

Evaluation of heme oxygenase 1 (HO 1) in Cd and Ni induced cytotoxicity and crosstalk with ROS quenching enzymes in two to four leaf stage seedlings of *Vigna radiata*

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Abstract Research on heme oxygenase in plants has received consideration in recent years due to its several roles in development, defense, and metabolism during various environmental stresses. In the current investigation, the role of heme oxygenase (HO) 1 was evaluated in reducing heavy metal (Cd and Ni) uptake and alleviating Cd and Ni toxicity effects in the hydroponically grown seedlings of *Vigna radiata* var. *PDM 54*. Seedlings were subjected to Cd- and Ni-induced oxidative stress independently at different concentrations ranging from 10 to 100 μM . After 96 h (fourth day) of treatment, the stressed plants were harvested to study the cellular homeostasis and detoxification mechanism by examining the growth, stress parameters (LPX, H_2O_2 content), and non-enzymatic and enzymatic parameters (ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and catalase (CAT)) including HO 1. At 50 μM CdCl_2 and 60 μM NiSO_4 , HO 1 activity was found to be highest in leaves which were 1.39 and 1.16-fold, respectively. The greatest HO 1 activity was reflected from the reduction of H_2O_2 content at these metal concentrations (50 μM CdCl_2 and 60 μM NiSO_4) which is correlated with the increasing activity of other antioxidant enzymes (CAT, APX). Thus, HO 1 works within a group that generates the defense machinery for the plant's survival by scavenging ROS which is confirmed by a time-dependent study. Hence, it is concluded that seedlings of *V. radiata* were more tolerant

towards metal-induced oxidative stress in which HO 1 is localized in its residential area (plastids).

Keywords Heme oxygenase · Reactive oxygen species · Cadmium · Nickel · *Vigna radiata* · Antioxidant · Oxidative stress · Abiotic stress

Abbreviations

ROS	Reactive oxygen species
CAT	Catalase
APX	Ascorbate peroxidase
GPX	Guaicol peroxidase
SOD	Superoxide dismutase
NPT	Non-protein thiol
CSI	Chlorophyll stability index
HO 1	Heme oxygenase 1
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)

Introduction

Heavy metal contaminants in the environment are the most significant abiotic stress which constrains the productivity and quality of crop plants. The availability and the toxicity of heavy metals in plants depend on the complex rhizospheric reaction between the soil, plants, and microbes. Among an array of heavy metals, cadmium and nickel are the most phytotoxic redox inactive metals which are released into the arable soil from farming and industrial processes and deteriorate the development process of plants by incorporating into the food chain leading to its biomagnifications (Gill and Tuteja 2011; Gill et al. 2011, 2012). Cadmium, one of the chief

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agents of the abiotic stress (Ona et al. 2006), is toxic to plants at almost all concentrations. The permissible limit of Cd in agricultural soil is 100 mg kg^{-1} of soil (Mwegoha and Kihampa 2010). Cd is a needless element for plants which is easily absorbed by the roots and transported to the aerial plant parts, thus entering into the food chain causing health problems in biological organisms. Its increasing concentration in soil leads to decline plant growth by affecting nutrient uptake, water uptake, loss of photosynthesis activity, degradation of chlorophyll activity, loss in enzymatic activity, and high production of reactive oxygen species (ROS) leading to oxidative stress (Gill and Tuteja 2011). Thus, due to elevated toxicity and high solubility in water, cadmium (Cd) is a significant environmental pollutant (Daud et al. 2009).

Nickel is the other essential transition metal import into the surroundings due to smelting wastes, extensive mining, and continuous use of pesticides and phosphate fertilizers. Ni is an essential element for plants as it is a component of a number of enzymes, such as glyoxalases (family I), peptide deformylases, methyl-CoM reductase, ureases, superoxide dismutases, and hydrogenases (Ermler et al. 1998; Kupper and Kroneck 2007) which plays an important role in various metabolic processes including ureolysis, hydrogen metabolism, methane biogenesis, and acidogenesis (Maier et al. 1993; Collard et al. 1994; Ragsdale 1998; Mulrooney and Hausinger 2003). But in greater concentration, it leads to disparity in nutrient uptake which results in interruption of important biological processes in plants leading to leaf chlorosis and necrosis, decline growth responses, and failure of cell membrane function (Ahmad et al. 2008; Siddiqui et al. 2011). Recent reports on Ni toxicity reveal that Ni induced lipid peroxidation and declines water uptake in plants which extensively hamper the membrane functionality. Enormously high concentrations of Ni in soil destroy the farmland and make it unsuitable for vegetation (Duarte et al. 2007). Thus, different heavy metals exert different toxic effects in plants (Sethy and Ghosh 2013).

Heavy metal-stimulated destruction in plants consist of a series of reactions, the major step of which includes linking of metal to the cysteine residue of proteins or enzymes and the production of ROS (Mehrag 1994; Zhang et al. 2004). In addition, heavy metals also promote the generation of ROS and lipid peroxidation which enhances the antioxidant defense system in plants (Sharma and Dietz 2009). Plant protection system to ROS comprises free oxygen radical detoxifying enzymes like catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), guaiacol peroxidase (GPX), and glutathione reductase (GR) as well as supplementary antioxidant molecules namely reduced glutathione (GSH), carotenoids, proline, thiols, and other compounds able of quenching ROS (Liu et al. 2009; Demirevska-Kepova et al. 2004). All the above antioxidant enzymes and molecules have biological role under non-stressed environment, but their activity or

quantity enhanced during oxidative stress. Induction of the antioxidant actions of enzymes via oxidative stress represents the response of a plant towards unfavorable conditions (Foyer et al. 1994) which vary with plant species, tissues analyzed, and the kind, intensity, and duration of the stress treatment (Schutzendubel and Polle 2002). ROS detoxification process begins with the activity of SOD, the first metalloenzyme which converts O_2^- radicals to H_2O_2 and O_2 (Alscher et al. 2002). Accumulation of H_2O_2 is prohibited inside the cell by CAT activity or through the ascorbate–glutathione cycle where ascorbate peroxidase reduces it to H_2O so that the deposition of O_2^- and H_2O_2 is effectively prevented (Sharma and Dietz 2009). Recently, Cui et al. (2013) reported that hydrogen-rich water (HRW) has the ability to improve the effects due to cadmium-induced oxidative stress in *Medicago sativa*, which is consistent with a significant enhancement of the ratio of reduced/oxidized (homo) glutathione (h) GSH).

Recent advances achieved in elucidating the role of heme oxygenase a novel antioxidant enzyme in plant cell defense against oxidative stress. Heme oxygenases are the universal and highly active family of enzymes which catalyzes the oxidative degradation of Fe(III) protoporphyrin IX (heme) to biliverdin, Fe(II), and CO in the presence of reducing equivalents (Shekhawat and Verma 2010). The enzyme was originally identified in the animal system where its role is in the bilirubin production in rat liver (Tenhunen et al. 1968). Now, the genes encoding HOs have been isolated from a wide variety of living system including animals, algae, cyanobacteria, cryptophyta, higher plants, and pathogenic bacteria. In photosynthetic organism, HO gene was first identified in red algae (*Cyanidium caldarium*) (Shekhawat and Verma 2010). Further studies on HO suggested that the enzyme exists in three isoforms: an inducible heme oxygenase 1 (HO 1) and constitutive heme oxygenases HO 2 and HO 3 (Maines 1988). Until now, gene encoding HO 1 has been isolated and strongly characterized as a defending element in cellular protection against oxidative stress in animal system (Tomaro and Batlle 2002; Otterbein et al. 2003). Conversely, fewer reports discussed the functions of HO 1 in quenching ROS in plants. HO 1 role in providing protection against the oxidative stress induced by cadmium has been well reported in *Glycine max* (Balestrasse et al. 2005, 2008; Shekhawat et al. 2011). The HO 1 role in soybean plants was also confirmed by pretreatment of ascorbic acid prior to UV B treatment where HO 1 expression is a dosage-dependent phenomenon (Yannarelli et al. 2006; Santa-Cruz et al. 2017). Other reports on HO 1 depicted its role in abiotic stress including wheat, *M. sativa*, and Chinese cabbage (Cui et al. 2011; Fu et al. 2011; Xu et al. 2011). Additionally, protection exerted by Jasmonic acid against Cd stress in soybean roots has also been reported (Noriega et al. 2012). Recent reports about heme-heme oxygenase 1 (heme-HO 1), a novel antioxidant system, which results in the regulation of

ammonium tolerance in rice *HO 1 (OsSE5)*, were investigated. Besides cellular defense mechanism, several other roles of HO 1 involvement have also been reported in plants, viz. in biosynthetic pathways leading to phytochrome chromophore synthesis (Terry and Kendrick 1999; Davis et al. 2001; Emborg et al. 2006; Linley et al. 2006) and chlorophyll synthesis (Terry et al. 2001; Li et al. 2014; Zhu et al. 2017), developmental role in lateral and adventitious root development (Xu et al. 2011; Cao et al. 2011; Li et al. 2015), and stomatal movement (Cao et al. 2007).

On the basis of the recent information on HO 1 concerning its antioxidant role, the present study is performed to evaluate the regulatory changes of the various physiological processes that occur due to the exposure of cadmium and nickel in the seedlings of *Vigna radiata* and to investigate the *HO 1* gene expression in the seedlings subjected to metal toxicity. The purpose of the research is to ensure the probable functions of HO 1 in cellular protection against Cd- and Ni-induced oxidative stress. Therefore, the current investigation is mainly based on the significance of the heme oxygenase 1 in plant response to metal exposure. The investigation may enhance our understanding to interpret the upregulation as well as downregulation activity of heme oxygenase with other enzymatic and non-enzymatic parameters. Additionally, the current study will be important as it discusses the leading role of HO 1 in providing defense against metal-induced oxidative stress which has been previously unexplored in *V. radiata*, a heavy metal-tolerant species. The crop is a rich source of protein (18–36%) and iron (Bains et al. 2003) and easily survives in the stress conditions which is the precondition of the Thar desert.

Materials and methods

Plant material and establishment of hydroponic culture

Seeds of *V. radiata* var. *PDM 54* were collected from NBPGR, Jodhpur, surface sterilized with 0.1% mercuric chloride for 1–2 min to avoid fungal contamination, and washed thoroughly four to five times with autoclaved distilled water to remove the remaining traces of mercuric chloride in the seeds. Sterilized seeds were sprouted in an autoclaved glass petri dish (10 cm) having autoclaved blotting paper imbibed with sterile deionized water (10–15 ml) at 25 °C in a BOD incubator (vaiometra) under dark conditions. Germinated seeds were shifted to light in thermo statically culture room sustained at 25 ± 2 °C and 50% relative humidity. After a week growth in petri dishes under the above mentioned condition, the hydroponic cultures were established by transferring the uniformly germinated seedlings in plastic pots (12 × 12 cm) containing 1000 ml of Hoagland nutrient solution (pH 6.8 to 6.9, Elico LI 120 pH meter). Ten seedlings

were transferred per pot. These pots were transferred to the thermostatically controlled culture room maintained at 25 ± 2 °C and 50% relative humidity (Fig. 1). The hydroponic solution was aerated twice a day with glass rod to supply adequate oxygen as well as to avoid precipitation of salts. On every third day, the Hoagland medium was changed to evade nutrient deficiencies to seedlings.

Heavy metal treatment in growth medium

The two to four leaf stage (1-week-old) adapted seedlings were further utilized for practical work. The seedlings were subjected to cadmium and nickel stress at different concentrations ranging from 10 to 100 µM. A control having only Hoagland nutrient solution was used to compare the effect of heavy metals on crop plant. After 96 h of treatment, the stressed plant harvested to study the various physiological parameters.

Plant growth parameters

Growth parameters were measured in terms of fresh weight, dry weight, root length, and shoot length of seedlings. For growth parameter studies, seedlings were harvested and washed with deionized water. To determine the dry weight, the fresh tissue was kept in a hot air oven (Nsico Hicon Oven) at 65 °C for drying and the weight (Adair Dutt 125 A SCS) of dried tissue was calculated.

Tolerance index of plant was determined from fresh weight (FW) by using the formula $(FW \text{ treated} / FW \text{ control}) \times 100$ and characterized in percent tolerance (Wilkins 1978).

