American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

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OPEN ACCESS American Journal of Plant Physiology

ISSN 1557-4539
DOI: 10.3923/ajpp.2017.58.70

Research Article
Cadmium Phytoextraction and Induced Antioxidant Gene Response in *Moringa oleifera* Lam.

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Abstract

Background and Objectives: Heavy metal contamination is the major problem spreading worldwide as non biodegradable pollutant. The toxicity of metals creates major threat for primary and secondary consumer in environment and reaches to the top level consumers in the ecosystem through food chain. Therefore, present study was focused on cadmium uptake from cadmium chloride ($CdCl_2$) contaminated soil using *Moringa oleifera* Lam. plant which is known for hyperaccumulation of heavy metals. Effects of various concentrations of $CdCl_2$ on plant morphology, biochemical and molecular parameters were determined in Moringa oleifera (*M. oleifera*). **Materials and Methods:** Three *M. oleifera* seeds/pot containing 1 kg sterilized soil were sown and placed under controlled photoperiod. Ten days old germinating seedlings were used to treat with different Cd concentrations. After treatment, the seedlings were used to analyze plant growth and morphology, bioconstituents and antioxidant genes (CAT and APX) expression at 10, 20 and 30 days. Data were analyzed by one way analysis of variance by SPSS. **Results:** This study showed maximum Cd uptake 168.563 mg kg^{-1} soil in 30 days old plant roots than 19.951 mg kg^{-1} Cd soil in shoots at 5 mM Cd concentration. Protein isolated from leaves of treated seedlings showed variation in differential pattern of polypeptides compared to protein in control leaves. High secretion of metallothioneins proteins, proline and polyphenol content induced with increasing metal concentrations (1 mM <2 mM <3 mM <5 mM). In addition Cd induced significant increase in antioxidant enzymes activity: APX (EC 1.11.11), CAT (EC 1.11.1.6) and GR (EC 1.6.4.2) with increasing metal level. Higher expression of CAT and APX gene transcripts upon increasing metal exposure provides more evidence for potential of Cd accumulation in *M. oleifera*. **Conclusion:** Retention of high level of Cd in roots indicates that *M. oleifera* has potential for phytoextraction of heavy metals by rhizofiltration.

Key words: Cadmium, metal accumulation, phytoextraction, *Moringa oleifera*, antioxidant genes

Citation: Shaili Yadav and Jyoti Srivastava, 2017. Cadmium phytoextraction and induced antioxidant gene response in *Moringa oleifera* Lam. Am. J. Plant Physiol., 12: 58-70.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The presence of heavy metals in the environment, soil and water leads to their bioaccumulation and deposition inside the plants, animals and human tissues causing several hazardous effects[1]. Heavy metals are included in the category of major toxic pollutant which can persist in the environment for longer duration and get biomagnified inside the organisms. Cadmium (Cd) is one of the toxic heavy metals which is neither included as essential element nor does biological functions[2]. Cd with 112.4 g mol^{-1} atomic mass is a lustrous, silver-white, ductile, very malleable metal and has a half life of 18 years. Sources of Cd are coal combustion, volcanic action, phosphate fertilizers, industrial uses such as Ni-Cd batteries, Zn production, plating, pigments, domestic effluents, sewage sludge and wastage incineration. According to Wanger[3], soil containing Cd concentrations ranging from 0.32-1 mM are considered potential less toxic for soil microorganisms and non hyperaccumulating plants. According to the World Health Organisation (WHO), the recommended permissible level of Cd concentration in plants is 0.02 mg kg^{-1}, while in water it is 0.01 mg L^{-1} [4]. Increased level of Cd in the soil causes disturbance in water potential and uptake of nutrient, decline in plant morphological growth, slow rate of photosynthesis, leaf chlorosis, wilting, metabolic irregularities and cell death due to oxidative stress in plants[5]. Several physical and bio-chemical efforts have been made for environmental clean-up process, phytoremediation is one of them. The prospect of eliminating toxic metals from contaminated water and soil using plants is known as phytoremediation[6]. Many hyperaccumulating plant species have been reported which either accumulate heavy metals in cytoplasm of root cells and aerial plant-parts or sequester heavy metals in vacuoles or tonoplast. Plants accumulate heavy metals, explosives, crude oil, leachates, polyaromatic hydrocarbons through roots and transport these pollutants in different plant-parts to stabilize/degrade in less toxic simpler form by phytoextraction, phytovolatilization or rhizofiltration[7]. *Moringa oleifera* Lam. (drumstick) belonging to family *Moringaceae*, is one of the efficient metal accumulating plant species[8]. *M. oleifera* is known to be tolerant to heavy metals: Cd, Pb, Hg, Cu, Cr and Zn[9,10]. The seeds of *M. oleifera* were considered as a naturally effective metal adsorbent for metal adsorption in contaminated soil and water. As an attempt in remediation *M. oleifera* seeds have been previously used to treat metal cations and the reported recoveries 70-89% for Pb, 76.59% for Cd, 60.21% for As, 68.85% for Cr, 60.52% for Ni and 66-92% for Fe from contaminated sites were generally high[11-13].

