HEME OXYGENASE-1 (BJHO-1) FUNCTIONS IN MODULATING ANTIOXIDANT DEFENCE RESPONSES AGAINST CADMIUM INDUCED OXIDATIVE STRESS: AN IN VITRO AND IN VIVO COMPARATIVE ANALYSIS

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ABSTRACT

Cadmium (Cd) is a non-essential and most harmful heavy metal pollutant which promotes the overproduction of reactive oxygen species (ROS). ROS can cause peroxidation of lipid membranes, cell death, nucleic acid damage, protein oxidation and inhibition of various vital enzymes. However, the functional mechanism of HO-1 as an antioxidant is not well understood. The present study is aimed to characterize the mechanism of HO-1 gene via in vitro approach. Here, we report that HO-1 works against oxidative stress via detoxifying H_2O_2, a potential ROS.

Effect of $CdCl_2$ has been observed and evaluated on HO-1 and H_2O_2 content in hemin efficient and deficient condition in hydroponic culture and callus medium. A comparative analysis of antioxidant mechanism has also been done in plant roots and callus system. We have found higher HO-1 expression on pretreatment with H_2O_2 in comparison to control, and we also observed higher H_2O_2 content on HO-1 inhibition. HO-1 is involved in H_2O_2 signaling in Brassica juncea, and this leads to detoxification of H_2O_2 which strengthen defense system against Cd induced oxidative stress.

KEYWORDS: Cadmium, Oxidative Stress, HO-1, CAT, APX, Brassica Juncea, H_2O_2.

Abbreviations: Cadmium; Cd, Hemeoxygenase 1; HO-1, Catalase; CAT, Ascorbate Peroxidase; APX.

INTRODUCTION

The large scale mining of gold, silver and untreated discard of electronic industries increasing several problems to the environment, their discards not only lead to contamination of ecosystem, but also affect the quality of food crops and their production rate. Major part of this discard is hazardous heavy metals. These metals deteriorate the soil productivity up to several extents. Cd is one of the 7 most hazardous heavy metals which cause soil pollution by

incorporating in to the food chain leading to its biomagnifications (Gill and Tuteja 2011; Gill et al., 2011, 2012). This all is due to the free radical production by Cd imposing oxidative stress (Foyer et al., 1997; Zhang et al., 2005; Gill and Tuteja, 2010). In plants also diverse strategies have been developed for detoxification of heavy metals (Cobett, 2000). To counteract the deleterious effects of Cd, plants possess enzymatic and non enzymatic antioxidant defense system (Gill and Tuteja, 2010; Gill et al., 2011, 2012). Among the various genes encoding antioxidant proteins, there has been a growing interest over the last years in the heme oxygenase enzyme (HO EC 1.14.99.3) which catalyses the oxidation of heme to billiverdin IXα (BV), CO, and Fe^{2+}. Further BV is reduced to BR via reaction is catalyzed by biliverdin reductase (He and He, 2013).

This HO-1 performs a wide variety of cellular functions and exhibit different enzymatic characteristics. In mammals, HO-1 and its catalytic products are important in neural function, protection against oxidative stress and tissue injury (Tomaro and Battle, 2002). In cyanobacteria and algae HO-1 is also required for the synthesis of the phycobilin chromophores (Shekhawat, 2010; Terry, 2002) and Triticum aestivum HO1 also play an important role in abiotic stress responses (Xu et al., 2011). Additionally, multiple stimuli in humans, including hemolysis, inflammatory agents, oxidative stress, and heat shock, can markedly increase HO activity and HO-1 gene expression in vitro and in vivo (Maines, 1988). Similarly, recent studies in plants have found that HO-1 gene could be induced by multiple stimuli and various abiotic stresses, such as heavy metals, glutathione depletion (Cui et al., 2011), UV radiation (Yannarelli et al., 2006), salinity stress (Balestrasse et al., 2008) and hydrogen peroxide (H_2O_2) (Chen et al., 2009). This response was thought to be a part of cellular defense mechanism, against various stresses triggered oxidative damage and also exhibit hormone-like responses (Wu et al., 2011). Hemin and Hematin were also observed to induce HO1 expression in wheat (Xuan et al., 2007), tomato (Xu et al., 2010), and rice germinating seeds (Liu et al., 2010). Activity of HO-1 as an antioxidant is well understood in plants but activity of HO-1 has not been studied in in vitro systems. Higher plants have multiple protective mechanisms against metal induced oxidative stress including ion homeostasis, ROS scavenging, and transducers of long-distance response coordination (Gill and Tuteja, 2010). But at cellular level or tissue level survival of the plant is very difficult. Earlier antioxidant property of HO-1 has been reported but its functional mechanism is still poorly understood. HO-1 might work in co-ordination with NO to alleviate salinity tolerance during wheat seed germination (Xu et al., 2010). Under stress HO up regulation also protects