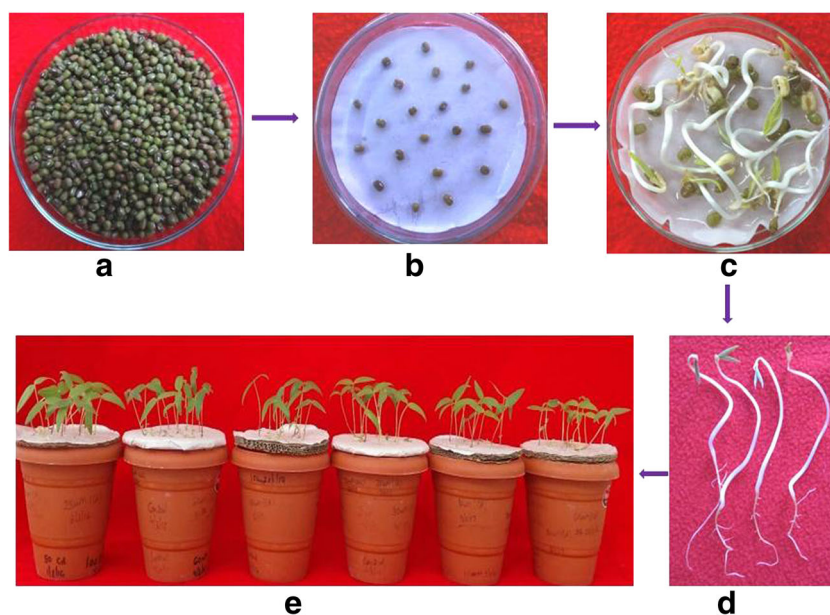
Estimation of chlorophyll content and CSI

Young leaves of *V. radiata* seedlings were assembled and processed for resolving chlorophyll pigments in which 0.1 g of fresh leaf tissues was homogenized in 80% chilled acetone in cold conditions; homogenate was centrifuged (Sigma 3K15) at 10,000×g for 15 min at 4 °C. After centrifugation, the absorbance of the supernatant was recorded at 645 and 663 nm, respectively. The chlorophyll a, chlorophyll b, and total chlorophyll content were determined by Arnon (1949) method. Chlorophyll stability index (CSI) was calculated by the formula $(\text{amount of chlorophyll under stress condition} / \text{amount of chlorophyll in control condition}) \times 100$.

Estimation of total protein content

Protein was estimated by using the method of Lowry et al. (1951). One milliliter of the extracted sample was taken which was homogenized in 50 mM phosphate buffer (pH 7.0). 4.5 ml of reagent I [48 ml of 2% Na₂CO₃ (sodium carbonate in 0.1 N NaOH), 1 ml of 0.5% CuSO₄·5H₂O (copper sulfate), and 1 ml

Fig. 1 Represents the schematic procedure of the successful establishment of hydroponic culture. Seeds of *V. radiata* (PDM 54) (A), sterilized seeds of *V. radiata* germinated in a petri dish under controlled conditions (25 ± 2 °C) (B), germinated seedlings (C), randomly selected seedlings of *V. radiata* at cotyledonary stage for the establishment of hydroponic culture (D), and successful establishment of hydroponic culture (E)



of 1% sodium potassium tartarate], was added. A measure of 0.5 ml of Folin phenol reagent was added after 10-min incubation. Thirty-minute incubation produces a blue color complex in the mixture. Absorbance was taken at 660 nm against a blank without sample (using Elico double beam SL 210 UV VIS spectrophotometer). Protein content was calculated from a standard curve prepared by using bovine serum albumin (BSA) as a standard.

Lipid peroxidation

Lipid peroxidation was predicted by computing the production of malondialdehyde (MDA) content with 2-thiobarbituric acid (TBA) according to De Vos et al. (1989). Plant tissue (0.5 g) was crushed in 10 ml of 0.25% TBA in 10% trichloroacetic acid. The extract was heated at 95 °C for half an hour in a water bath and then brings it to the room temperature by immediately cooling it on ice. Centrifuge it at $10,000\times g$ for 15 min, the absorbance of supernatant was recorded at 532 and 600 nm. The MDA content ($\text{nmol cm}^{-1} \text{g}^{-1}$ fresh weight of tissue) was calculated through Beer Lambert's law by applying extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$. The specific absorbance was determined by subtracting the absorbance at 600 nm from absorbance at 532 nm.

Determination of H_2O_2 content

H_2O_2 production in seedlings of plant was determined spectrophotometrically by the method described by Alexieva et al. (2001). For H_2O_2 determination, 0.5 g fresh plant tissue was homogenized in 5 ml of 0.1% (w/v) TCA in an ice bath. Homogenate was centrifuged at $12,000\times g$ for 15 min. The reaction mixture consists of 0.5 ml of the supernatant, 0.5 ml

of 10 mM potassium phosphate buffer (pH 7.0), and 1 ml of 1 M KI. Reaction was carried out for 1 h in darkness, and absorbance was measured at 390 nm (using Elico double beam SL 210 UV VIS spectrophotometer). Amount of H_2O_2 was calculated by using the extinction coefficient of $0.28 \mu\text{M}^{-1} \text{ cm}^{-1}$.

Non-enzymatic parameters

Proline estimation

Proline estimation was performed by the procedure given by Bates et al. (1973). Plant material (500 mg) was homogenized in 5 ml of freshly prepared 3% sulfosalicylic acid. Centrifuge it at $3000\times g$ for 20 min, 2 ml of supernatant was reacted with 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml of glacial acetic acid. Incubate it at 100 °C for 60 min in a water bath and immediately keep it on ice to terminate the reaction. To this, 4 ml of toluene was introduced and then spin for 1 min. Absorbance of chromophore was recorded at 520 nm. The amount of proline was calculated by using a standard curve made by L-proline. Proline content was expressed in micrograms per gram fresh weight of tissue.

Estimation of thiol content

The thiol content (reduced GSH and oxidized GSSG) was estimated following the method of Anderson (1985). For total glutathione determination, 0.5 g of fresh plant tissue was homogenized in 3 ml of 5% (w/v) sulfosalicylic acid under cold conditions. The homogenate was centrifuged at $1000\times g$ for 10 min. To 0.5 ml of supernatant, 0.5 ml of reaction mixture

[100 mM potassium phosphate buffer (pH 7.0) and 3 mM Na₂EDTA] and 50 µl of 0.15% DTNB (in absolute methanol) were added. After 5 min, the absorbance was recorded at 412 nm using Elico double beam SL 210 UV VIS spectrophotometer. GSSG was assayed by the same method in the presence of 2-vinylpyridine. Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated from the difference between total GSH and GSSG.

For non-protein thiol (NPT) determination, 0.5 g plant tissue was homogenized in 5 ml of 5% (w/v) TCA. Incubate it on ice for 10 min, and further centrifuge it at 8000×g for 10 min. One milliliter of supernatant was reacted with 3 ml of 0.2 M Tris-HCl (pH 8.5) and 0.1 ml of 10 mM DTNB (in absolute methanol). Incubate it at room temperature for 2 min, and absorbance was calculated at 412 nm (Sedlak and Lindsay 1968). Non-protein thiol content was calculated in millimolar per gram fresh weight of tissue by using the extinction coefficient 13.6 mM⁻¹ cm⁻¹.

Enzymatic parameters

For extorting antioxidative enzyme, 0.5 g of plant tissue was crushed in 5.0 ml of 50 mM phosphate buffer, pH 7.0. The extract was centrifuged at 5000×g for 20 min at 4 °C. The supernatant was preserved at -20 °C for the antioxidative enzyme assay.

CAT (EC 1.11.1.6) activity was measured by the method of Aebi (1974). The assay system comprises of 50 mM phosphate buffer (pH 7.0), 9 mM H₂O₂, 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 H₂O₂ at 240 nm was taken as 0.039 mM⁻¹ cm⁻¹.

Activity of APX (EC 1.11.1.11) is the rate of H₂O₂-dependent oxidation of ascorbic acid. Its activity was estimated by the procedure given by Chen and Asada (1989). Ascorbic acid oxidation rate was determined by recording the decrease in absorbance at 290 nm for 3 min (extinction coefficient 2.8 mM⁻¹ cm⁻¹).

Guaicol peroxidase (GPX) (EC 1.11.1.7) was calculated by the procedure of Putter (1974). The assay mixture comprises of 50 mM phosphate buffer (pH 7.0), 20 mM guaicol, 3.7 mM H₂O₂, and a suitable aliquot of enzyme in the final volume 3 ml. Increase in the absorbance was recorded at 436 nm (extinction coefficient 26.6 mM⁻¹ cm⁻¹).

Heme oxygenase assay

HO (EC 1.14.99.3) was determined by the procedure given by Balestrasse et al. (2005). Plant sample (0.3 g) was crushed in 4 ml of extraction buffer (ice-cold solution of 0.25 M sucrose solution containing 1 mM PMSF, 0.2 mM EDTA, and 50 mM potassium phosphate buffer (7.4)). Mixture was centrifuged at

20,000×g for 20 min, and supernatant was used for estimating HO activity. The reaction mixture consists of 50 mM potassium phosphate buffer (pH 7.4), 60 nM NADPH, 250 µl HO extract (0.5 mg of protein), and 200 nM hemin up to a final volume of 500 µl. Samples were incubated at 37 °C for 1 h, and absorbance was recorded at 650 nm. Activity of HO was estimated by calculating the production of biliverdin (extinction coefficient 6.25 µM⁻¹ cm⁻¹).

Intracellular metal (Cd and Ni) content determination

Uptake of intracellular metal ion was estimated by the procedure given by Bates et al. (1982). Plant tissues were oven dried at 80 °C (Nsico Hicon Oven) overnight, and dry weight was calculated. One hundred milligrams of dried plant sample was digested in 5 ml of digestion mixture containing HNO₃ (70%) + H₂O₂ (30%) + deionized water in 1:1:3 ratio until the solution becomes colorless. Residual solution was dissolved in 2% (v/v) nitric acid to a final volume of 5 ml, and Cd and Ni concentration was estimated by atomic absorption spectrophotometer.

The bioconcentration factor (BCF) is used to reflect the ability of the corresponding plant species to take up a particular metal into root/leaf with respect to its concentration in the associated environment. It is the ratio of root/leaf metal to whole metal concentration present in the nutrient solution and was calculated according to the formula given by Zayed et al. (1998). The translocation factor (TF) shows the metal translocation properties of corresponding metal species (Stoltz and Greger 2002). The TF were calculated by the formula $TF = [\text{metal } \mu\text{g g}^{-1} \text{DW}]_{\text{leaf}} / [\text{metal } \mu\text{g g}^{-1} \text{DW}]_{\text{root}} \times 100$.

Statistical analysis

Data were statistically analyzed in experimental observations using Sigma 12.0 version software, and the results were expressed as mean (±standard error) of three independent replicates of each independent experiment.

Results

Growth parameters of two to four leaf stage seedlings of *V. radiata* were analyzed by measuring fresh weight, dry weight, shoot length, and root length. As shown in Table 1, increasing concentration of cadmium progressively decreases the fresh weight and dry weight up to 33.86 and 55.32% with respect to control. The effect of cadmium on fresh and dry weight was noticed maximum at 50 µM concentration after 96 h of treatment. Root length on exposure to cadmium decreases progressively with increasing concentration of CdCl₂, whereas no significant effect of cadmium was observed on shoot length after fourth day of

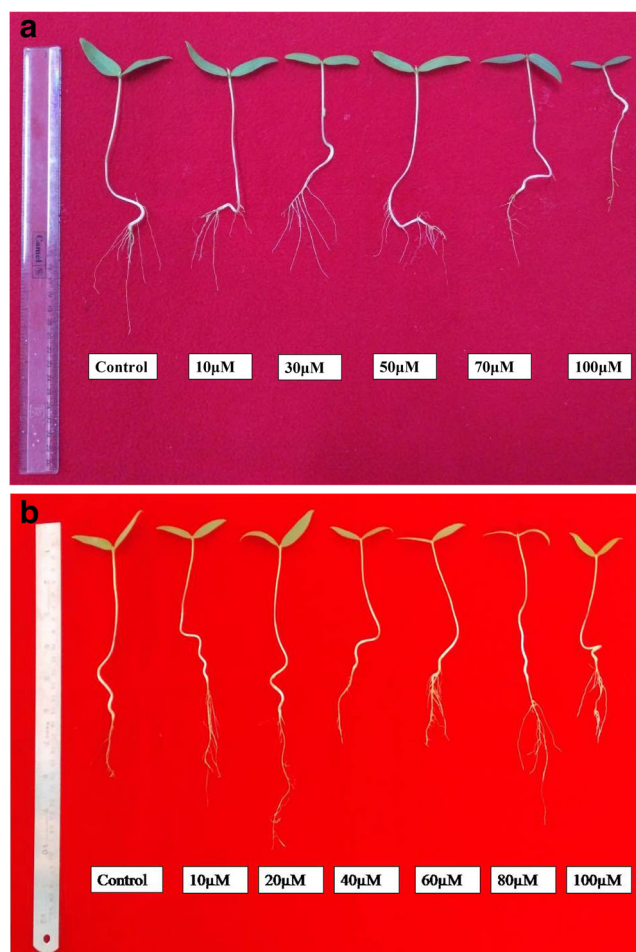


Fig. 2 **a** Morphological alteration in root and shoot length in *Vigna radiata* seedlings after treated it with different concentrations of CdCl_2 (10–100 μM) for a period of 96 h. **b** Morphological alteration in root and shoot length in *V. radiata* seedlings after treatment for a period of 96 h with different concentrations of NiSO_4 (10–100 μM)

treatment (Fig. 2a) (Table 1). Similar results of fresh weight and dry weight were observed on treating the seedlings with NiSO_4 (Table 2). Shoot length of *V. radiata* seedlings initially increases and then decreases progressively on increasing the concentration of nickel (Fig. 2b). The maximum shoot length was observed at 20 μM which was 1.8-fold with respect to control. Effect of nickel on root length

was noticed at initial concentration of NiSO_4 , 20 μM at which root length was 1.62-fold in comparison to control (Fig. 2b) (Table 2).