Among all species of *Moringa* only *M. oleifera* is cultivated widely, which is indigenous to South Asia, where it grows in the Himalayan foothills to Northern West Bengal, India[14]. The active dimeric cationic polyelectrolyte proteins, proline and cysteine amino acids, organic acids, polysaccharides, many functional groups (viz methoxyl, hydroxyl-aliphatic, carboxyl and phenolic) present in *M. oleifera* make it more efficient for its water coagulating[15,16,8]. Furthermore, antioxidative enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and nonenzyme antioxidant ascorbic acid in *M. oleifera* plants contribute in Cd induced production of excessive reactive oxygen species and free radicals[17]. For enhancing efficiency of phytoremediation or metal uptake several genes as, metal chelator, metal transporter, metallothioneins (MT), phytochelatins (PCs) and enzymatic genes CAT, APX, SOD and GR have been identified and transferred to plants through genetic engineering[18]. The upregulation of SOD, CAT, APX and GR gene expression in the presence of heavy metals promotes the metal oxygen radical scavenging and maintenance of the integrity of cellular membranes[19]. The present study was carried out to investigate accumulation of Cd in different parts of *M. oleifera* seedlings from soil as this plant may further be useful in soil reclamation through the process of phytoremediation. Moreover, the present experiment shows a change in growth and biochemical aspects, soluble protein and metallothioneins protein content and antioxidant enzymes activity in *M. oleifera* seedlings treated with Cd concentrations in order to contribute to understand the potential of *M. oleifera* against Cd stress. The metal induced expression of antioxidant genes CAT and APX has also been studied in *M. oleifera* as defence against Cd.

MATERIALS AND METHODS

Plant sample and growth conditions: Seeds of *M. oleifera* Lam. were collected from Krishi Vigyan Kendra Banasthali University, Rajasthan (India). About three seeds/pot were sown containing 1 kg sterilized soil under controlled conditions. Pots were placed in green house with a photoperiod of 13 h light and 11 h dark at 35±2°C. Ten days old uniform seedlings were selected for the treatment of cadmium chloride ($CdCl_2$) at concentrations 1, 2, 3 and 5 mM along with control (0) in 5 replicates. Changes in morphology, bioconstituents and induced antioxidant enzymes (CAT, APX and GR) activity were recorded on 10th, 20th and 30th days in *M. oleifera*. (Fig 1). The accumulated concentration of Cd (mg kg^{-1}) in plant-parts and translocation factor of Cd from root into shoot were also

Fig. 1: Growth in 30 days old *M. oleifera* seedlings

calculated at each stress period. The expression of CAT and APX gene transcripts were analyzed in the root and leaf samples of *M. oleifera*.