also protects nitrogen metabolism in nodules of soybean plants (Zilli et al., 2008). On metal induced oxidative stress, HO-1 induction is mediated by ß-cyclo dextrin. Hemin provides critical defense by lowering down level of metal accumulation (Fu et al., 2011). HO-1 found to be upregulated in a dose dependent manner as a mechanism of cell protection against oxidative damage. CO, NO and H_2O_2 network have a regulatory role in HO-1 expression. Conversely CO, NO and H_2O_2 metabolism are also regulated by HO-1. CO being a by product of HO-1 catalytic degradation, decreases the level of endogenous H_2O_2 by enhancing the activities of CAT and APX (Wu et al., 2011)

Here, we observed that HO-1 participates in defense process by decreasing toxic H_2O_2. We have chosen Brassica juncea as a model plant and have seen activity of BjHO-1 because Brassica can be potentially used as material for phytoremediation since its metal accumulating capacity and develops sufficient biomass. Genetic transformation is also well established with this plant for detoxification of heavy metals and plant species within the Brassicaceae family have been observed to be easily grown and manipulated in vitro (George et al. 1987). The study is important, as it helps in selecting a suitable experimental plant material for the study of Cd tolerance mechanisms and to develop metal tolerant cell lines through in vitro approaches. With the aim of obtaining a better comprehension of the in vitro and in vivo activity of BjHO-1 enzyme on Cd induced oxidative stress; we have analyzed oxidative stress parameters along with HO-1 and tried to find out mechanism lying under antioxidant action of BjHO-1.

Material and Method:

Plant material

The seeds of B. juncea cv. Varuna (Bio-902) were obtained from Agriculture Division, Banasthali University. These seeds were used for in vitro cultivation of the species via callus and hydroponic cultures. Seeds of B. juncea were surface sterilized for 20s with 90% ethanol and for 10 min with 5% sodium hypochlorite. The seeds were then rinsed several times with sterile distilled water and germinated on filter paper soaked in petriplates (Fig.1). The equal number of seeds was germinated on blotting paper moistened with distilled water in Petri plates (Fig.1). These were placed in a dark cabinet for 3 days and then cultivated in a growth chamber at 25/22°C day/night temperature, 50–55% relative humidity, and 14/16 h light/dark with an illumination of 175 µmol m^{-2} s^{-1} light intensity. After 5 days of explants cultivation, the leaves of aseptically grown seedlings were excised and cultured on modified MS medium

for callus induction, and seedlings germinated on blotting paper were transferred to Hoagland's nutrient solution for hydroponic culture under the mentioned environmental conditions.

Callus induction, growth, and Cd treatment

Callus induction was obtained from leaf explants of B. juncea on MS medium with a combination of α 6-benzyl aminopurine (BAP) and α-naphthalene acetic acid (NAA). Before autoclaving at 121°C for 15 min, pH of the media was adjusted to 5.8. On this combination, a friable callus developed within a week (Fig.1). Callus after a 3-week growth on the above combination was subcultured on to MS medium containing increasing concentrations of $CdCl_2$ (0, 10, 50, 100, 150 or 200 µM) and maintained under the same environmental conditions as above for 10 days. Samples, which comprised of the entire callus mass in a flask from each $CdCl_2$ treatment, were collected after 15 days of metal treatment and used for further studies. The experimental design was randomized with three replicates for each treatment/time interval. The total callus mass in a culture vessel was used for fresh weight and dry weight determination and enzyme extraction.

Seedling growth and cadmium treatment

After 5 days cultivation in Petri plates, the seedlings were transferred to Hoagland's nutrient solution having pH of 6.4 (Hoagland and Arnon, 1949). The nutrient solution was changed every third day. Subsequent to day 15, seedlings were subjected to different concentrations of cadmium (0, 10, 50, 100, 150, and 200 µM) and maintained in 200 ml of Hoagland's nutrient solution.

Plant growth parameters

Growth was measured in terms of fresh weight of seedlings and callus. Tolerance index of roots and callus was calculated from fresh weight (FW) by using the formula, (FW treated/FW control) ×100, and represented in percent tolerance (Wilkins, 1978).