Photosynthetic pigments increase progressively with increasing concentration of cadmium up to 30 μM ; at 50 μM , a significant decrease was observed in Chl a, Chl b, and total chlorophyll in the case of leaves (Table 3). Whereas in the case of shoots, different results were observed; at initial concentration of cadmium up to 30 μM , a significant decline in photosynthetic pigment was observed, but on further increasing the concentration up to 100 μM , a gradual increase in chlorophyll content was observed. Percentage decrease at 30 μM concentration was more in Chl a in comparison to Chl b in shoots (Table 3). Stability index of chlorophyll was also calculated, and differing results were obtained in the case of leaves and shoots. It was observed that at 50 μM , 66.05% increase and 9.8% decrease in leaves and shoots, respectively, in CSI were noticed as compared to control after 96 h of Cd treatment (Table 3). The effect of nickel on photosynthetic pigments was also determined. Chlorophyll content decreases gradually with increasing concentration of nickel which results in chlorosis of the leaves. At 60 μM concentration, approximately 25.19% inhibition in total chlorophyll pigment was noticed in leaves (Table 4). In shoots, a noticeable increase was observed in Chl a and total chlorophyll content. At 60 μM Ni, 25% decline in chlorophyll stability index of leaves was observed after 96 h (Table 4).

Protein content in seedlings of *V. radiata* treated with Cd increases at initial concentration, but a significant increase was observed at 50 μM concentration of CdCl_2 (Fig. 3a). At 50 μM Cd treatment, 117.6, 174, and 87.58% increase in protein content was observed in leaves, shoots, and roots, respectively. However, after 50 μM concentration, a slight decline in protein quantity was noticed in leaves, but the recorded concentrations were notably elevated with respect to control. Except 50 μM concentration, no significant change was observed in protein content in the case of shoots and roots (Fig. 3a). The protein content increases progressively with increase in Ni concentrations. At 60 μM Ni treatment, 59.2 and 49.7% increase in protein content was recorded in leaves and roots, respectively. No significant effect of Ni was noticed in the protein content of shoots (Fig. 3b).

Table 1 Effect of cadmium on fresh weight, dry weight, shoot length, and root length of seedlings of *V. radiata*

Conc. of CdCl_2	Fresh weight (g)	Dry weight (g)	Shoot length (cm)	Root length (cm)	Tolerance index (%)
Control	0.378 ± 0.00629	0.188 ± 0.00374	11.13 ± 0.966	11.78 ± 1.481	100.0 ± 0.00
10 μM	0.350 ± 0.0252	0.173 ± 0.0066	12.38 ± 0.687	16.3 ± 0.877	92.593 ± 6.97
30 μM	0.300 ± 0.0197	0.112 ± 0.00583	11.2 ± 0.603	13.3 ± 0.628	79.365 ± 8.49
50 μM	0.237 ± 0.0413	0.060 ± 0.00316	10.4 ± 0.579	12.6 ± 0.812	62.698 ± 4.05
70 μM	0.282 ± 0.0111	0.070 ± 0.00913	11.2 ± 0.490	12.2 ± 0.860	74.603 ± 8.58
100 μM	0.250 ± 0.00913	0.084 ± 0.00510	12.6 ± 0.812	10.4 ± 0.663	66.137 ± 5.21

Table 2 Effect of nickel on fresh weight, dry weight, shoot length, and root length of seedlings of *V. radiata*

Conc. of NiSO ₄	Fresh weight (g)	Dry weight (g)	Shoot length (cm)	Root length (cm)	Tolerance index (%)
Control	0.707 ± 0.0145	0.307 ± 0.00333	10.167 ± 1.129	12.500 ± 0.404	100.0 ± 0.000
10 µM	0.697 ± 0.0639	0.290 ± 0.0057	8.1330 ± 0.0882	12.667 ± 1.202	98.585 ± 9.624
20 µM	0.690 ± 0.0173	0.263 ± 0.00333	18.333 ± 2.028	20.333 ± 0.167	97.595 ± 7.279
40 µM	0.620 ± 0.0513	0.253 ± 0.00333	16.733 ± 0.897	12.567 ± 0.825	87.694 ± 8.913
60 µM	0.520 ± 0.0451	0.233 ± 0.0333	14.833 ± 0.333	11.100 ± 0.586	73.550 ± 6.42
80 µM	0.440 ± 0.0208	0.157 ± 0.00333	13.100 ± 0.808	14.933 ± 2.466	62.235 ± 4.45
100 µM	0.273 ± 0.0233	0.193 ± 0.00633	9.400 ± 0.265	10.567 ± 0.829	38.614 ± 7.79

The responses of the MDA content to the applied concentrations of cadmium and nickel are depicted in Fig. 4a, b. A substantial elevation in MDA amount in *V. radiata* seedlings treated with different cadmium concentrations was observed after 96 h of treatment. Plant tissue treated with 50 µM Cd shows 161.49% increase in MDA content with respect to control in leaves. Whereas, MDA content at 50 µM Cd was 4.02 times in comparison to control in root tissues. However, after 50 µM Cd concentration, a slight decrease in MDA content (at 70 µM Cd) was recorded (Fig. 4a). Considerable increase in the MDA content was also depicted with increasing concentration of Ni in Fig. 4b. At 60 µM Ni treatment, 150.48, 122.2, and 67.49% increase in MDA content in comparison to control was observed in leaves, shoots, and roots, respectively. But, after 60 µM concentration, a minute decline in MDA content was noticed (at 80 µM Ni) which again rises at higher concentration (100 µM Ni treatment). To understand the time-dependent effect of Cd and Ni on MDA content, a study was made after 0.5, 6, 15, 24, 48, 72, and 96 h for which 50 µM Cd and 60 µM Ni concentrations were selected, as maximum effects were observed at these concentrations. As shown in Fig. 4c at 50 µM Cd and 60 µM Ni, the MDA content increases significantly till 24 h and then decreases. MDA content at 50 µM Cd and 60 µM Ni at 24 h was 1.79 and 1.72-fold higher in comparison to initial time interval in leaf tissue. Maximum increase in MDA content in leaves at 50 µM Cd was observed after 96 h of treatment which is 3.2 times than MDA content at 0.5 h. The linear increase in MDA content after individual treatment of 50 µM Cd and 60 µM Ni was also observed in roots, but no significant change in MDA content was noticed in shoots. The increased accumulation of lipid peroxides in both Cd and Ni is an indication of enhanced production of reactive oxygen species like H₂O₂. H₂O₂ showed a significant increase in leaves of Cd-treated seedlings (Fig. 5a). The level of H₂O₂ was 1.69 times (at 10 µM Cd) in comparison to control over a period of 96 h, and thereafter, no significant change was observed

at high Cd treatment. Effect of Ni on H₂O₂ content was also noticed in leaves of *V. radiata*. H₂O₂ content initially increases with increase in Ni concentration. At the highest Ni treatment, the H₂O₂ content was found to increase. At 10 µM Ni, the H₂O₂ content was increased by 1.24-fold as compared to control (Fig. 5b). H₂O₂ content was also studied with respect to the time duration of Cd and Ni exposure. The studies were conducted after 0.5, 6, 15, 24, 48, 72, and 96 h. Treatment with 50 µM Cd induced a rapid increase in H₂O₂ content at initial hours. The threshold level of H₂O₂ was observed at 6 h which is 1.29-fold higher than the H₂O₂ content at 0.5 h (Fig. 5c). Similar results were obtained with 60 µM Ni. The peak value of H₂O₂ with 60 µM Ni was noticed at 6 h which is 1.15-fold higher than the H₂O₂ content at initial hours (Fig. 5c).

Proline content in leaf tissue of *V. radiata* on exposure to CdCl₂ increased significantly with increasing concentration of Cd (Fig. 4a). At 70 µM Cd treatment, proline content was found to be maximum which was 2.79-fold with respect to control in leaves. Whereas in root tissues, 166.5% increase in proline content was found at initial Cd concentration (10 µM Cd). In shoots, no significant change was observed in proline content on treating the seedlings with Cd (Fig. 4a). However, the effect of Ni on proline content was only observed in leaves in which the significant increase was observed at 60 µM Ni concentration which was 6.15-fold in comparison to control (Fig. 4b).

V. radiata seedlings on exposure to cadmium show progressive increase in glutathione content with increasing concentration of CdCl₂. The oxidized and reduced glutathione (GSSG and GSH) content increases with elevated CdCl₂ concentration till 50 µM and then decreases at higher Cd treatment (Table 5). At 50 µM Cd, the GSSG content in leaves, shoots, and roots was 1.13, 1.17, and 1.27-fold with respect to control, while percent increase in GSH content at this concentration was 28, 19.68, and 5.27% than control in all the three tissues, viz. leaves, shoots, and roots (Table 5). The GSH/

Table 3 Chlorophyll a, chlorophyll b, and total chlorophyll content in seedlings of *Vigna radiata* exposed to different concentrations of cadmium chloride after 96 h

Conc. of CdCl ₂	Shoots							
	Leaves			Shoots				
	Chl a	Chl b	Total Chl	CSI	Chl a	Chl b	Total Chl	CSI
Control	0.615 ± 0.00481	0.250 ± 0.00247	0.866 ± 0.00732	100 ± 0.00	0.0849 ± 0.00181	0.0310 ± 0.000193	0.116 ± 0.00177	100 ± 0.00
10 µM	1.043 ± 0.0114	0.467 ± 0.00293	1.510 ± 0.0142	174.474 ± 0.169	0.0802 ± 0.000388	0.0278 ± 0.000003	0.108 ± 0.000400	93.289 ± 1.740
30 µM	1.100 ± 0.0127	0.535 ± 0.000624	1.635 ± 0.0129	188.856 ± 0.682	0.0726 ± 0.00024	0.0258 ± 0.00031	0.0984 ± 0.00025	85.003 ± 1.307
50 µM	0.983 ± 0.00443	0.455 ± 0.000306	1.437 ± 0.00464	166.054 ± 1.138	0.0754 ± 0.00029	0.0290 ± 0.000784	0.104 ± 0.000987	90.163 ± 2.046
70 µM	0.853 ± 0.000306	0.359 ± 0.00113	1.212 ± 0.000895	140.003 ± 1.205	0.0829 ± 0.00069	0.0285 ± 0.000560	0.111 ± 0.00123	96.249 ± 2.511
100 µM	1.145 ± 0.000333	0.558 ± 0.00160	1.702 ± 0.00133	196.682 ± 1.499	0.1000 ± 0.00065	0.0382 ± 0.000654	0.139 ± 0.00120	119.728 ± 2.609

Table 4 Chlorophyll a, chlorophyll b, and total chlorophyll content in seedlings of *Vigna radiata* exposed to different concentrations of nickel after 96 h

Conc. of NiSO ₄	Shoots							
	Leaves			Shoots				
	Chl a	Chl b	Total Chl	CSI	Chl a	Chl b	Total Chl	CSI
Control	1.001 ± 0.00463	0.424 ± 0.00384	1.425 ± 0.00404	100 ± 0.00	0.0720 ± 0.00025	0.0337 ± 0.00089	0.106 ± 0.00089	100 ± 0.00
10 µM	0.804 ± 0.00535	0.311 ± 0.00265	1.115 ± 0.00780	78.270 ± 0.328	0.0717 ± 0.00009	0.0328 ± 0.00030	0.105 ± 0.00006	98.873 ± 0.862
20 µM	0.865 ± 0.00220	0.344 ± 0.00247	1.209 ± 0.00468	84.857 ± 0.117	0.0746 ± 0.00117	0.0396 ± 0.00102	0.114 ± 0.00215	108.074 ± 1.505
40 µM	0.820 ± 0.00428	0.331 ± 0.00504	1.151 ± 0.00930	80.770 ± 0.433	0.0988 ± 0.00073	0.0446 ± 0.00067	0.143 ± 0.00140	135.562 ± 1.060
60 µM	0.761 ± 0.00157	0.304 ± 0.00127	1.066 ± 0.00181	74.773 ± 0.126	0.0806 ± 0.00017	0.0367 ± 0.00019	0.117 ± 0.00027	110.977 ± 0.933
80 µM	0.946 ± 0.00375	0.385 ± 0.00246	1.332 ± 0.00618	93.445 ± 0.194	0.0872 ± 0.00038	0.0393 ± 0.00024	0.127 ± 0.00016	119.691 ± 1.020
100 µM	1.055 ± 0.00058	0.452 ± 0.00312	1.507 ± 0.00258	105.78 ± 0.151	0.1112 ± 0.001010	0.0499 ± 0.00145	0.162 ± 0.00245	153.041 ± 2.352