Morphological analysis: The effect of $CdCl_2$ on length of root and shoot, surface area of leaf, weight of fresh and dry leaf was observed in treated as well as control seedlings of *M. oleifera*. Relative water content (RWC) in leaf was checked using the given formula[20].

$$RWC\ (\%) = \frac{(FW\text{-}DW)}{(TW\text{-}DW)} \times 100$$

Where,
FW = Fresh weight of leaf
DW = Dry weight of leaf
TW = Turgid weight of leaf

Determination of Cd content: Cd treated root, shoot and leaf samples of 10, 20 and 30 days old seedlings were excised and washed in millipore water. Samples were immersed in 5 mM $CaCl_2$ solution for 3-4 min to remove any surface bound metal without affecting internal metal content. Roots were washed thoroughly in cold millipore water and its particles were translocation of metal from root to the shoot. The samples were dried in filter paper and soon after oven at 50°C. Dried samples were digested with mixture of HNO_3/H_2O_2 in muffle furnace (Tempo instruments and equipment Pvt. Ltd.) at 150°C for 6 h. Thereafter, acidified sample was diluted to 20 cm^{-3} with 2% HNO_3/H_2O_2. The mixture was filtered through Whatman filter paper No. 42 and analyzed for metal analysis using atomic absorption spectrophotometer (AAS, Solaar, ICE3000 Series, Thermo Scientific, India) with standard values of Cd in solution[21]. Translocation of Cd from root to shoot was calculated by the given formula[22]. TF>1 represent the effective translocation of metal from root to the shoot.

$$TF = \frac{\text{Concentration of metal in shoot}}{\text{Concentration of metal in root}}$$

Where, concentration of metal is in mg kg^{-1}.

Estimation of proline content: Estimation of proline was done by employing the method given by Bates *et al.*[23]. Proline concentration was measured at 520 nm by spectrophotometer.

Polyphenol analysis: Polyphenol content was analyzed using the method given by Bray and Thorpe[24]. About 0.1 g leaf tissue was homogenized in 75% methanol (HPLC grade). The supernatant was incubated at room temperature after adding 25% sodium carbonate. Absorption was taken at 725 nm by spectrophotometer.

Antioxidant activity assay: APX (EC 1.11.11) activity in treated and control leaf samples was checked using the method given by Nakano and Asada[25]. The APX activity was recorded at 290 nm using the extinction coefficient 0.28 $mM^{-1}cm^{-1}$. The activity of CAT (EC 1.11.1.6) was determined by the method given by Aebi[26]. The CAT activity was assayed at 240 nm following the extinction coefficient 39.4 $mM^{-1}cm^{-1}$. Activity of GR (EC 1.6.4.2) in Cd treated and control leaf samples were determined by Ghisla and Massey[27] method, while the CAT activity was assayed at 412 nm using the extinction coefficient 13.6 $mM^{-1}cm^{-1}$.

Estimation of protein: Extraction and estimation of protein in control and treated leaves were done by Lowry *et al.*[28]. Concentration of proteins was determined by Nanodrop spectrophotometer (NanoDrop 2000C, Thermo Scientific) at 660 nm. Isolated protein was separated using 30% poly-acrylamide gel in SDS-PAGE electrophoresis apparatus (vertical gel electrophoresis system, Genei) Laemmli[29]. Qualitative analysis of bands in gels was done by gel documentation software (Alphalmager MINI protein sample).