Estimation of total protein

The protein content of the Brassica juncea (L). Czern., roots and callus were determined at different Cd, Cu concentrations after 72 hours of exposure period following the method of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the standard. 0.5 ml of 1N

NaOH was followed by 0.1 ml of extracted sample. After 10 minutes digestion on boiling water bath, 2.5 ml of reagent B (48 ml of 5 % Na_2CO_3 and 2 ml of 0.5 % $CuSO_4.5H_2O$ in 1 % sodium potassium tartarate) was added. 0.5 ml Folin-phenol reagent was added after 10 minutes. After 30 minutes of incubation a blue colour complex was developed in the mixture. Absorbance was taken at 700 nm against a blank without sample. Protein content was expressed as percent of control.

Extraction and assays of enzymatic antioxidants

For extraction, 500 mg fresh root and callus sample was homogenized in 5.0 mL extraction buffer containing 1 mM EDTA, 0.05% Triton-X-100, 2% PVP, 1 mM ascorbate in 50 mM phosphate buffer, pH-7.8. This mixture was centrifuged at 13000 rpm for 20 minutes at 4 °C. Resulting Supernatant was stored at -20 °C for the assay of different antioxidative enzymes.

Superoxide dismutase Assay

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium according to the method of Beauchamp and Fridovich (1971). Three mL reaction mixture containing 50 mM phosphate buffer (pH-7.8), 13 mM Methionine, 75 µM NBT, 2 mM Riboflavin, 0.1 mM EDTA and a 100 µl aliquot of enzyme extract was incubated for 30 minutes under fluorescent lamp. A tube containing enzyme kept in dark served as blank while the control tube without enzyme kept in light served as control. The absorbance was taken at 560 nm. One unit of activity is the amount of enzyme required to inhibit 50% initial reduction of NBT under light.

Catalase Assay

The CAT activity was measured by the method of Aebi (1974). The assay system comprised of 50 mM phosphate buffer (pH-7.0), 20 mM H_2O_2 and a suitable aliquot of enzyme in the final volume of 3 mL. Decrease in the absorbance was taken at 240 nm. The molar extinction coefficient of H_2O_2 at 240 nm was taken as 0.039 mM^{-1} cm^{-1}. The enzyme activity was expressed as unit mg^{-1} of proteins.

Ascorbate peroxidase assay

Ascorbate peroxidase activity, the rate of hydrogen peroxide-dependent oxidation of ascorbic acid, has been determined in a reaction mixture that contained 50 mM phosphate buffer (pH

7.0), 0.6 mM Ascorbic acid, and enzyme extract (Chen and Asada 1989). The reaction was initiated by addition of 10 µL of 10% H_2O_2, and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm for 3 min (ε-2.8 mM^{-1} cm^{-1}).

Hemeoxygenase preparation and assay

HO activity was assayed by following the method (Yannarelli et al., 2006). 300 mg fresh sample were homogenized in a Potter-Elvehejm homogenizer using 1.2 mL of ice-cold 0.25 M sucrose solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.2 mM EDTA, and 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20, 000 × g for 20 m and supernatant used for activity determination. The assays (1 mL final volume) contained 250 µL of extract (500 ng protein), 10 µM hemin, 0.15 mg mL^{-1} bovine serum albumin, 50 µg mL^{-1} (4.2 µM) spinach (Spinacia oleracea) ferredoxin (Sigma,USA), 0.025 units mL^{-1} spinach ferredoxin-$NADP^+$ reductase (Sigma, USA). The reaction was started by adding NADPH to a final concentration of 100 µM, samples were incubated at 37 ºC for 30 m and BV formation was calculated using the absorbance change at 650 nm. The concentration of BV was estimated using a molar absorption coefficient at 650 nm of 6.25mM^{-1} cm^{-1} in 0.1 M HEPES-NaOH buffers (pH 7.2). One unit of the enzyme forms 1 nmol of biliverdin in 30 m under assay conditions.

RNA isolation from GITC method

Total RNA was isolated from fresh-weight samples of callus and roots by grinding with mortar and pestle in liquid nitrogen until a fine powder appeared after that 5 ml Guanidium Isothiocynate buffer containing β mercaptoethanol was added and some more grinded. 2.5 ml water saturated phenol and 2.5 ml Chloroform: isoamyl alcohol has been used for removal of protein. After proper mixing and centrifugation at 10,000 rpm for 15 minutes, supernatant has been transferred to fresh falcon and same procedure is repeated. To the supernatant, $1/10^{th}$ volume of Sodium Acetate and 1 volume of chilled Isopropanol have been added to precipitate RNA. Keep it at -20^0 C for overnight. RNA has been precipitated by centrifugation. Washing has been done by 75 % ethanol, dried and dissolved in Diethylpyro carbonate (DEPC) treated water.