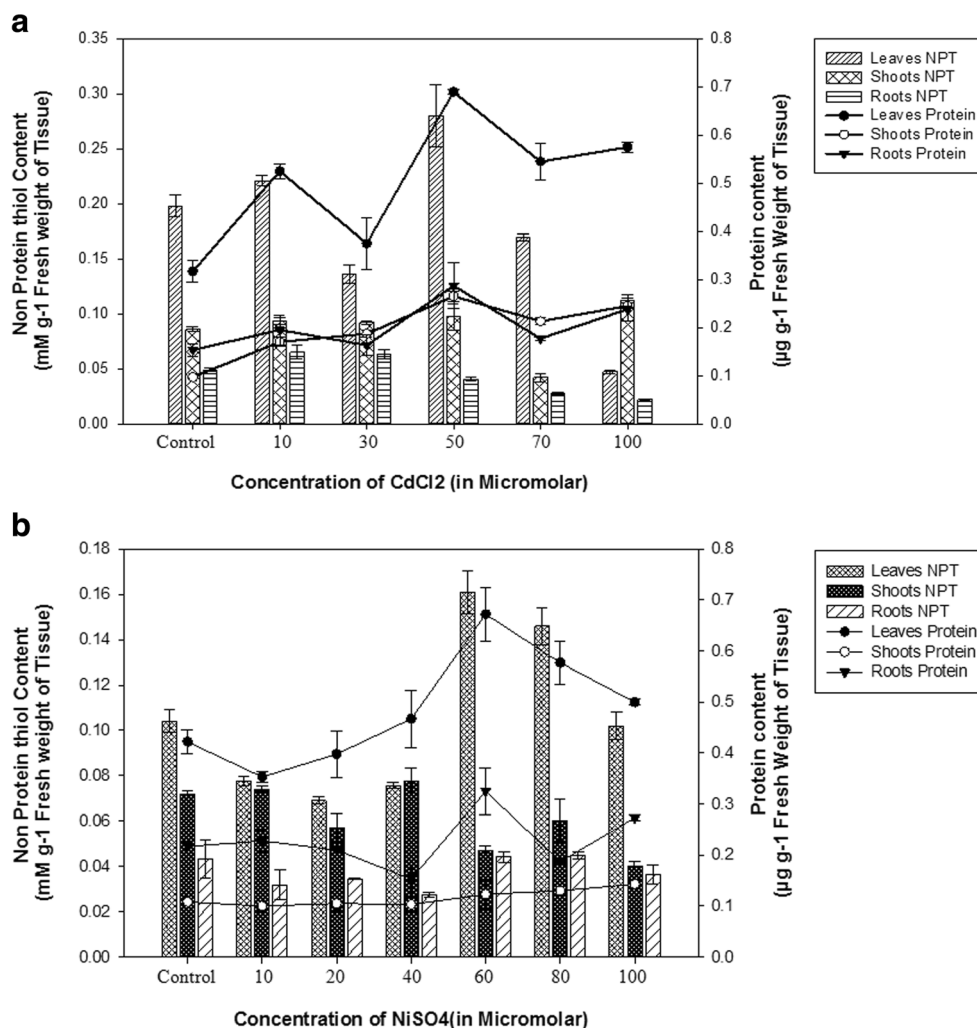
Table 5 Oxidized glutathione (GSSG) and reduced glutathione (GSH) content and GSH/GSSG ratio in seedlings of *Vigna radiata* exposed to different concentrations of cadmium after 96 h

Conc. of CdCl ₂ (μM)	GSSG (μM g ⁻¹ fresh weight of tissue)						GSH (μM g ⁻¹ fresh weight of tissue)						GSH/GSSG						
	Leaves		Shoots		Roots		Leaves		Shoots		Roots		Leaves		Shoots		Roots		
Control	22.167 ± 0.481	15.778 ± 0.434	12.444 ± 0.530	24 ± 0.441	18.056 ± 0.401	17.944 ± 0.20	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.146 ± 0.043	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035
10	24.556 ± 0.20	18.5 ± 0.673	14.056 ± 0.588	25.166 ± 0.763	19.056 ± 0.294	17.5 ± 0.167	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.034 ± 0.051	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035
30	22.778 ± 0.401	17 ± 0.674	14.889 ± 0.455	24.611 ± 0.242	19.444 ± 0.547	17.722 ± 0.147	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.034 ± 0.051	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035
50	25.111 ± 0.242	18.444 ± 0.242	15.889 ± 0.242	30.722 ± 1.029	21.611 ± 0.222	18.889 ± 0.556	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.034 ± 0.051	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035
70	23.111 ± 0.530	14.50 ± 0.509	12.999 ± 0.586	24.944 ± 0.147	20.50 ± 0.385	18.668 ± 0.348	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.034 ± 0.051	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035
100	17.333 ± 0.347	12.833 ± 0.50	10.777 ± 0.400	25.722 ± 0.278	20.223 ± 0.294	17.834 ± 0.167	1.084 ± 0.0347	1.011 ± 0.0297	1.114 ± 0.0322	1.149 ± 0.074	1.192 ± 0.035	1.034 ± 0.051	1.034 ± 0.051	1.149 ± 0.074	1.172 ± 0.006	1.190 ± 0.041	1.447 ± 0.064	1.234 ± 0.061	1.192 ± 0.035

GSSG ratio after treated *V. radiata* seedlings with CdCl₂ for a period of 96 h progressively increases with rise in Cd concentration in leaves and shoots. However, in the case of roots, the GSH/GSSG ratio decreases significantly till 50 μM Cd treatment. After 50 μM, the GSH/GSSG ratio was found to increase in root tissue (Table 5). Moreover, non-protein thiol content was observed maximum at 50 μM Cd which is 41.4% in comparison to control in leaves. Whereas, no significant change was observed in shoot and root tissues (Fig. 3a). The effect of Ni on glutathione and non-protein thiol content was also recorded. The GSSG content initially increases at initial Ni concentration till 40 μM and then decreases with further increase in concentration in leaf tissue. While in shoot and root tissues, the GSSG content declines significantly with elevated NiSO₄ concentration. Similarly, GSH content decreases progressively with increase in Ni concentration in shoot and root tissues. But no significant effect of Ni on GSH content in leaf tissue was noticed (Table 6). Furthermore, the GSH/GSSG ratio in seedlings of *V. radiata* after NiSO₄ treatment decreases with rise in metal concentration till 60 μM treatment and then increases with further increase in metal concentration in leaf tissue. While in shoots and roots, the GSH/GSSG ratio decreases progressively with increase in NiSO₄ concentration (Table 6). Non-protein thiol content initially decreases and then increases with increasing concentration of Ni. At 60 μM Ni, non-protein thiol was found to be maximum which was 54.8% with respect to control in leaves. No considerable change was observed in non-protein thiol in both shoot and root tissues, respectively (Fig. 3b).

Changes in the activity of antioxidant enzymes were found in the two to four leaf stage seedlings of *V. radiata* treated with CdCl₂ and NiSO₄. GPX activity increased significantly after Cd exposure up to 50 μM concentration in leaves and shoots which was 2.46 and 3.11-fold with respect to control at 50 μM (Fig. 6a). In roots, GPX activity after Cd treatment was found maximum at 10 μM concentrations in which 102.3% increase in GPX activity was recorded with respect to control (Fig. 6a). Whereas, GPX activity in the NiSO₄-treated seedlings of *V. radiata* increases significantly with increase in concentration. GPX activity in roots at 10 μM concentration was found to be maximum which was 65.49% in comparison to control (Fig. 6b). Catalase activity on Cd exposure was found to increase with increase in Cd concentration in leaves, and at 50 μM Cd treatment, the catalase activity was found to be 48.03% with respect to control. After 50 μM concentration, the activity of catalase was found to be almost constant in leaves. No significant change was observed in catalase activity in roots and shoots on exposure to Cd metal (Fig. 6a). Effect of Ni on catalase activity was observed in leaves of *V. radiata* which was 36.5% with respect to control at 60 μM concentration (Fig. 6b). An increase of 36.3

Fig. 3 **a** NPT and total protein content in seedlings of *Vigna radiata*. Seedlings were treated with 10, 30, 50, 70, and 100 μM cadmium for a period of 96 h. **b** NPT and total protein content in seedlings of *V. radiata*. Seedlings were treated with 10, 20, 40, 60, 80, and 100 μM nickel for a period of 96 h. *Vertical bars* represent SE ($n = 3$)



and 10.23% in the activity of ascorbate peroxidase was observed in roots and leaves, respectively, on exposure to Cd metal at 50 μM (Fig. 5a). No significant change was observed in shoots after Cd treatment (Fig. 5a). An increase of 194.7% in APX activity was observed in roots with respect to control at 60 μM Ni treatments. No significant change was observed in APX activity in shoots and leaves on exposure to Ni (Fig. 5b).

Antioxidant enzyme activity was also calculated in a time-dependent manner which is based on the duration of the interaction of Cd and Ni with the seedlings. The studies were conducted after 0.5, 6, 15, 24, 48, 72, and 96 h on 50 μM CdCl₂ and 60 μM NiSO₄. GPX activity in *V. radiata* seedlings was noticed from the beginning of the 50 μM Cd and 60 μM Ni treatment. The increase in GPX activity at 50 μM Cd lasted for 96 h, but the linear increase was found to be after 48 h, and the maximum activity was noticed at 72 h in leaves, shoots, and roots which was 2.02, 1.43, and 2.08 times higher than the activity observed just after the treatment (Fig. 6c). Time-dependent effect of 60 μM Ni treatment on GPX activity

was found to increase till 72 h in leaves and shoots which was 2.03 and 1.78 times higher than the GPX activity at 0.5 h, but in roots, the GPX activity was found to decrease after 6 h up till 48 h, and then, a sudden increase was observed at 72 h at which the activity was 1.60 times higher than the activity at 0.5 h (Fig. 6c). Treatment with 50 μM Cd induced a rapid increase in catalase activity. The peak level of catalase was observed at 24 h which were 2.02, 2.64, and 2.35 times higher than the study conducted at 0.5 h of treatment in leaves, shoots, and roots, respectively (Fig. 6d). Catalase activity after exposing the seedlings of *V. radiata* with 60 μM Ni linearly increases with increase in time intervals. The threshold catalase activity was observed at 24 h which were 1.24, 2.69, and 4.71-fold higher than the activity observed just after the treatment in leaves, shoots, and roots (Fig. 6d).

Heme oxygenase activity was higher in leaf tissue in comparison to shoots and roots on treating the seedlings with CdCl₂. In leaf tissue, HO significantly increased up to 50 μM CdCl₂, and after that, constant HO activity was observed. At 50 μM CdCl₂ concentration, a 38.8% increase in

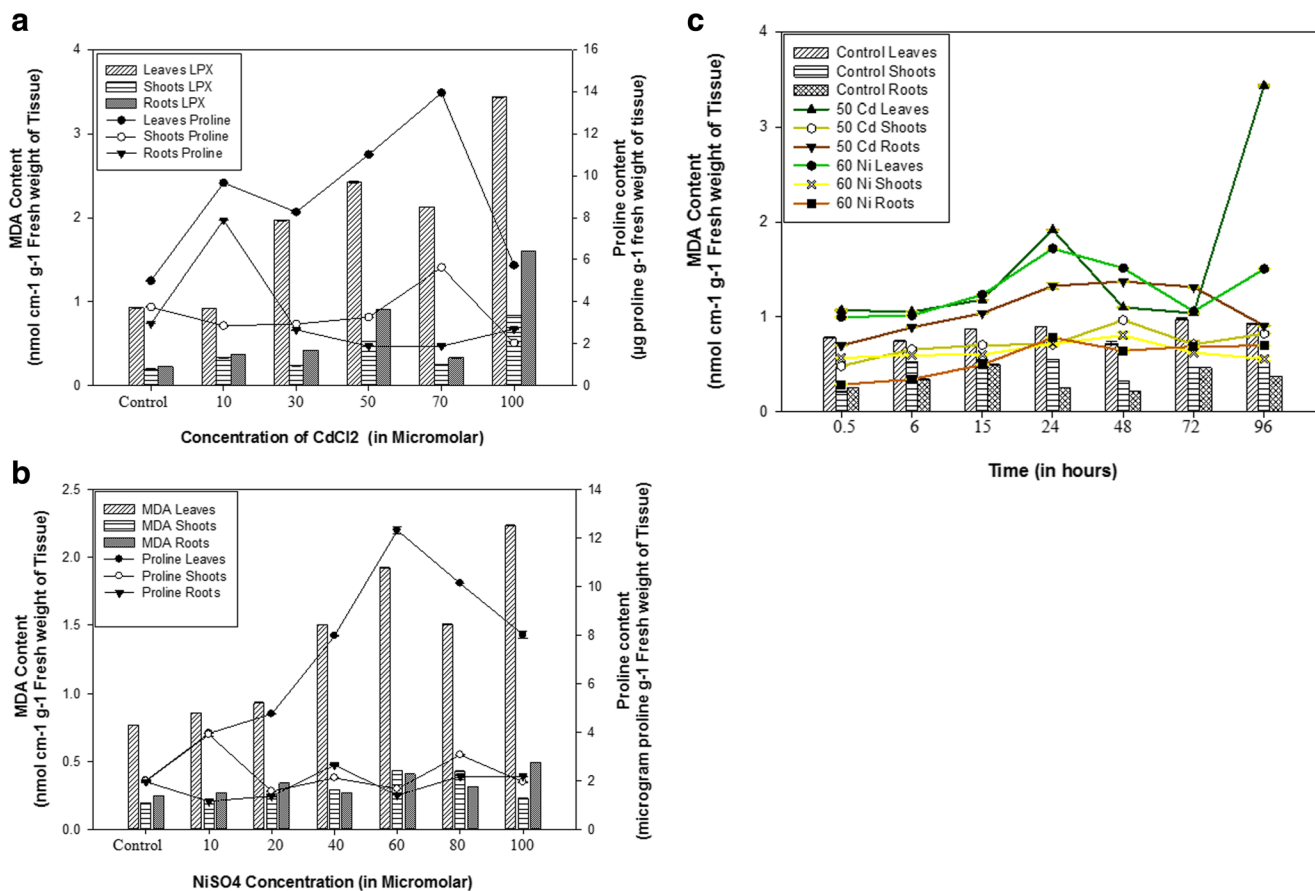


Fig. 4 a Proline and MDA content in seedlings of *Vigna radiata*. Seedlings were treated with 10, 30, 50, 70, and 100 μM cadmium for a period of 4 days. Vertical bars represent SE ($n = 3$). b Proline and MDA content in *V. radiata* seedlings. Seedlings were treated with 10, 20, 40, 60,