Estimation of metallothioneins proteins: The level of total metallothioneins proteins in treated and control leaf tissues was measured by Cataldo *et al.*[30]. 0.1 g leaf tissues were homogenized in 0.3 mL of the homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, 0.01% β-mercaptoethanol, pH-8.6) and centrifuged at 10,000 rpm for 30 min. Then 1 mL of chilled 90% ethanol and 80 µL of chloroform were added to the supernatant and mixture was centrifuged at 6000 rpm for 10 min (4°C). Three volumes of cold ethanol were added to the resulting supernatant and stored at -20°C for 1 h. Thereafter the supernatant was centrifuged at 6000 rpm for 10 min to purify metallothionein pallets. The resulting pellets were washed with an ethanol: chloroform: homogenization buffer (87:1:12) and soon after centrifuged at 6000 rpm for 10 min. The pellets were air dried and resuspended in 100 µL of 5 mM Tris-HCl, 1 mM EDTA (pH-7). The dissolved metallothionein fraction was added to 420 µL of 0.43 mM 5,5-dithiobis (nitrobenzoic acid) in a 0.2 M phosphate buffer (pH-8) and placed for 30 min at 25±2°C, thereafter absorbance was taken at 412 nm. The concentration of reduced sulfhydril in samples was calculated with standard curve of glutathione at 412 nm. Glutathione contains one cysteine/molecule. Thus it can be used as a standard for quantifying cysteine in protein assuming that 1 mole of metallothionein contains 20 mole of cysteine[31,32].

Antioxidant gene expression: Gene expression in various Cd treated plant samples was observed based on a quantitative real time polymerase chain reaction (qRT-PCR) technique using specific primers[33]. For qRT-PCR technique the total RNA was extracted from 1, 2, 3 and 5 mM Cd treated leaf and 5 mM Cd treated root samples (0.2 g) using Trizol reagent (RNAiso Plus, Takara). The total RNA was then diluted using nuclease free water and quantified by measuring absorbance at 260 nm using the formula: concentration [µg cm^{-3}] = A260×40×dilution factor. Three µg of total RNA was used for reverse transcription using Thermo Scientific Verso cDNA Synthesis Kit and the volume made upto 20 µL using nuclease free water for 20 µL reactions. Different reagents were added as in the indicated order, 5x cDNA synthesis buffer 4 µL, 500 µM dNTP Mix 2 µL, RNA primer 1 µL, RT enhancer 1 µL, Verso enzyme mix 1 µL, template RNA 3 µL, nuclease free water to 20 µL mixed gently and spun down at 1000 rpm for 15 sec. The tubes were incubated for 30 min at 42°C in gradient RT-PCR (SimpliAmp Thermal Cycler). The reaction was terminated by heating at 95°C for 2 min. Primer pairs specific to *M. oleifera* CAT (NCBI accession number GQ365164.1) cDNA (sense primer 5′-CATGAATTTCATGCACAGGG-3′ and antisense primer 5′-TGAAATTGTTCTCCTTCTCA-3′) and APX (NCBI accession number JQ284377.1) cDNA (sense primer 5′-TCGAGCCGATCAAGGAGCAG-3′ antisense primer 5′-GCAAAGAAAGCATCCTCATC-3′) were used[34]. Primers for drumstick actin gene cDNA (sense primer 5′-TGGAAAGTGTCAAAGTGGGG-3′ antisense primer 5′-CGATAATAACAACAGTAATGGCAGC-3′) were used as control[35]. All the qRT-PCR experiments were conducted in 5 replicates. The expressions of CAT and APX were determined following the method given by Rivera-Becerril *et al.*[36] at various time intervals (10, 20 and 30 days) after the Cd treatment. For the qRT-PCR, 2 mm^3 of 1:10 diluted cDNA was mixed with a SYBR Green PCR master mix (Thermo Scientific

DyNAmo Flash SYBR Green qPCR Kit) and primers in a final volume of 20 mm^3. The PCR was performed using a Rotor Gene Q Real-Time system (Qiagen Gmbh, Hilden, Germany) with the following conditions: An initial activation step at 95°C for 4 min, denaturation at 95°C for 15 sec annealing at 58°C for 15 sec and extension at 70°C for 20 sec. Melt curve analysis of the PCR product was carried out as 72°C for 1 min and ramped from 72-95°C with a rise by 1°C every 5 sec. The specificity of the reaction was confirmed by gel electrophoresis. Relative gene expressions were calculated in terms of log fold changes using the ΔCt method.