Semi-quantitative RT-PCR

DNA-free total RNA (1µg) from different treatments was used for first-strand cDNA synthesis in a 20 µL reaction volume containing 2.5 U of RT enhancer and Verso Enzyme Mix (Genei) and l M oligo (dT) primer. PCR reactions were performed using 2 µl of a 2-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 U of Taq polymerase (Genei) in a 25 µl reaction volume. Primers used were from following sequence: Brassica juncea HO1 (accession number JX275832.1). The cycle numbers of the PCR were 35 for all the genes. To standardize the results, the relative abundance of actin was determined and used as the internal standard. Aliquots from the PCR were loaded on 0.8% agarose gels with the use of ethidium bromide (EB). Specific amplification products of the expected size were observed, and their identities were confirmed by sequencing.

Primer Name	**Primer Sequence**
Bj HO-1 Forward Primer	ATGGCTTACTCAGCTCCCATATCTCCATCCCT
Bj HO-1 Reverse Primer	TTAAGCTTTGAAGCAAATAAGAG
Bj CAT Forward Primer	ATGGATCCTTACAAGTATCGGC
Bj CAT Reverse Primer	TTAGATGCTTGGTCTCACGTTC
Bj APX Forward Primer	ATGACGAAGAACTACCCAACC
Bj APX Reverse Primer	TTAAGCATCAGCAAACCCG
Bj SOD Forward Primer	ATGGGCAAGGGAGTTCGA
Bj SOD Reverse Primer	TTAGCCCTGAAGACCAATAATACC
Actin Forward Primer	CTGGAATGGTGAAGGCTGGGTT
Actin Reverse Primer	CGGAGGATAGCGTGAGGAAGAG

Cloning of BjHO-1 cDNA

The nucleotide sequences of plant heme oxygenases were acquired from NCBI Database (http://www.ncbi. nlm.nih.gov/) and the highly conserved regions were analyzed using BLAST. Based on the conserved sequence of these genes, primers were designed from conserved region. cDNA was amplified with designed gene specific primers and amplified fragment was cloned in to pGEMT vector (Promega India). Plasmid was isolated from positive clones and has been sequenced from Banglore Genei India. Sequence of HO-1 was confirmed by using BLAST and submitted in to NCBI (Accession No. JX275832.1).

Analysis of HO gene relationships within plants

To analyse the evolutionary relationships between BjHOs and those from other plant species, the amino acid sequences of Arabidopsis thaliana, Arabidopsis lyrata subsp. Lyrata, Brassica napus, Brassica rapa subsp. pekinenis were retrieved from NCBI. Alignment of amino acid sequences was performed using CLUSTALW (Version 2.0). The phylogenetic tree was constructed byneighbour-joining (NJ) method with MEGA 5.

Histochemical Detection of H_2O_2

To analyze H_2O_2 generation, roots were excised and immersed in a 1% solution of 3,3-diaminobenzidine (DAB) in Tris–HCl buffer (pH 6.5), vacuum-infiltrated for 5 min, and then incubated at room temperature for 16 h in the absence of light. Roots were illuminated until the appearance of a brown color, characteristic of the reaction of DAB with H_2O_2.

Statistical analysis

Data were statistically analyzed in experimental observations using student's t-test, and the results were expressed as mean (±standard error) of three independent replicates of each independent experiment which is repeated three times. The significance of differences between control and each treatment was analyzed using analysis of variance at $P \leq 0.05$.

Results

Effect on growth

Callus cultures were induced from leaves on MS medium containing 1 mg L^{-1} BAP and 0.2 mg L^{-1} NAA. Callus cultures and plant seedlings of B. juncea were exposed to increasing concentrations of $CdCl_2$ (0, 10, 50, 100, 150, and 200 µM), showed reduction in growth rate. In Table 1, the variation of FW is presented for five concentrations of $CdCl_2$. It can be seen that 150 µM and 200 µM $CdCl_2$ inhibited growth of callus after 9 days and more dramatically after 15 days. Fresh weight and dry weight of roots and callus decreases significantly on increasing concentration of Cd (Table-1). Moreover, red-brown patches were also observed in calluses exposed to above concentrations. Callus treated with 200 µM cadmium showed 81.31% reduction in percent tolerance in comparison to controls (Fig. 1; i). At 200 µM cadmium; a 78.32% reduction in tolerance was seen in seedlings in comparison to control, which proves higher tolerance of callus (Fig. 1; i).

