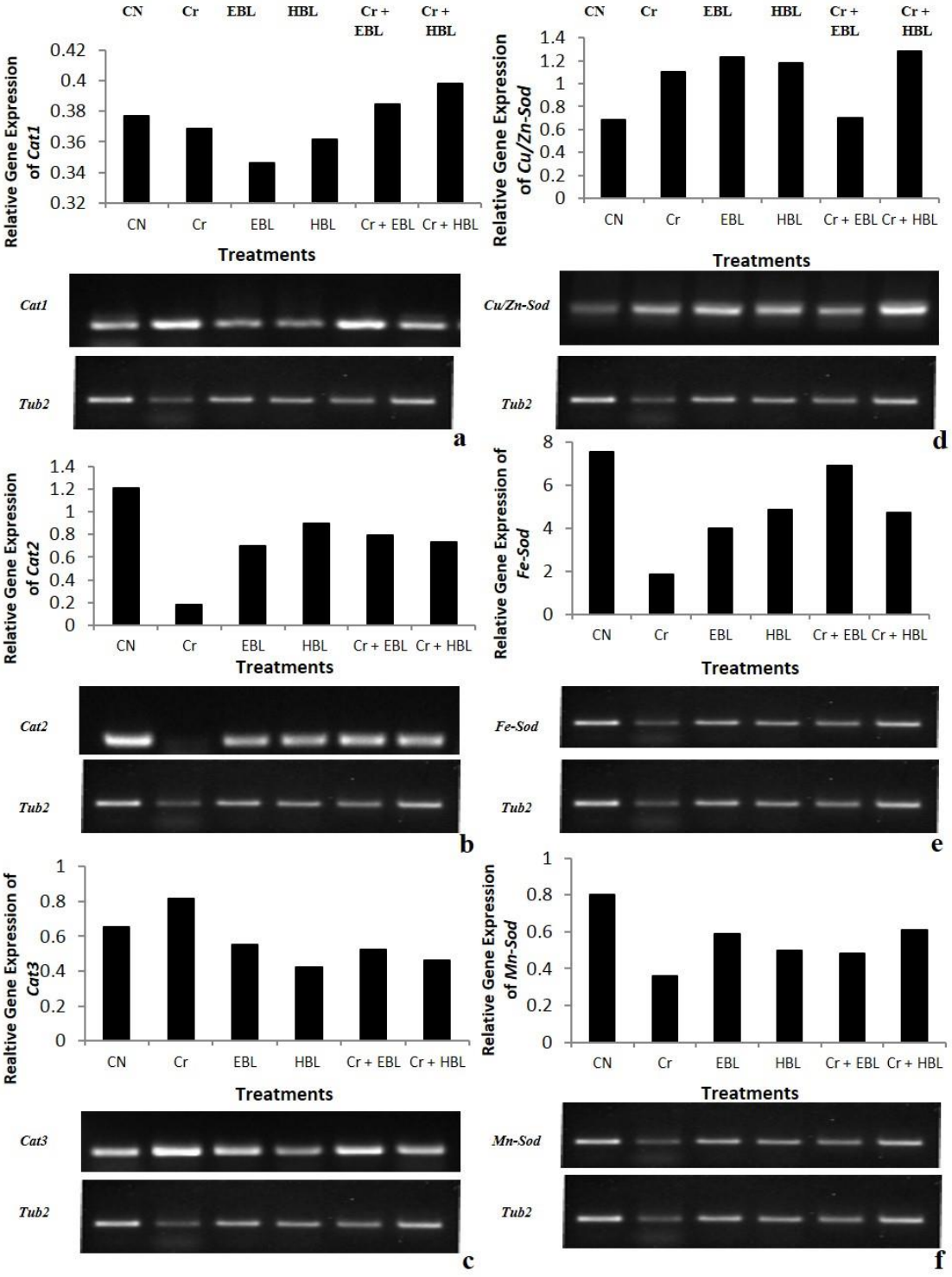

Figure 2. An ethidium bromide stained agarose gel electrophoresis products from reverse transcriptase PCR of 7 days old radish seedlings exposed to distilled water (Control, CN), 1.0 mM Cr (Cd), 10^{-7} M EBL (EBL), 10^{-7} HBL (HBL), both 1.0 mM Cr and 10^{-7} M EBL (Cr+EBL), both 1.0 mM Cr and 10^{-7} M HBL (Cr+HBL) for various key antioxidant genes: **(a)** *Cat1*, **(b)** *Cat2*, **(c)** *Cat3*, **(d)** *Cu/Zn-Sod*, **(e)** *Fe-Sod* and **(f)** *Mn-Sod*. Results were first normalized to the housekeeping gene *Tub2*, and then the relative expression of genes under various treatments was determined.