Statistical analysis: All morphological and biochemical experiments and differential gene expression were performed in 5 replicates (n = 5) for each treatment. One way analysis of variance was applied to all data by SPSS version 16. The data given here is mean of 5 replicates±standard deviation (SD). The treatment means were employed to Tukey's multiple comparisons to determine significant ($p \leq 0.05$) difference in treatment. Sigma Plot™ v.10 was used to prepare graphs.

RESULTS

Plant growth and morphology: Variation in root and shoot length of *M. oleifera* was observed with increasing Cd concentration at 10, 20 and 30 days (Fig. 2a and b). Significant ($p \leq 0.05$) decline in root length and shoot length was recorded in treated seedlings, though, there was substantial increase in treated shoot length and root length with 10, 20 and 30 days of stress period. But among all the treated plants, maximum root length 5.37 cm and shoot length 25 cm was measured on 5 mM Cd with 30 days. With increasing concentration of

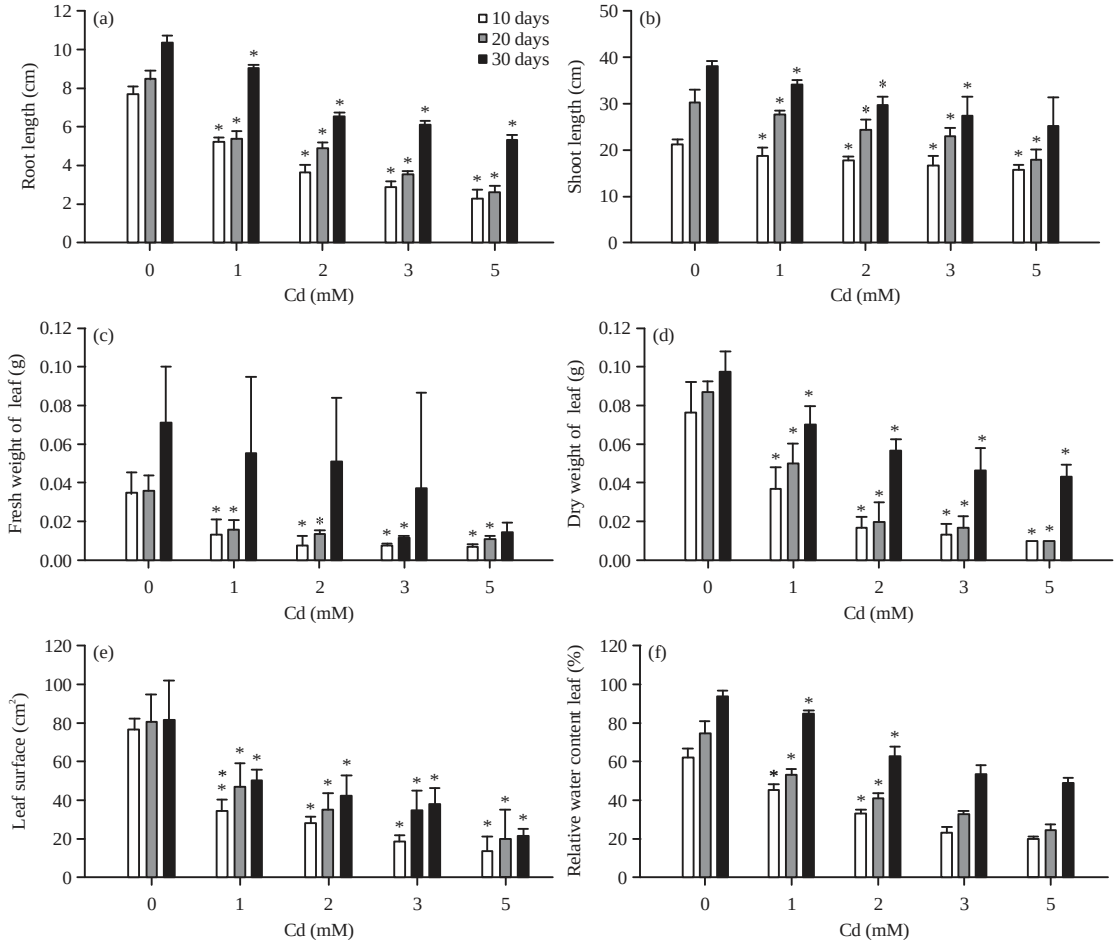

Fig. 2(a-f): Effect of Cd concentrations on (a) root length (b) shoot length (c) leaf fresh weight (d) leaf dry weight (e) leaf surface area and (f) leaf relative water content in *M. oleifera* at 10, 20 and 30 days
Values indicate the Mean±SD (n = 5). *Denotes mean value significantly from control (0) at $p \leq 0.05$

Table 1: Accumulation of different Cd concentrations in *M. oleifera* roots and shoots (mg kg^{-1}) at 10, 20 and 30 days of stress period

Concentration	Cd in roots (mg kg^{-1})			Cd in shoots (mg kg^{-1})		
Cd (mM)	10 Days	20 Days	30 Days	10 Days	20 Days	30 Days
0	0.066±0.04	0.104±0.04	0.123±0.02	0.057±0.00	0.085±0.57	0.091±0.02
1	1.761±0.22	11.237±15.01	21.236±5.11*	0.428±0.08*	0.761±0.03*	0.904±0.29
2	21.903±2.97*	32.853±6.55*	45.712±8.7*	1.142±0.25*	1.237±0.17*	1.566±0.15