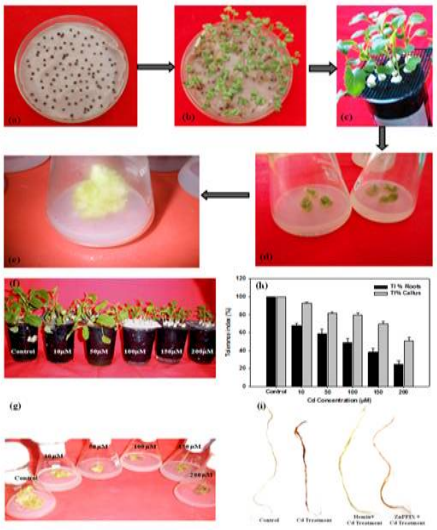

Figure 1

Fig. 1 Seed germination, hydroponic culture, and callus of Brassica juncea (A) Planting of seeds (B) 5-day-old seedlings (C) Planting of seedlings in Hydroponic culture (D) Callus formation from leaves (E) Callus proliferation (F) Morphological alteration in hydroponic cultures of B. juncea treated with Cd concentration (0-200 µM Cd) for 72 h (G) Effect of Cd concentrations (0-200 µM Cd) on leaflet-derived callus after 15-day exposure (I) Tolerance index (percentage) of callus and seedlings of B. juncea. Callus and seedlings were treated with 0, 10, 50, 100, 150, and 200 µM of cadmium. Treatment was given for a period of 15 days to callus and 3 days to seedlings. Vertical bars show standard errors (n=3). (J) H_2O_2 localization in situ in Roots treated with 0 µM Cd (Control), 200 µM Cd, 200 µM Cd + Hemin, 200 µM Cd + ZnPPIX. Experiments were performed as described in "Materials and Methods" Section. The figure is representative of four different experiments.

Metal Content Estimation

Metal content has been estimated in roots and callus of Brassica juncea. Metal accumulation on various treatments has been shown in both the tissues (Table 2). In case of roots at 200 µM Cd metal content has been found 54 times higher than control, where as in callus it was 9.27 times higher than control.

Activity of antioxidant enzymes

SOD activity analysis was also carried out with extracts isolated from callus and roots exposed to $CdCl_2$. The effect of varying concentrations of Cd on SOD activity of callus and seedlings of B. juncea was shown in Fig. 2b. SOD activity of callus and roots showed a significant ($P \leq 0.05$) increase in its activity in concentration-dependent manner up to 150 µM cadmium (Fig. 2b). The SOD activity slightly decreased with further increase in the cadmium concentration to 200 µM in callus and seedlings. Exposure of plants up to 150 µM Cd for 72 h led to an increase in SOD mRNA in roots (54%) and callus (63%) (Fig. 3b). Exposure to 200 µM Cd for 72 h resulted in a 34% decrease in SOD transcript level in roots and 28 %

decrease in callus. (Fig. 3b). However, total SOD activity was higher in callus in comparison to seedlings.

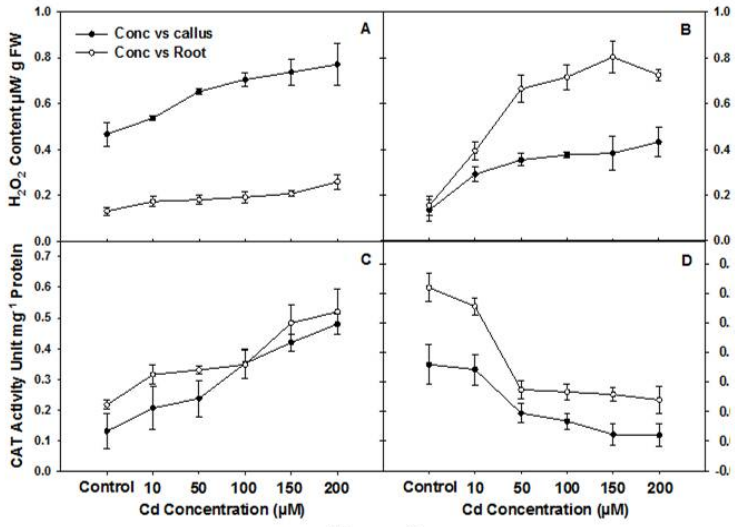

Figure 2

Fig. 2 (A) Effect of Cd on hydrogen peroxide (H_2O_2) content in Brassica roots and callus. (B-D) Changes in antioxidants activity in callus and seedlings of B. juncea. (B) Superoxide dismutase (SOD) Activity, (C) Catalase (CAT) activity, (D) Ascorbate peroxidase (APX) activity. Callus and seedlings were treated with equimolar concentrations of Cd. Treatment was given for a period of 15 days to callus and 3 days to seedlings. Vertical bars show standard errors (n=3).