80, and 100 μM nickel for a period of 4 days. Vertical bars represent SE ($n = 3$). c The time duration of MDA content at 50 μM cadmium and 60 μM nickel at equal time interval for a period of 96 h. Values are mean \pm SE ($n = 3$)

HO activity was observed in leaf tissue. HO activity in roots was found maximum at 100 μM CdCl₂, whereas no significant change was observed in HO activity in shoots (Fig. 7a). Effect of Ni on heme oxygenase activity was found to be higher in leaves at 60 μM concentration which was 43.67% in comparison to control. No significant change was observed in shoot and root tissues on treating the seedlings with NiSO₄ (Fig. 7b). Time-dependent measurements of HO content which depend on the duration of Cd and Ni exposure were examined. The study was conducted after 0.5, 6, 15, 24, 48, 72, and 96 h. At 50 μM Cd treatment, a rapid increase in HO activity with increase in time exposure was observed. The peak value of HO activity was observed at 24 h which is 2.12, 2.08, and 2.74 times higher than the HO activity just after the treatment (Fig. 7c). Similar results were obtained with 60 μM Ni. The HO activity observed at 24 h with 60 μM Ni was 1.18, 3.18, and 7.17 times higher than the activity of HO at 0.5 h (Fig. 7c).

The intracellular Cd and Ni content in leaves and roots of *V. radiata* seedlings was investigated (Table 7). Intracellular Cd accumulation decreases with increase in Cd content till

50 μM Cd and further increases at higher concentration in leaf tissue. The highest Cd accumulation was noticed at 10 μM Cd (9.86 times) and minimum at 50 μM Cd (1.45 times). However, in roots, the intracellular Cd content increases, with rise in Cd concentration. The maximum Cd accumulation was at 70 μM Cd which is 54.71-fold, and minimum accumulation was at 10 μM Cd that is 9.38 times as compared to control (Table 7). Intracellular Ni content in leaf and root tissues of *V. radiata* seedlings increases with elevated concentration of Ni till 50 μM and then decreases. The intracellular Ni content gradually decreased from 53.7-fold (at 50 μM Ni) to 45.3-fold (at 60 μM Ni) in leaf tissue over a period of 4 days (96 h). Ni accumulation in roots was greatest at 50 μM Ni which is 57.03 times and lowest at 40 μM Ni concentration which is 14.49 times in comparison to control (Table 7).

Besides, intracellular metal accumulation BCF has been suggested as the other parameter for recognizing the metal extraction and accumulation ability of *V. radiata*. The BCF in different tissues of *V. radiata* seedlings was evaluated for Cd and Ni after 96 h of treatment. BCF decreases progressively with increasing concentration of Cd in the nutrient solution

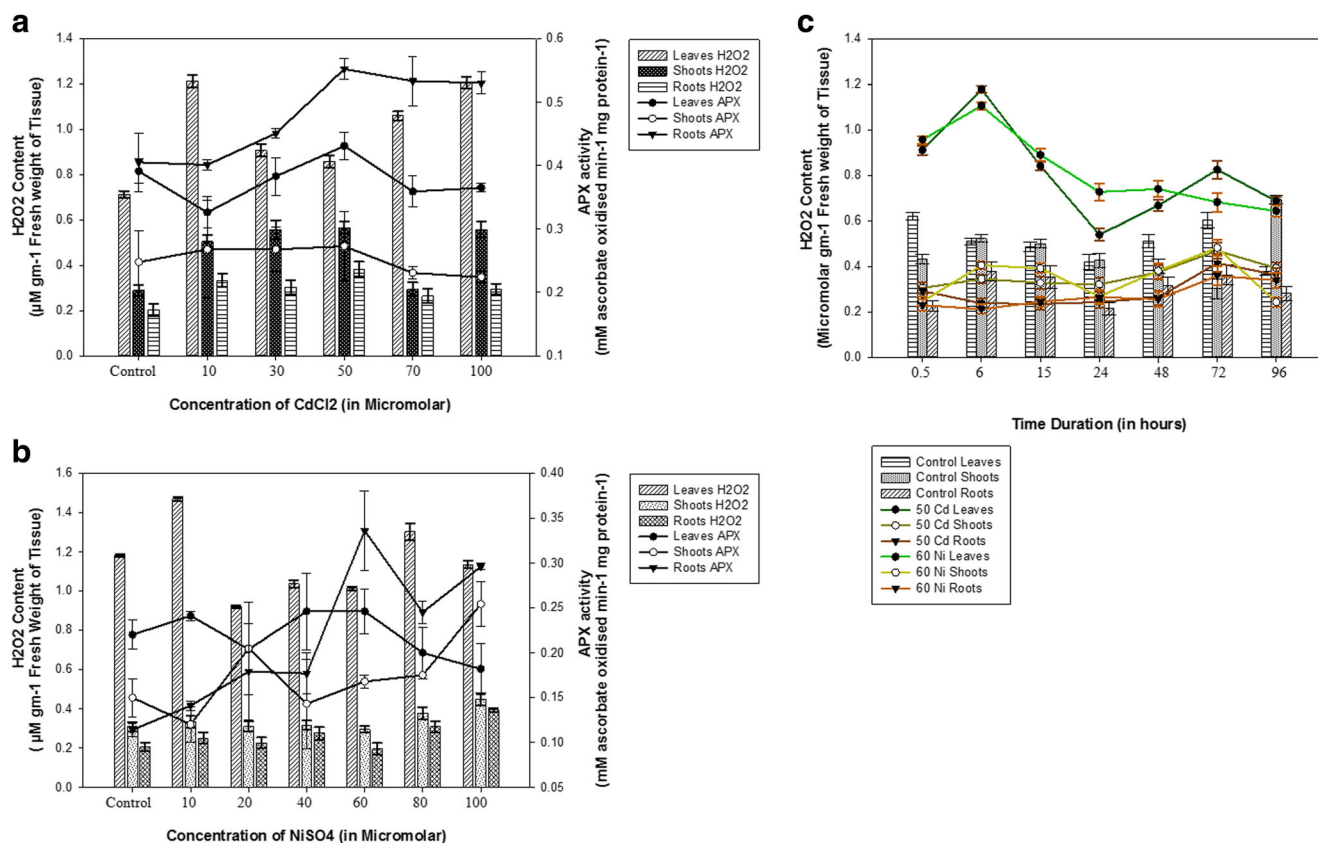


Fig. 5 **a** H₂O₂ content and APX activity in seedlings of *Vigna radiata*. Seedlings were treated with equimolar concentration of cadmium chloride for a period of 96 h. Vertical bars represent SE ($n = 3$). **b** H₂O₂ content and APX activity in seedlings of *V. radiata*. Seedlings

were treated with 10, 20, 40, 60, 80, and 100 μM concentration of nickel sulfate for a period of 96 h. Vertical bars represent SE ($n = 3$). **c** The effect of time on H₂O₂ content at 50 μM cadmium and 60 μM nickel at 0.5, 6, 15, 24, 48, 72, and 96 h. Values are mean \pm SE ($n = 3$)

up to 50 μM and then increases in the case of leaves. But the increase in BCF at 70 μM Cd was 92.7% less than 10 μM Cd. The BCF of Cd in root is significantly higher at low Cd concentrations (at 10 μM Cd) (Table 7). Similar pattern was obtained for BCF in Ni which was ranged from 0.3309 to 0.12013 (from 10 to 70 μM) in leaves and 0.7553 to 0.2171 (from 10 to 70 μM) in roots.

The TF signifies the internal metal transportation. In the current study, the TF of Cd decreases at initial concentration till 50 μM CdCl₂ and then increases with further increase in concentration. The maximum TF was observed at 10 μM Cd which was 31.4 (Table 7). However, similar pattern of TF was observed in the case of NiSO₄. The peak value of TF was observed at 70 μM Ni which was 55.33 (Table 7).

Discussion

In plants, heavy metals (Cd and Ni) in a micromolar concentration promote toxicity by generating ROS and lipid peroxidation (Hsu and Kao 2004; Shekhawat et al. 2008) which results in the reduction of photosynthetic rate,

growth inhibition, and disturbances in antioxidant defense system (Sandalo et al. 2001). The produced ROS (H₂O₂, OH⁻ radicals) acts as a messenger for the induction of multiple stress-responsive genes responsible for the heavy metal acclimatization and repair. Hence, the aim of the present study is to investigate the possible role of HO 1 in providing cellular defense to the hydroponically grown *V. radiata* seedlings which is subjected to different Cd- and Ni-induced oxidative stress. The study is conducted by examining the different morphological parameters and non-enzymatic as well as enzymatic parameters associated to the antioxidant defense mechanism. The research is significant as it is helpful in selecting an appropriate crop species to study the leading role of HO 1 in antioxidant defense mechanism and to produce a metal-tolerant crop plant in the near future.

Plant primarily responds to metal toxicity by inhibiting its overall development which is in terms of decline in fresh weight and dry weight, shoot length reduction, and root length reduction. The present study on two to four leaf stage *V. radiata* seedlings treated with varying concentrations of Cd and Ni illustrates a regular reduction in the overall

Table 6 Oxidized glutathione (GSSG) and reduced glutathione (GSH) content and GSH/GSSG ratio in seedlings of *Vigna radiata* exposed to different concentrations of nickel after 96 h

Conc. of NiSO ₄ (μM)	GSH (μM g ⁻¹ fresh weight of tissue)				GSH/GSSG					
	Leaves		Shoots		Leaves		Shoots		Roots	
	Leaves	Shoots	Leaves	Shoots	Leaves	Shoots	Leaves	Shoots	Leaves	Shoots
Control	22.940 ± 0.242	8.694 ± 0.0199	8.317 ± 0.0095	20.667 ± 0.976	15.972 ± 0.487	11.906 ± 0.40	0.902 ± 0.051	1.837 ± 0.057	1.432 ± 0.049	
10	22.222 ± 0.147	8.361 ± 0.0242	8.428 ± 0.0434	22.667 ± 1.302	12.139 ± 0.457	12.628 ± 0.48	0.899 ± 0.051	1.451 ± 0.051	1.498 ± 0.050	
20	25.222 ± 0.147	8.545 ± 0.0642	8.217 ± 0.0441	20.722 ± 0.564	14.011 ± 0.563	10.617 ± 0.34	0.822 ± 0.023	1.639 ± 0.054	1.291 ± 0.035	
40	27.0 ± 0.0964	8.222 ± 0.0579	7.972 ± 0.0495	23.778 ± 0.484	10.666 ± 0.522	8.694 ± 0.433	0.881 ± 0.020	1.296 ± 0.054	1.090 ± 0.048	
60	24.555 ± 0.434	7.955 ± 0.0434	7.738 ± 0.0482	20.111 ± 0.242	8.432 ± 0.389	7.207 ± 0.388	0.819 ± 0.005	1.059 ± 0.043	0.931 ± 0.044	
80	19.944 ± 0.530	7.944 ± 0.0588	7.621 ± 0.0448	20.222 ± 0.484	8.111 ± 0.569	5.878 ± 0.492	1.014 ± 0.019	1.020 ± 0.064	0.772 ± 0.066	
100	18.833 ± 0.254	7.639 ± 0.0529	7.6 ± 0.0348	21.0 ± 1.229	6.139 ± 0.478	5.233 ± 0.351	1.116 ± 0.073	0.803 ± 0.057	0.677 ± 0.033	