Cd metal treatments at 0.5 mM concentration revealed significant up-regulation of all genes of *SOD* and *CAT* except the expression of *Cat2* (Fig. 1a) and *FeSod* (Fig.1e). Furthermore, no such remarkable change in the expression of *Tub2* gene was observed

under Cd or BRs treatments (Fig. 1). The folds change in gene expression of *Cat1* (1.129 times), *Cat3* (1.291 times) and *MnSod* (1.599 times) was high, while maximum change was recorded in gene expression of *Cu/ZnSod* (3.646 times) during Cd Stress in comparison to untreated seedlings (Fig. 1a, c, d and f). When compared with house-keeping gene, the maximum up-regulation in the expression of *Cu/ZnSod* gene was recorded under Cd stress (Fig.1d). Also, seed-presoaking treatments were observed to up-regulate the gene expressions of *Cat1*, *Cat2* and *Cat3* in Cd stressed radish shoots (Fig. 1a, b and c).

Cd-phytotoxicity up-regulated the gene expressions of *Cu/ZnSod* (Fig. 1d) and *MnSod* (Fig. 1f) which was further stimulated by seed pre-sowing applications of EBL and HBL at 10^{-7} M. However, no such enhancing impact of BRs pre-treatment was observed in the expression of *FeSod* in radish seedlings subjected to Cd toxicity (Fig. 1e). The maximum fold change (3.256 times) in expression of *Cu/ZnSod* was induced by EBL when compared to untreated seedlings. Also, a similar up-regulating effect of HBL on gene expression of *Cu/ZnSod* was observed. The applications of HBL and EBL showed 1.741 and 1.369 folds change in expression of *Cu/ZnSod* gene in Cd stressed radish seedlings. Also, 0.404 and 0.662 times respective change in gene expressions of *FeSod* and *MnSod* was observed in EBL treated radish shoots during Cd toxicity. Also, HBL pre-sowing application resulted in 0.808 and 0.353 folds change in gene expressions of *FeSod* and *MnSod* in Cd stressed radish seedlings (Fig. 1e and f).

The Cd metal down-regulated the expression of *Cat2* gene which was further enhanced significantly by treatments of EBL and HBL in comparison to only metal treated radish seedlings (Fig. 1b). Though both EBL and HBL up-regulated the gene expression of *CAT* but HBL induced highest up-regulation (1.794 times) in expression of *Cat3* when compared with untreated seedlings (Fig. 1c). With respect to *Tub2* gene, expression of *Cat2* was up-regulated to 2.85 folds and 3 folds under influence of EBL and HBL respectively. Also, 1.535 and 1.214 times change in expression of *Cat1* and *Cat3* was recorded in EBL-treated seedlings subjected to Cd stress. Similarly, 1.309 and 1.741 folds change in gene expression of *Cat1* and *Cat3* was noticed under the influence of HBL treated seedlings exposed to Cd toxicity. Thus, expression of *Cat2* gene was observed to be maximum up-regulated under Cd stressed seedlings pre-treated with HBL and EBL (Fig. 1b).

In radish seedlings, Cr metal at 1.0 mM concentration resulted in up-regulation of *Cat1*, *Cat3* and *Cu/ZnSod* genes, however gene expressions of *Cat2*, *FeSod* and *MnSod* were observed to be down-regulated remarkably (Fig. 2). About 0.166, 0.267, 0.490 folds change in expression of *Cat2*, *FeSod* and *MnSod* was observed in Cr-stressed radish seedlings with respect to untreated seedlings (Fig. 2b, e and f). Whereas 1.072, 1.365 and 1.761 times change in expressions of *Cat1*, *Cat3* and *Cu/ZnSod* were observed (Fig. 2a, c and d). In comparison to *Tub2* gene, the expression of *FeSod* was reported to show maximum (1.8 folds) change under Cr stress (Fig. 2e). However, 1.6 times fold change in expression of *Cu/ZnSod* was recorded during Cr toxicity in comparison to control. Though expression of *CAT* genes and *SOD* genes were regulated under Cr stress yet, maximum folds change in the expressions of *Cu/ZnSod* and *FeSod* were observed in comparison to *Tub2* gene (Fig.2).

The down-regulated expressions of *FeSod* (Fig. 2e) and *MnSod* (Fig. 2f) were remarkably reversed by pre-sowing applications of EBL and HBL at 10^{-7} M to 1.0 mM Cr stressed radish seedlings. Applications of EBL and HBL showed 4.867 and 6.875 times change in expression of *FeSod* under Cr when compared with only Cr treated (1.836 folds) radish shoots. In addition to this, a stimulating effect of HBL was observed on the expression of *Cu/ZnSod* gene in shoots of radish seedlings under Cr toxicity (Fig. 2d). In comparison to untreated seedlings the maximum increase with HBL treatment was recorded in the gene expression of *Cu/ZnSod* in Cr stressed radish shoots.