Fig. 3b shows the changes in the activity of CAT. CAT activity and its gene expression increased throughout all cadmium treatments in both callus cultures and the seedlings of B. juncea. The increase in its activity was significant ($P \leq 0.5$) in comparison to control up to the 200 µM concentration of cadmium. However, similar to the above results, the total CAT activity and expression was observed to be more in callus.

Fig. 2d shows decrease in APX Activity. APX activity is decreasing continuously on increasing concentration of Cd up to 50 µM. Then it became stable up to 200 µM. APX activity is higher in callus in comparison to roots.

H_2O_2 Content

H_2O_2 a product of SOD reaction showed a significant increase (Fig. 2; a). On the other hand the level of H_2O_2 in Cd treated callus gradually increased at 10 µM to 24% but at 50 µM it increased up to 54.38 % and at 200 µM up to 94.61 %. A different trend has been also observed in H_2O_2 content as it was higher in callus in comparison to roots (Fig. 2; a). To

analyze whether HO-1 is working on detoxification of H_2O_2 or not, we have carried out treatments of Hemin (HO-1 inducer) and ZnPPIX (HO-1 inhibitor) which clearly demonstrates that treatment of Hemin reduces the H_2O_2 content up to 77% (Table 3). To assure the detoxification role of HO-1 we have given treatment of ZnPPIX which inhibits HO-1 activity, and we have found H_2O_2 content same as control. Hence, it provides evidence that HO-1 shows strong antioxidant effect against Cd stress by detoxification of H_2O_2 (Table 3).

Table 1: Effect of cadmium on fresh weight and dry weight of callus and Roots of B. juncea

Cd Concentration (µM)	Fresh weight		Dry weight	
	Roots	Callus	Roots	Callus
Control	0.442 ± .013	1.64 ± .005	0.043 ± .001	0.327 ± .033
10 µM	0.434 ± .034	1.52 ± .027	0.039 ± .003	0.208 ± .024
50 µM	0.376 ± .042	1.34 ± .014	0.028 ± .010	0.083 ± .016
100 µM	0.312 ± .021	1.31 ± .023	0.021 ± .013	0 .065 ± .014
150 µM	0.245 ± .013	1.14 ± .021	0.015 ± .007	0.143 ± .026
200 µM	0.183 ± .015	0.834 ± .015	0.013 ± .009	0.062 ± .011

Data recorded after 15 days treatment in callus and 3 days treatment in seedlings.

Table 2: Intracellular Cd content in roots and callus of B. juncea

Cd Concentration (µM)	Intracellular Cd content (µg / g FW)	
	Root	Callus
Control	25.4360 ± 0.20	11.3583 ± 0.06
10 µM	446.5600 ± 0.03	39.0067 ± 0.01
50 µM	804.8027 ± 0.12	49.0113 ± 0.11
100 µM	1076.0833 ± 0.09	49.5143 ± 0.07
150 µM	1092.8076 ± 0.04	97.8033 ± 0.36
200 µM	1352.2473 ± 0.15	102.0857 ± 0.25

Data recorded after 15 days treatment in callus and 3 days treatment in seedlings.

Table 3: H_2O_2 Content estimation in B. juncea upon different treatments

Cd Concentration (µM)	H_2O_2 Content (µM /g FW)					
	Cd Treatment		Cd + Hemin Treatment		Cd + Zn Protoporphyrin IX Treatment	
	Callus	Roots	Callus	Roots	Callus	Roots
Control	0.466 ± 0.12	0.131 ± 0.05	0.323 ± 0.11	0.127 ± 0.06	0.45 ± 0.13	0.123 ± 0.04
50 µM	0.653 ± 0.17	0.181 ± 0.052	0.448 ± 0.13	0.132 ± 0.07	0.586 ± 0.14	0.168 ± 0.12
200 µM	0.770 ± 0.19	0.258 ± 0.074	0.489 ± 0.12	0.117 ± 0.05	0.556 ± 0.15	0.176 ± 0.14

Data recorded after 15 days treatment in callus and 3 days treatment in seedlings.