Table 7 Intracellular metal ion content in root and leaf tissues of *V. radiata* seedlings exposed to different concentrations of Cd and Ni after 96 h

S. no.	Conc. of metal ion (CdCl ₂ and NiSO ₄) (μM)	Intracellular Cd		BCF of Cd in		Translocation factor (TF) of Cd		Intracellular Ni		BCF of Ni in		TF of Ni	
		content in leaves (μg l ⁻¹)	content in roots (μg l ⁻¹)	leaves	roots	Cd	Cd	leaves (μg l ⁻¹)	content in roots (μg l ⁻¹)	leaves	roots	leaves	roots
1.	Control	27.85 ± 2.233	93.17 ± 2.745	0.00 ± 0.0	0.00 ± 0.00	29.89 ± 2.245	27.85 ± 2.233	93.17 ± 2.745	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	29.89 ± 2.245	
2.	10	274.6 ± 2.978	874.1 ± 8.341	0.1364 ± 0.0014	0.4342 ± 0.0025	31.42 ± 1.347	869.7 ± 2.127	1985 ± 1.989	0.3309 ± 0.00032	0.7553 ± 0.00045	43.81 ± 5.826		
3.	40	71.37 ± 2.583	1653 ± 5.572	0.0089 ± 0.0009	0.2053 ± 0.0031	4.32 ± 0.588	1784 ± 9.879	1350 ± 223.03	0.1697 ± 0.00015	0.1284 ± 0.00021	132.15 ± 16.25		
4.	50	40.49 ± 3.954	2470 ± 9.316	0.0040 ± 0.0002	0.2454 ± 0.0015	1.64 ± 0.0057	1496 ± 4.154	5313 ± 2.732	0.11385 ± 0.00011	0.4043 ± 0.00018	28.16 ± 3.251		
5.	60	89.51 ± 1.406	4053 ± 22.56	0.0074 ± 0.00015	0.3355 ± 0.0008	2.21 ± 0.0132	1262 ± 17.73	5191 ± 435.8	0.0800 ± 0.00054	0.3292 ± 0.0007	24.31 ± 1.154		
6.	70	139.8 ± 8.314	5097 ± 7.174	0.0099 ± 0.0003	0.3617 ± 0.0074	2.74 ± 0.0107	2210 ± 254.4	3994 ± 57.647	0.12013 ± 0.00028	0.2171 ± 0.00061	55.33 ± 9.243		

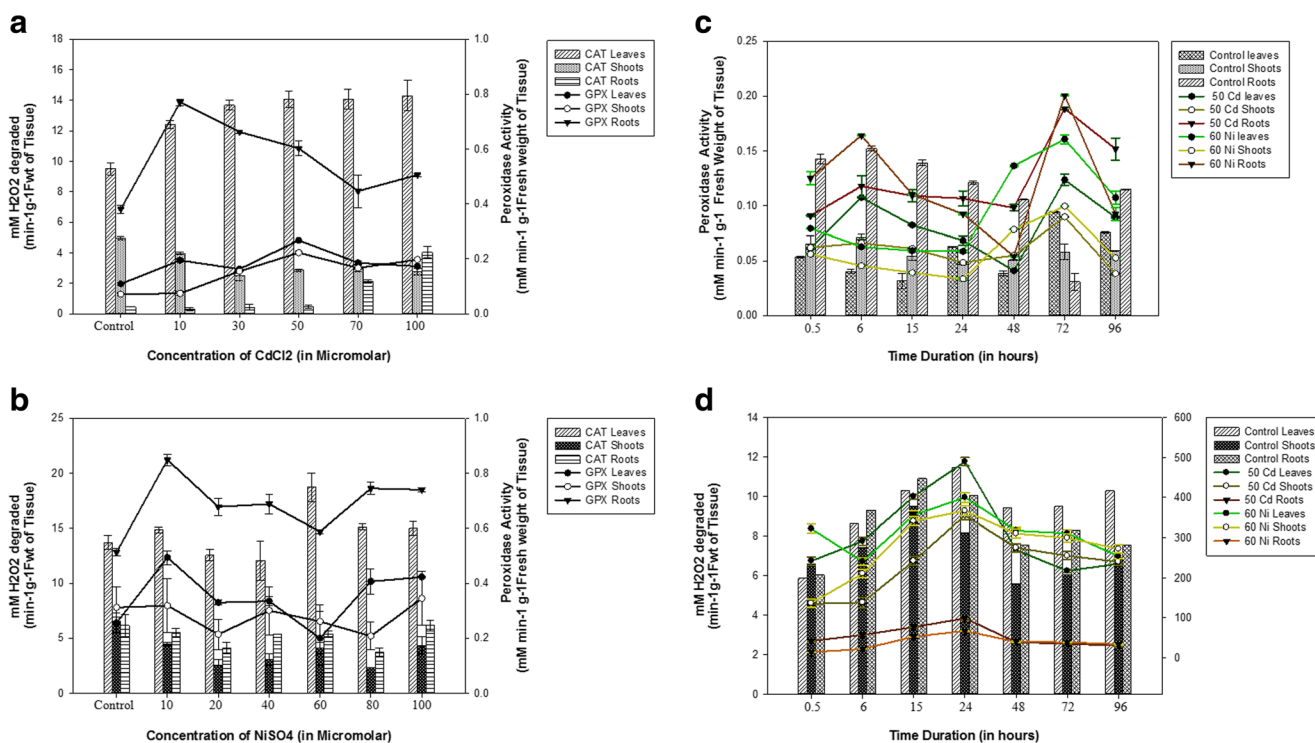


Fig. 6 **a** Catalase and guaiacol peroxidase activity in *Vigna radiata* seedlings. Seedlings were treated with 10, 30, 50, 70, and 100 μM cadmium for a period of 4 days. Vertical bars represent SE ($n = 3$). **b** Catalase and guaiacol peroxidase activity in *V. radiata* seedlings. Seedlings were treated with 10, 20, 40, 60, 80, and 100 μM cadmium for a period of 4 days. Vertical bars represent SE ($n = 3$). **c** The effect of time on guaiacol

peroxidase activity in *V. radiata* seedlings at 50 μM cadmium and 60 μM nickel at equal time interval for a period of 96 h. Values are mean \pm SE ($n = 3$). **d** The effect of time on catalase activity in *V. radiata* seedlings at 50 μM cadmium and 60 μM nickel at equal time interval for a period of 96 h. Values are mean \pm SE ($n = 3$)

development. This was in agreement with other studies on inhibitory effect of Cd using different plant species (Verma et al. 2008). The inhibitory effect on the growth of seedlings by Cd has earlier been reported by Ferreira et al. 2002 and by Ni has recently been reported by Hu et al. (2015).

In the present study, reduction in chlorophyll contents (Chl a, Chl b, and total Chl) with increasing concentration of Cd and Ni was observed (Tables 3 and 4). Heavy metal causes strong oxidation of chlorophyll machinery, hence reduces the size and density of chloroplast (Sidlecka and Baszynsky 1993). Decrease quantity of photosynthetic pigments in leaves subjected to Cd stress was also discussed in *Phragmites australis* (Pietrini et al. 2003). Protein content initially increased in the leaves with increasing concentration of Cd and Ni in the nutrient media and decreased at higher concentration (Fig. 7a, b). Elevated protein content at lower metal concentration is recognized in stimulation of stress proteins (Sanita di Toppi and Gabbriellini 1999). Production of ROS like H₂O₂ and other species has been straightly interrelated with protein destruction (Romero-Puertas et al. 2002). Thiol content (glutathione and NPT) is associated to the sequestration of heavy metals which maintain the level of ROS and hence considered as an essential element of cellular antioxidant defense system

(Noctor and Foyer 1998). The higher thiol content (GSSG, GSH, and NPT) in foliar tissue exposed to sublethal metal concentration (50 μM Cd and 60 μM Ni) was interpreted as an acclimation which strengthens the defense system (Tausz et al. 2004).

Accumulation of proline has been found to increase with increasing concentration of Cd and Ni. Proline deposition in the current study has been comparable as reported earlier with the study conducted on Cd (Hare and Cress 1997; Shekhawat et al. 2009). Higher proline concentration has been related with the enhanced tolerance against metal-stimulated oxidative stress (Siripornadulsil et al. 2002). Resistance towards oxidative stress was projected to relate with ROS detoxifying capability of proline (Tripathi et al. 2004, 2013).

The MDA content level, one of the major TBA reactive metabolites, increased with increase in Cd and Ni concentration in leaf tissue. The similar pattern of results was reported by Shekhawat et al. (2009) and Verma et al. (2013). Increase in MDA content with time (Fig. 4c) recommended that the MDA content in leaves was associated to the exposure period of heavy metal. Heavy metal enhances the production of ROS which results in the elimination of hydrogen from unsaturated fatty acid, and thus forms

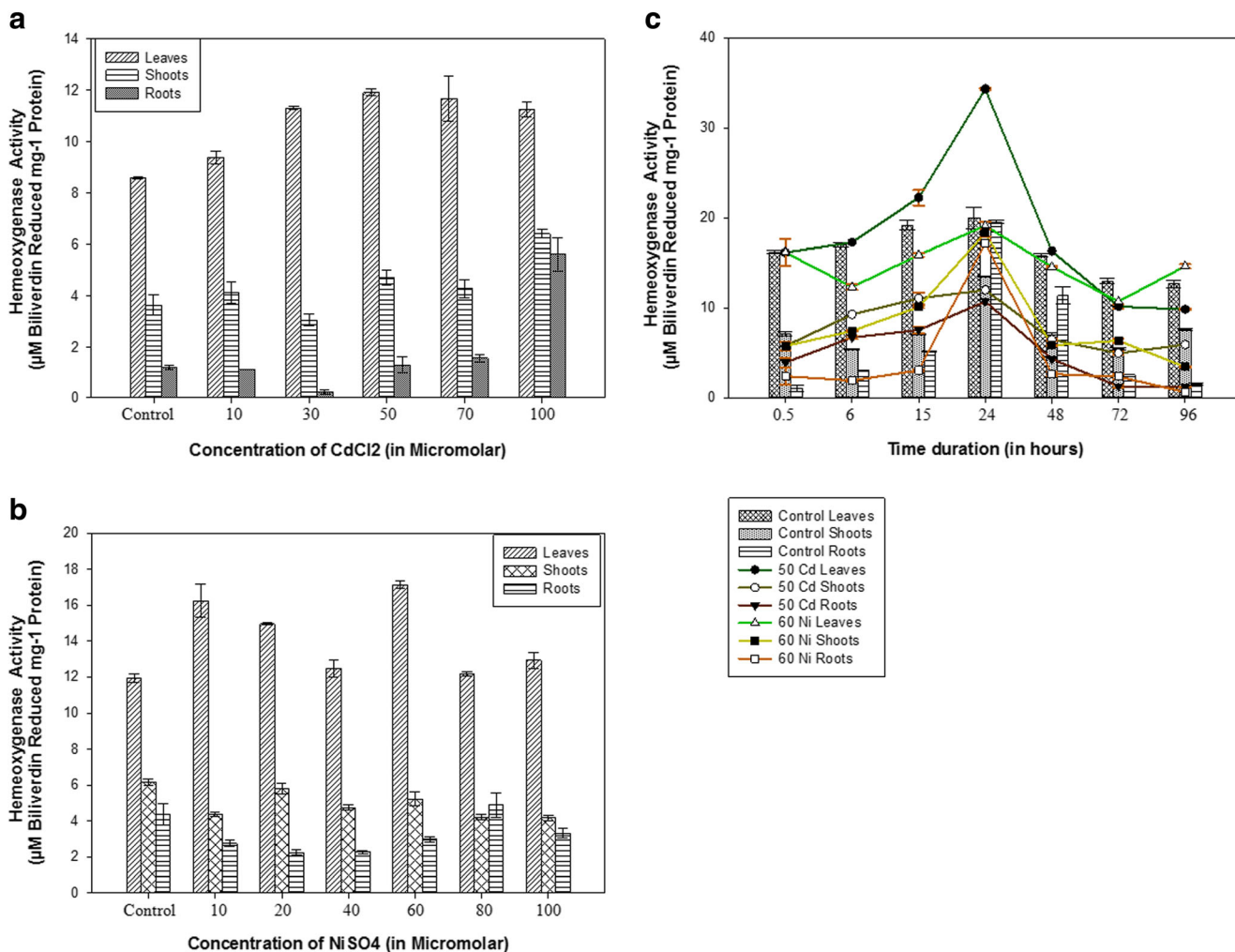


Fig. 7 **a** Heme oxygenase 1 activity in seedlings of *Vigna radiata*. Seedlings were treated with 10, 30, 50, 70, and 100 μM cadmium for a period of 96 h. Vertical bars represent SE ($n = 3$). **b** Heme oxygenase 1 activity in seedlings of *V. radiata*. Seedlings were treated with 10, 20, 40,

60, 80, and 100 μM nickel for a period of 96 h. Vertical bars represent SE ($n = 3$). **c** Effect of time on HO 1 activity in *V. radiata* seedlings at 50 μM cadmium and 60 μM nickel at equal time interval for a period of 96 h. Values are mean \pm SE ($n = 3$)

various lipid radical and reactive aldehydes which is the basis of cell membrane distraction (Reinheckel et al. 1998). Further lipid peroxidation depends on the activity of lipoxygenase which oxidizes lipid to produce lipid hydroperoxides (Arvind and Prasad 2003) that was also demonstrated under heavy metal stress in *Phaseolus vulgaris* (Somashkaraiah et al. 1992) and *Arabidopsis thaliana* (Skórzynska-Polit et al. 2006). In plant cells, H_2O_2 is produced in a wide range of biotic and abiotic stresses (Laloi et al. 2004). In the current investigation, we observed that increasing concentration of Cd and Ni accumulated more amount of H_2O_2 in the leaf tissue of *V. radiata* seedlings. The accumulation of H_2O_2 in *Brassica juncea* in the presence of Cd has been well reported by Verma et al. (2013) and Verma et al. (2008). Increase in H_2O_2 contents at high concentration of heavy metal may be due to the toxic effects of metals that cause metabolic imbalance and finally lead to the cell death (Verma et al. 2013). Because Cd-stimulated cell

death was correlated to the H_2O_2 , burst has been studied by Schutzenubel et al. (2001) in *Pinus sylvestris*.