Seed pre-soaking treatments significantly up-regulated the gene expression of *Cat1* and *Cat2* except the expression of *Cat3* in shoots of radish exposed to Cr toxicity (Fig. 2 a-c). However, HBL induced maximum change (1.412 folds) in the gene expression of *Cat1* when compared to Cr (1.072 times) in comparison to untreated seedlings (Fig. 2a). In case of *Cat2*, maximum increase was recorded under HBL (0.790 folds) and EBL (0.731 folds) treatments when compared with only Cr stressed (0.182) seedlings (Fig. 2b).

During various abiotic stresses, cellular homeostasis is altered and ROS production is elevated. Heavy metals like chromium induce oxidative stress in plant that is mediated by NADPH oxidase[10, 11]. An up-regulation in heavy metal-specific, superoxide ($O_2^{\bullet -}$) generating NADPH oxidase genes have also been recorded[12]. Among various antioxidant enzymes involved in antioxidant defence system, SOD and CAT enzymes are most significant for the removal of superoxide radicals and its conversion into H_2O and O_2[1, 5]. We have previously reported that seed-presoaking treatments of BRs significantly improved the specific activities of SOD and CAT enzymes under Ni, Cd and Cr stress in radish[3, 4, 5]. Both EBL and HBL were found to be most effective at the dose of 10^{-7} M in ameliorating the heavy metals induced oxidative stress by modulating the activities of antioxidant enzymes, antioxidants and various others important biochemical components. Therefore, the molecular studies on the key antioxidant enzymes are pre-requisite to understand further underlying mechanism of action of BRs in stress alleviation. Hence, the present study is an insight into the molecular mechanism of differential gene expression of *SOD* and *CAT* genes under influence of BRs during metals induced oxidative stress in radish.

In the present study, seed pre-soaking treatments of HBL up-regulated the expression of *Cu/ZnSod*, *MnSod*, *Cat1*, *Cat2* and *Cat3* whereas EBL also stimulated *FeSod* expression in radish seedlings under Cd stress (Fig. 1). Further in Cr stressed seedlings, gene expression of SOD and CAT was up-regulated except *Cat2* gene (Fig. 2) Since *Cu/ZnSod* is most common SOD and acts in cytosol, chloroplast and peroxisomes therefore, metal induced generation of ROS may have enhanced its expression under stress [1, 5]. Further, to negate the effect of ROS on plasma-membranes or plants cells, BRs may have directly enhanced the expression of *Cu/ZnSod*. Also, *FeSod* dismutates the superoxide radical to H_2O_2 in chloroplasts [5]. It is feasible that role of *FeSod* was also partly carried out by *Cu/ZnSod* thus resulting in stimulation of its gene expression. However, *Cat1*, *Cat2* and *Cat3* genes are localized in peroxisomes where they convert H_2O_2 into H_2O thereby reducing oxidative damage in plant cells [1, 5]. All *CAT* genes at same time or in co-ordination contribute for detoxification of H_2O_2 in peroxisomes.

A recent study[13] indicated that exogenous EBL increased the expression of the *dehydroascorbate reductase* (*Dhar*), *glutathione reductase* (*Gr*), *monodehydroascorbate reductase* (*Mdhar*), and *glutathione synthetase* (*Gs*) in the leaves of zinc stressed *Solanum melongena* L. Wu and co-workers[13] also suggested that EBL can potentially regulate the regeneration of related antioxidants at the transcriptional level. Similarly, the exogenous EBL treatment up-regulated the expression of *Sod*, *Cat*, *ascorbate peroxidase (Apx)* and *Gr* under Cr stress in rice[14]. Therefore, it is possible that the stress responses in BRs treated or untreated shoots of radish might be regulated by antioxidant enzymes and some other signals[15]. Although the signaling pathway of action of BRs have been studied by using diverse genomic and proteomic approaches, but still there are various unknown components are unsolved [16]. Therefore, detailed understanding of mechanism of BRs mediated stress-mitigation is pre-requisite. Such thorough studies might reveal the complex sequence of metabolic shifts, like enhancement or suppression of key enzymatic reactions, induction of protein synthesis and the production of various defence related bio-chemicals in plants through the application of BRs.

Brassinosteroids (BRs) when applied at specific dose can ameliorate the heavy metal toxicity in plants which is mediated by regulation of the expressions of key antioxidant genes. Therefore, BRs may be implicated in plant stress-protection and enhancing their productivity under heavy metal stress conditions. Further studies on underlying mechanisms of interactions of BRs with other plant growth regulators might provide more efficient phytohormone-based agronomic practices to reduce the risk of crops being exposed to metal contaminated soils.

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