HO-1 Activity

To evaluate whether HO-1 is involved in the Cd induced stress-mediated acclimation to oxidative stress, we also investigated the effects of Cd treatment on HO-1 activity and its gene expression in plant seedlings and callus system. As shown in Fig. 3(a) less than 50 µM Cd there was no significant increase in the HO-1 activity respect to controls in roots. Concentration of 50 µM Cd was capable of inducing HO-1 activity (7 fold), while 200 µM Cd increased enzyme activity up to the maximum in plant roots. A gradual increase of HO-1 activity was also found in callus up to 200 µM Cd treatment. When gene expression of HO-1 assayed with BjHO-1 specific primers (Fig. 3; b) were assayed, a positive correlation with enzyme activity was found. On one hand, findings demonstrated the protective role that HO-1 plays against oxidative stress in Cd-treated plants, and on the other hand, the present data, which is a comparative analysis of HO-1 activity in callus and plant seedlings, indicate that HO-1seems to neutralize the effects brought about by Cd via detoxifying H_2O_2. These results prompted us to investigate the behavior of HO-1 as a consequence of H_2O_2 treatment via analyzing enzyme activity and transcript level analysis. Table 3 shows that application of Hemin caused the deterioration of H_2O_2 content (with respect to controls) in both callus and seedlings. Plants subjected to ZnPPIX (HO-1 inhibitor) showed an increase of H_2O_2 content with respect to controls. Pre-treatment with 0.5 mM H_2O_2 before the addition of Cd provoked a major enhancement of HO-1 activity and transcript level (Fig. 3; b). HO-1 has been described as a feature of plant response to stress conditions. To assess whether HO-1 is involved in the protection against Cd with other enzymes, we have measured transcript level of HO-1 with SOD, CAT and APX, Which shows coordinating behavior of HO-1 with other enzymes (Fig. 3; b).

Histochemical analysis of H_2O_2

To obtain clues about the mechanism of HO-1 in its defense against Cd, H_2O_2 localization in situ was performed. Histochemical method was applied as shown in Fig. 3(b). Cd produces red colour in roots, whereas pretreatment with 10 µM Hemin prevented this effect and the Roots were similarly devoid of red brown colour as that of controls. Pre-treatment with 200µM ZnPPIX restored H_2O_2 accumulation (Fig. 3; b).

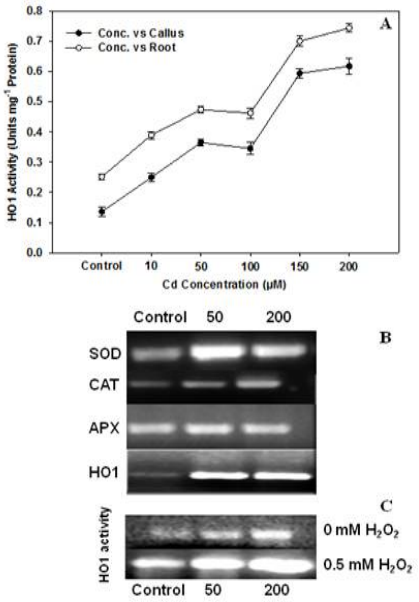

Fig. 3(A) Effect of Cd on HO-1 activity in equimolar concentration in roots and callus. Values are the mean of three independent experiments and bars indicate SE. (B) SOD, CAT, APX and HO-1 gene expression in soybean leaves subjected to different Cd concentrations (50 or 200μM Cd) for 3 days in roots and 15 days in callus. SOD, CAT, APX and HO-1 transcript expression was analyzed by semi-quantitative RT-PCR. (C) Transcript expression of HO-1 with or without H_2O_2 treatment. Values are the mean of three independent experiments.

Isolation of full-length BnHO family genes

According to the method of homologous-gene cloning and rapid amplification of cDNA ends (RACE), B. juncea cDNAs coding heme oxygenase (designated BjHO-1) was isolated. Alignment of the deduced amino acid sequence of BjHO-1 revealed the common features, such as a non-conserved N-terminal region of various residues and a highly conserved C-terminal extension with approximate 220-amino acids (Fig. S1).

Fig. S1 The evolutionary history was inferred using the Neighbor-Joining method for BjHO-1. The optimal tree with the sum of branch length = 0.31364545 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 7 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 790 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Discussion

Success of employing plant tissue culture techniques for selection and isolation of metal tolerant lines and evaluation of the same depends on an exact correlation between in vitro metal tolerance and whole plant tolerance. In some cases response to stress has been the same in cultures and in whole plants (Tal et al., 1978), but in most of the cases; in vitro responses have been quite different from in vivo responses (Flowers et al., 1985). In plants Cd toxicity alters metabolism in plants (Bergman et al., 2001) which can include the formation of ROS, chlorosis, reduced growth rate, and discoloration in seedlings and callus. In B. juncea callus discoloration occurs due to Fe deficiency caused by competition between Fe and Cd for uptake sites in the membrane. Earlier also the inhibitory effect of Cd on growth of seedlings has been reported in other plant species (Vitoria et al., 2001). Although 200µM $CdCl_2$ was required to inhibit seedlings growth after 72 hours and callus growth after 15 days indicates that callus may be able to uptake Cd more slowly in comparison to roots in seedlings. In addition the cell environment may become more oxidized during callus growth as compare to seedlings growth. We have found more metal accumulation in seedlings in comparison to callus, which probes B. juncea being a strong metal accumulator develops its feature at plant level. We have found higher activity of Hemeoxygenase (HO-1) in seedlings as compare to callus. This observation indicates that there are possibly several physiological factors operating at the whole plant level which confer metal tolerance in seedlings which are lacking