The present investigation shows the outcome of HO 1 on the metabolic process of *Vigna* under Cd and Ni stress. Our data signify that plants produce elevated level of HO 1 in leaves in comparison to roots with increasing concentration of both Cd and Ni. The increase in HO 1 level depends on the concentration and duration of metal (Fig. 7a, b). The previous studies demonstrated the role of HO 1 in providing defense against the reactive oxygen species due to the oxidative stress generated by the cadmium-treated soybean plants (Balestrasse et al. 2005, 2008; Yannarelli et al. 2006). Accumulation of HO under stress indicates that heavy metal provokes the antioxidant defense mechanism which might be arbitrated by HO. In support to this, numerous reports have revealed the defensive role of HO against abiotic stress which is strongly associated to the HO-

arbitrated reduction of ROS in plants (Yannarelli et al. 2006; Noriega et al. 2012; Xie et al. 2015). High dosage as well as exposure time of metal did not induce endogenous HO 1 production which might be due to the cell metabolism destruction. However, lower concentration of metal in the hydroponic nutrient medium elevates the HO 1 activity as a result of metal-induced oxidative stress.

Results reveal that seedlings treated with sublethal metal concentration (50 μM Cd and 60 μM Ni) exhibit visible changes in antioxidant defense machinery in comparison to control. To evaluate that metal toxicity reduction may be credited to antioxidant properties of HO 1, the antioxidant enzyme activities like CAT, APX, and GPX were studied. The convincing outline was observed for these antioxidant enzymes where the HO 1 activity is increased in seedlings of *V. radiata* which were induced by metal stress (in both Cd and Ni stress). In the current study at sublethal concentration of the studied metal (50 μM Cd and 60 μM Ni), the highest activity of antioxidant enzymes along with HO 1 was found in different parts of the plant. In higher plants, indication has been provided that surplus metal in the nutrient medium stimulates GPX, APX, and CAT activity has been provided (Shekhawat et al. 2009). However, the activities of all these antioxidant enzymes were lowest at the highest metal concentration (100 μM) probably due to deactivation. The prohibition of enzyme activities at greater concentrations of stress might be attributed to ROS-stimulated alteration such as transductional alterations, DNA damage, improved vulnerability, and protein disintegration to proteolysis (Smirnov 1998).

ROS (H_2O_2) level increases with increasing concentration of metal and time exposure that is at 10 μM CdCl_2 and 10 μM NiSO_4 as well as after 6 h of metal exposure (50 μM Cd and 60 μM Ni), but a decrease was observed at sublethal metal concentrations and on increasing exposure time. Reduction in H_2O_2 intensity might be due to scavenging through APX since at these concentrations of metal, APX activity was highest in root tissue. Similarly in *B. juncea*, Shekhawat et al. (2009) reported a rise in APX activity with declining H_2O_2 content. Histochemical analysis conducted by Zilli et al. (2009) of H_2O_2 in soybean leaves under salinity stress demonstrated a decrease in H_2O_2 content at 100 mM concentration, although the activity of other antioxidant enzymes including HO 1 was highest at this concentration. Time-dependent increase in antioxidant enzyme activity including HO 1 (Figs. 6c, d and 7c) suggests that accumulation of antioxidant enzymes was apparently related to the plant metal exposure period. At 24 h, the highest activity of antioxidant enzymes including HO 1 was observed. At this time period, H_2O_2 content was found to be lowest. The low activity of antioxidant enzymes along with increase in H_2O_2 content at high metal concentration (100 μM) may be due to the participation of H_2O_2 in the

signal transduction needed for the action of antioxidant enzymes to prevail over the effects of stress (Vranova et al. 2002; Zilli et al. 2009). Due to decrease in antioxidant enzyme activity at high metal concentration, H_2O_2 accumulation increases in all plant parts.

In the recent study, leaf tissue elucidates enhanced ROS scavenging and avoidance of membrane damage than root tissue which is confirmed from the AAS study on different plant parts of *V. radiata* seedlings. The current investigation reveals that at sublethal concentrations of heavy metals (50 μM Cd and 60 μM Ni), the intracellular accumulation of Cd and Ni in leaf tissue decreases 85.26% (from 10 to 50 μM) and 29.26% (from 40 to 60 μM) in Cd and Ni, respectively. While at the same concentrations, intracellular heavy metal (Cd and Ni) accumulation is higher in roots in comparison to leaves. This indicates greater level of HO 1 in leaf tissue (Fig. 7a, b). High level of HO 1 in leaf tissue is based on the fact that heme oxygenase activity is localized in plastids (Cornejo et al. 1998; Muramoto et al. 1999). HO 1 localized in plastid is highly inducible by H_2O_2 (Chen et al. 2009) which suggests that HO 1 might be using H_2O_2 as a substrate along with CAT and APX-mediating complex signal transduction network. Higher HO 1 activity in leaves in comparison to roots may be due to the fact that plants are adapted to cope up with metal stress through various physiological adaptations including changes in membrane permeability which exclude excess of metal, ability to sequester excess ions in cellular compartments including vacuole, ability to transport ions in the older leaves that undergo senescence, and operate in order to reduce the metal concentration or to alleviate the deleterious effects of metal (Dixit et al. 2014). Due to high mobility, Cd and Ni are readily uptaken by plants. Majority of the metal ions (Cd and Ni) are retained in the root tissues, and minute quantity is translocated to foliar tissues (Cataldo et al. 1983). Compared to the control, *V. radiata* seedlings exposed to metal stress illustrate high intracellular metal content in root tissues. Decline in BCF and TF with elevated Cd and Ni concentration indicates the defensive role of HO 1. Nahar et al. (2016) demonstrated similar results while studying the exogenous effect of spermine in mung bean exposed to Cd-induced oxidative stress.

In conclusion, the consequences of the study signify that heavy metal (Cd and Ni) are the basis of oxidative stress in *V. radiata* ensuing in overall growth inhibition. HO 1 plays an imperative role in series of reactions dependable for metal tolerance by regulating antioxidant stimulation or diminution. Thus, based on previous study, HO 1 works within an assembly of antioxidant enzymes in which some enzymes upregulate while other downregulate with HO 1 that generate the defense mechanism for plant's existence. Recent outcomes specify an elementary plant reaction through antioxidant enzymes (APX, CAT, and GPX) that is widespread to the majority of stress response cycle and a particular response of HO

1 that was earlier not known in *V. radiata*. As these antioxidant enzymes including HO 1 became tolerant, the H₂O₂ level was plagued. Activation of HO 1 illustrates that HO 1 has a chief function in the defense system towards metal stress. The study enhances our understanding of the intricacy of the defense group against metal stress which will be useful in developing metal-tolerant varieties for future research.

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References

- Aebi H (1974) Catalases. In: Bergmeyer HU (ed) Methods of enzymatic analysis, Verlag Chemie, Weinheim. Academic Press Inc, New York, p 680
- Ahmad P, Sarwat M, Sharma S (2008) Reactive oxygen species, antioxidants and signaling in plants. *J Plant Biol* 51:167–173
- Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ* 24:1337–1344
- Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases [SODs] in controlling oxidative stress in plants. *J Exp Bot* 53:1331–1341
- Anderson ME (1985) Determination of glutathione and glutathione disulfides in biological samples. *Methods Enzymol* 113:548–570
- Amon DI (1949) Copper enzymes in isolated chloroplasts: polyphenol oxidases in *Beta vulgaris*. *Plant Physiol* 24:1–15
- Arvind P, Prasad MNV (2003) Modulation of cadmium-induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate–glutathione cycle and glutathione metabolism. *Plant Physiol Biochem* 43:107–116
- Bains K, Yang R, Shanmugasundaram S (2003) High iron mung bean recipes for North India, Shanhua, Taiwan. AVRDC Publication 562: 34
- Balestrasse KB, Noriega GO, Batlle A, Tomaro ML (2005) Involvement of heme oxygenase as antioxidant defense in soybean nodules. *Free Radic Res* 39:145–151
- Balestrasse KB, Yannarelli GG, Noriega GO, Batlle A, Tomaro ML (2008) Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress. *Biometals* 21:433–441
- Bates LS, Waldren RP, Tear ID (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–207
- Bates SS, Tessier A, Campbell PGC, Buffle J (1982) Zinc adsorption and transport by *Chlamydomonas variabilis* and *Scenedesmus subspicatus* [Chlorophyceae] grown in semicontinuous culture. *J Phycol* 18:521–529
- Cao XY, Xuan W, Liu ZY, Li XN, Zhao N, Xu P, Wang Z, Guan RZ, Shen WB (2007) Carbon monoxide promotes lateral root formation in rapeseed. *J Integr Plant Biol* 49:1070–1079
- Cao Z, Geng B, Xu S, Xuan W, Nie L, Shen W, Liang Y, Guan R (2011) *BnHO 1*, a haem oxygenase-1 gene from *Brassica napus*, is required for salinity and osmotic stress-induced lateral root formation. *J Exp Bot* 62:4675–4689
- Cataldo DA, Garland R, Wildung RE (1983) Cadmium uptake kinetics in intact soybean plants. *Plant Physiol* 73:844–848
- Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant Cell Physiol* 30:987–998
- Chen XY, Ding X, Xu S, Wang R, Xuan W, Cao ZY, Chen J, Wu HH, Ye MB, Shen WB (2009) Endogenous hydrogen peroxide plays a positive role in the upregulation of hemeoxygenase and acclimation to oxidative stress in wheat seedling leaves. *J Int Plant Biol* 51:951–960
- Collard JM, Corbisier P, Diels L, Dong Q, Jeanthon C, Mergeay M, Taghavi S, Van der Lelie D, Wilmotte A, Wuertz S (1994) Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: mechanisms and applications. *FEMS Microbiol Rev* 14:405–414
- Cornejo J, Willows RD, Beale SI (1998) Phytobilin biosynthesis: cloning and expression of a gene encoding soluble ferredoxin-dependent heme oxygenase from *Synechocystis* sp. PCC 6803. *Plant J* 15:99–107
- Cui W, Fu G, Wu H, Shen W (2011) Cadmium-induced heme oxygenase-1 gene expression is associated with the depletion of glutathione in the roots of *Medicago sativa*. *Biometals* 24:93–103
- Cui W, Gao C, Fang P, Lin G, Shen W (2013) Alleviation of cadmium toxicity in *Medicago sativa* by hydrogen-rich water. *J Hazard Mater* 260:715–724
- Daud MK, Sun YQ, Dawood M (2009) Cadmium-induced functional and ultrastructural alterations in roots of two transgenic cotton cultivars. *J Hazard Mater* 161:463–473
- Davis SJ, Bhoo SH, Durski AM, Walker JM, Vierstra RD (2001) The heme-oxygenase family required for phytochrome chromophore biosynthesis is necessary for proper photomorphogenesis in higher plants. *Plant Physiol* 126:656–669
- De Vos CHR, Schat H, Vooijs R, Ernst WHO (1989) Copper-induced damage to the permeability barrier in roots of *silene cuiubalus*. *J Plant Physiol* 135:164–179
- Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Holzer L, Feller U (2004) Biochemical changes in barley plants after excessive supply of copper and manganese. *Environ Exp Bot* 52:253–266
- Dixit S, Venma K, Shekhawat GS (2014) In vitro evaluation of mitochondrial–chloroplast subcellular localization of heme oxygenase1 [HO 1] in *Glycine max*. *Protoplasma* 251:671–675
- Duarte B, Delgado M, Cacador I (2007) The role of citric acid in cadmium and nickel uptake and translocation, in *Halimione portulacoides*. *Chemosphere* 69:836–840
- Emborg TJ, Walker JM, Noh B, Vierstra RD (2006) Multiple heme oxygenase family members contribute to the biosynthesis of the phytochrome chromophore in Arabidopsis. *Plant Physiol* 140:856–868
- Ermiler U, Grabarse W, Shima S, Goubeaud M, Thauer RK (1998) Active sites of transition-metal enzymes with a focus on nickel. *Curr Opin Struct Biol* 8:749–758
- Ferreira RR, Fornazier RF, Vitoria AP, Lea PJ, Azevedo RA (2002) Changes in antioxidant enzyme activities in soybean under cadmium stress. *J Plant Nutr* 25:327–342
- Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. *Physiol Plant* 92:696–717
- Fu GQ, Xu S, Xie YJ, Han B, Nie L, Shen WB, Wang R (2011) Molecular cloning, characterization, and expression of an alfalfa [*Medicago sativa* L.] heme oxygenase-1 gene, *MshOI*, which is prooxidants regulated. *Plant Physiol Biochem* 49:792–799
- Gill SS, Tuteja N (2011) Cadmium stress tolerance in crop plants: probing the role of sulfur. *Plant Signal Behav* 6:215–222
- Gill SS, Khan NA, Tuteja N (2011) Differential cadmium stress tolerance in five Indian mustard [*Brassica juncea* L.] cultivars: an evaluation of the role of antioxidant machinery. *Plant Signal Behav* 6:293–300
- Gill SS, Khan NA, Tuteja N (2012) Cadmium at high dose perturbs growth, photosynthesis and nitrogen metabolism while at low dose it up regulates sulfur assimilation and antioxidant machinery in garden cress [*Lepidium sativum* L.]. *Plant Sci* 182:112–120
- Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul* 21:79–102
- Hsu YT, Kao CH (2004) Cadmium toxicity is reduced by nitric oxide in rice leaves. *Plant Growth Regul* 42:227–238

- Hu J, Deng Z, Wang B, Zhi Y, Pei B, Zhang G, Luo M, Huang B, Wu W, Huang B (2015) Influence of heavy metals on seed germination and early seedling growth in *Crambe abyssinica*, a potential industrial oil crop for phytoremediation. *Am J Plant Sci* 6:150–156
- Kupper H, Kroneck PMH (2007) Nickel in the environment and its role in metabolism of plants and cyanobacteria. *Met Ions Life Sci* 2:31–62
- Laloi CH, Apel K, Danon A (2004) Reactive oxygen signaling: the latest news. *Curr Opin Plant Biol* 7:323–328
- Li Q, Zhu F, Gao X, Sun Y, Li S, Tao Y, Lo C, Liu H (2014) Young leaf chlorosis 2 encodes the stroma-localized heme oxygenase 2 which is required for normal tetrapyrrole biosynthesis in rice. *Planta* 240:701–712
- Li J, Zhu D, Wang R, Shen W, Guo Y, Ren Y, Shen W, Huang L (2015) β -Cyclodextrin–hemin complex-induced lateral root formation in tomato: involvement of nitric oxide and heme oxygenase 1. *Plant Cell Rep* 34:381–393
- Linley PJ, Landsberger M, Kohchi T, Cooper JB, Terry MJ (2006) The molecular basis of heme oxygenase deficiency in the *pcd1* mutant of pea. *FEBS J* 273:2594–2606
- Liu D, Zou J, Meng Q, Zou J, Wusheng J (2009) Uptake and accumulation and oxidative stress in garlic [*Allium sativum* L.] under lead phytotoxicity. *Ecotoxicology* 18:134–143
- Lowry OH, Rosenberg NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. *J Biol Chem* 193:265–275
- Maier T, Jacobi A, Sauter M, Bock A (1993) The product of the *hypb* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. *J Bacteriol* 175:630–635
- Maines MD (1988) Heme oxygenase: function, multiplicity, regulatory mechanism and clinical applications. *FASEB J* 2:2557–2568
- Mehrag AA (1994) Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. *Plant Cell Environ* 17:989–993
- Mulrooney SB, Hausinger RP (2003) Nickel uptake and utilization by microorganisms. *FEMS Microbiol Rev* 27:239–261
- Muramoto T, Kohchi T, Yokota A, Hwang I, Goodman HM (1999) The *Arabidopsis* photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *Plant Cell* 11:335–348
- Mwegoha WJS, Kihampa C (2010) Heavy metal contamination in agricultural soils and water in Dares Salaam city, Tanzania. *Afr J Environ Sci Technol* 4:763–769
- Nahar K, Rahman M, Hasanuzzaman M, Alam MM, Rahman A, Suzuki T, Fujita M (2016) Physiological and biochemical mechanisms of spermine-induced cadmium stress tolerance in mung bean (*Vigna radiata* L.) seedlings. *Environ Sci Pollut Res* 23:21206–21218
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279
- Noriega G, Cruz DS, Batlle A, Tomaro M, Balestrasse K (2012) Heme Oxygenase is involved in the protection exerted by jasmonic acid against cadmium stress in soybean roots. *J Plant Growth Regul* 31:79–89
- Ona LF, Alberto AM, Prudente JA, Sigua GC (2006) Levels of lead in urban soils from selected cities in a central region of the Philippines. *Environ Sci Pollut Res* 13:177–183
- Otterbein LE, Soares MP, Yamashita K, Bach FH (2003) Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24:449–455
- Pietrini F, Iannelli MA, Pasqualini S, Massacci A (2003) Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* [Cav.] Trin ex Steudel. *Plant Physiol* 133:829–837
- Putter J (1974) Peroxidase. In: Bergemeyer HU (ed) *Methods of enzymatic analysis*. Academic Press, London, pp 685–690
- Ragsdale SW (1998) Nickel biochemistry. *Curr Opin Chem Biol* 2:208–215
- Reinheckel T, Noack H, Lorenz S, Wissedel I, Augustin W (1998) Comparison of protein oxidation and aldehyde formation during oxidative stress in isolated mitochondria. *Free Radic Res* 29:297–305
- Romero-Puertas MC, Palma JM, Gomez M, Del Rio LA, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. *Plant Cell Environ* 25:677–686
- Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, Del Rio LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot* 52:2115–2126
- Sanita di Toppi L, Gabbriellini R (1999) Responses to cadmium in higher plants. *Environ Exp Bot* 41:105–130
- Santa-Cruz D, Pacienza N, Zilli C, Pagano E, Balestrasse K, Yannarelli G (2017) Heme oxygenase up-regulation under ultraviolet-B radiation is not epigenetically restricted and involves specific stress-related transcription factors. *Redox Biol* 12:549–557
- Schutzendubel A, Polle A (2002) Cadmium and H₂O₂ induced oxidative stress in *Populus canescens* roots. *Plant Physiol Biochem* 40:577–584
- Schutzendubel A, Schwanz P, Teichmann T, Gross K, Lanthenfeld-Heyser R, Godbold DL, Polle A (2001) Cadmium induced changes in antioxidant system, hydrogen peroxide content and differentiation in Scots pine roots. *Plant Physiol* 127:887–898
- Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound and non protein sulphhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25:192–205
- Sethy SK, Ghosh S (2013) Effect of heavy metals on germination of seeds. *J Nat Sci Biol Med* 4:272–275
- Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci* 14:43–50
- Shekhawat GS, Verma K (2010) Heme oxygenase [HO]: an overlooked enzyme of plant metabolism and defence. *J Exp Bot* 61:2255–2270
- Shekhawat GS, Prasad A, Verma K, Sharma A (2008) Changes in growth, lipid peroxidation and antioxidant system in seedlings of *Brassica juncea* (L.) Czern. *Biochem Cell Arch* 8:145–149
- Shekhawat GS, Verma K, Jana S, Singh K, Teotia P, Prasad A (2009) In vitro biochemical evaluation of cadmium tolerance mechanism in callus and seedlings of *Brassica juncea*. *Protoplasma* 239:31–38
- Shekhawat GS, Dixit S, Verma K, Nasybullina EI, Kosmachevskaya OV, Topunov AF (2011) Heme oxygenase: enzyme with functional diversity. *J Stress Physiol Biochem* 7:88–94
- Siddiqui MH, Al-Whaibi MH, Basalah MO (2011) Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. *Protoplasma* 248:503–511
- Sidlecka A, Baszynsky T (1993) Inhibition of electron flow around photosystem I in chloroplasts of cadmium-treated maize plants in due to cadmium-induced iron deficiency. *Physiol Plant* 87:199–202
- Siripornadulsil S, Traina S, Verma DPS, Sayre RT (2002) Molecular mechanisms of proline mediated tolerance to toxic heavy metals in transgenic micro algae. *Plant Cell* 14:2837–2847
- Skórzynska-Polit E, Pawlikowska-Pawlega B, Szczuka E, Drazkiewicz M, Krupa Z (2006) The activity and localization of lipoygenases in *Arabidopsis thaliana* under cadmium and copper stress. *Plant Growth Regul* 48:29–39
- Smimoff N (1998) Plant resistance to environmental stress. *Curr Opin Biotechnol* 9:214–219
- Somashekaraiah BV, Padmaja K, Prasad ARK (1992) Phytotoxicity of cadmium ions on germinating seedlings of mung bean [*Phaseolus vulgaris*]: involvement of lipid peroxides in chlorophyll degradation. *Physiol Plantarum* 85:85–89
- Stoltz E, Greger M (2002) Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. *Environ Exp Bot* 47:271–280

- Tausz M, Sircelji H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress response concept valid. *J Exp Bot* 55:1955–1962
- Tenhunen R, Marver HS, Schmid R (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *PNAS* 61:748–755
- Terry MJ, Kendrick RE (1999) Feedback inhibition of chlorophyll synthesis in the phytochrome chromophore-deficient *aurea* and *yellow-green-2* mutants of tomato. *Plant Physiol* 119:143–152
- Terry MJ, Ryberg M, Raitt CE, Page A (2001) Altered etioplast development in phytochrome chromophore-deficient mutants. *Planta* 214:314–325
- Tomaro ML, Battle A (2002) Bilirubin: its role in cytoprotection against oxidative stress. *Int J Biochem Cell Biol* 34:216–220
- Tripathi BN, Mehta SK, Gaur JP (2004) Recovery of uptake and assimilation of nitrate in *Scenedesmus* sp. previously exposed to elevated leaves of Cu^{+2} and Zn^{+2} . *J Plant Physiol* 161:543–549
- Tripathi BN, Singh V, Ezaki B, Sharma V, Gaur JP (2013) Mechanism of Cu and Cd induced proline hyperaccumulation in *Triticum aestivum* [wheat]. *J Plant Growth Regul* 32:799–808
- Verma K, Shekhawat GS, Sharma A, Mehta SK, Sharma V (2008) Cadmium induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3–4 leaf stage plants of *Brassica juncea* [L.] Czern. *Plant Cell Rep* 27:1261–1269
- Verma K, Mehta SK, Shekhawat GS (2013) Nitric oxide [NO] counteracts cadmium induced cytotoxic processes mediated by reactive oxygen species [ROS] in *Brassica juncea*: cross-talk between ROS, NO and antioxidant responses. *Biometals* 26:255–269
- Vranova E, Inze D, Breusegem FV (2002) Signal transduction during oxidative stress. *J Exp Bot* 53:1227–1236
- Wilkins DA (1978) The measurement of tolerance to edaphic factors by means of root growth. *New Phytol* 80:623–633
- Xie Y, Mao Y, Xu S, Zhou H, Duan X, Cui W, Zhang J, Xu G (2015) Heme-heme oxygenase 1 system is involved in ammonium tolerance by regulating antioxidant defence in *Oryza sativa*. *Plant Cell Environ* 38:129–143
- Xu S, Zhang B, Cao ZY, Ling TF, Shen WB (2011) Heme oxygenase is involved in cobalt chloride-induced lateral root development in tomato. *Biometals* 24:181–191
- Yannarelli GG, Noriega GO, Battle A, Tomaro ML (2006) Heme oxygenase up regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species. *Planta* 224:1154–1162
- Zayed A, Gowthaman S, Terry N (1998) Phytoaccumulation of trace elements by wetlands plants: I. Duckweed *J Environ Qual* 27:715–721
- Zhang YY, Liu J, Liu YL (2004) Nitric oxide alleviates the growth inhibition of maize seedlings under salt stress. *J Plant Physiol Mol Biol* 30:455–459
- Zhu L, Yang Z, Zeng X, Gao J, Liu J, Yi B, Ma C, Shen J, Tu J, Fu T, Wen J (2017) Heme oxygenase 1 defects lead to reduced chlorophyll in *Brassica napus*. *Plant Mol Biol* 93:579–592
- Zilli CG, Santa-Cruz DM, Yannarelli CG, Noriega GO, Tomaro ML, Balestrasse KB (2009) Heme oxygenase contributes to alleviate salinity damage in *Glycine max* L. leaves. *Int J Cell Bio* 1–9. <https://doi.org/10.1155/2009/848516>