in dedifferentiated and unorganized callus cultures. Dose dependent increase in content of H_2O_2 in callus and seedlings indicated that accumulation of H_2O_2 was an early event. H_2O_2 is also a product of superoxide dismutase reaction. A significant increase in activity of H_2O_2 was also observed up to 200 µM Cd. H_2O_2 content was higher in case of callus as stress mediated toxic responses because in callus culture cells forms an unorganized growth where each cell has to fight for its own against oxidative stress.

H_2O_2 a famous reactive and toxic molecule has been reported to be a multifunctional trigger to induce defense system to maintain redox balance in plant cells (Foyer et al., 1997). Occurrence of H_2O_2 causes damage to plant cells and membranes. This could be one of the possible reasons of high MDA content because higher accumulation of lipid peroxides.

Cadmium induces different responses of antioxidant enzymes in B. juncea. Increase in SOD activity may be due to increased level of ROS, which causes increase in the expression of gene responsible for encoding SOD (Bowler et al., 1992). The decrease in level of SOD activity at higher cadmium concentration may be due to inactivation of enzyme by H_2O_2, produced in different cell compartments and from a number of nonenzymatic and enzymatic processes in cells (Luna et al. 1994). Increase in activity of SOD under conditions of cadmium toxicity is in consonance with several earlier observations (Hsu and Kao 2004). Increase in SOD activity has also been reported in adapted callus of sunflower cultivated under cadmium treatment (Gallego et al., 2002).

H_2O_2 produced by SOD catalyzed reaction is further reduced to water with the help of enzymes APX and CAT. Increase in activity of APX in cadmium-treated plant and callus appears to be in response of increased accumulation of H_2O_2, and the results are similar to earlier observations given by Sharma et al., (2004). An increased level of APX in response to cadmium treatment has been reported in barley (Sharma et al. 2004), Sesbania callus (Israr et al. 2006), and similarly in sunflower callus (Gallego et al. 2002). Apart from APX, other H_2O_2 scavenging enzyme is catalases. In the present study, CAT activity decreased continuously on increasing cadmium concentrations up to 200 µM. CAT is sensitive to superoxide radicals, thus, cadmium stress leads to inactivation of enzyme (Cakmak 2000). The decrease may also be related with degradation caused by peroxisomal proteases or may be due to photoinactivation of enzymes (Sandalio et al. 2001).

H_2O_2 content has been proved to increase under abiotic stress and enhanced gene expression of ROS scavenging enzymes. H_2O_2 is highly toxic mediator of stress induced signaling process and much evidence also emerged the fact that increase concentration of endogenous

H_2O_2 cannot completely scavenged by CAT and APX as in plants H_2O_2 is continuously generated in organelles such as chloroplast, mitochondria, microbodies and peroxisomes. HO-1 localized in chloroplast is highly inducible by H_2O_2 (chen et al., 2009). We observed increased transcript level with pretreatment of H_2O_2. So it can be suggested that HO-1 might be using H_2O_2 as a substrate along with CAT and APX mediating complex signal transduction network. We have found confirming results through histochemical analysis of H_2O_2 which shows decreased H_2O_2 accumulation with pretreatment of Hemin. HO-1 induces acclimation or tolerance against oxidative stress through decaying H_2O_2 via its byproduct billirubin. A lack of direct scavenging of H_2O_2 appears to contrast the protective affect seen with exogenously applied billirubin and increased HO-1 derived billirubin on H_2O_2 mediated damage in mammalian vascular endothelial cells. Inspite of higher content of H_2O_2 in callus we have found lesser HO-1 activity in callus as compare to roots because plants are adapted to cope up with metal stress with several physiological adaptations such as changes in membrane permeability causing exclusion of excess metal, ability to sequester excess ions in cellular compartments such as vacuole, ability to transport ions into the older leaves which undergo senescence, etc, operate in order to reduce the concentration of metal or to alleviate the deleterious effects of metal. By virtue of unorganized structure of callus such mechanisms possibly cannot operate in callus cultures.

The study is important, as it helps in selecting a suitable experimental plant material for the study of HO-1 mediated cadmium tolerance mechanisms and to develop metal tolerant cell lines through in vitro approaches. Success of employing plant tissue culture techniques for selection and isolation of HO-1 expressing metal tolerant lines and evaluation of the same depends on an exact correlation between in vitro metal tolerance and whole plant tolerance.

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