3	28.093±0.82*	57.616±5.02*	101.899±9.18*	1.618±0.17*	2.332±0.08*	2.485±0.21*
5	89.519±3.29*	146.658±7.19*	168.563±10.3*	2.380±0.08*	2.571±0.01*	19.951±14.93*

Values indicate the Mean±SD (n = 5). *Denotes mean value significantly from control (0) at $p \leq 0.05$

Table 2: Accumulation of different Cd concentrations (mg kg^{-1}) in *M. oleifera* leaves and Cd translocation into shoots from roots at 10, 20 and 30 days of stress period

Concentration	Cd in leaves (mg kg^{-1})			Cd translocation into shoots		
Cd (mM)	10 Days	20 Days	30 Days	10 Days	20 Days	30 Days
0	0.037±0.02	0.041±0.02	0.060±0.02	1.312±0.77	0.755±0.22	0.413±0.18
1	0.123±0.04*	0.161±0.02*	0.285±0.05	0.245±0.03*	0.044±0.01	0.245±0.19
2	0.190±0.09*	0.237±0.04*	0.380±0.11*	0.053±0.02*	0.034±0.02	0.039±0.01
3	0.237±0.04*	0.266±0.03*	0.571±0.08*	0.237±0.31*	0.089±0.11*	0.040±0.03*
5	0.285±0.01*	0.476±0.08*	0.999±0.14*	0.026±0.01	0.119±0.09	0.402±0.01

Values indicate the Mean±SD (n = 5).*denotes mean value significantly from control (0) at $p \leq 0.05$

Cd tolerance index in roots appeared to be decreasing. Decline in tolerance index ranged from 1 mM and reached maximum at 5 mM followed by 2 and 3 mM concentration during each stress period. The statistical analysis demonstrated that treated leaves showed concentration depended reduce in fresh weight but no major variation in dry weight in comparison to control leaves (Fig. 2c and d). Similarly, concentration depended decline in relative water content (%) and surface area of leaves was measured at each stress time interval. The reduce in surface area and leaf water content started from 1 mM followed by 5 mM at 10, 20 and 30 days. Among all Cd concentrations and stress periods the insignificant maximum water content 49.25% at 5 mM was reported high on 30 days (Fig. 2e and f). Whereas maximum significant ($p \leq 0.05$) surface area 0.53 cm^2 at 5 mM Cd was measured high on 30 days in all treated leaves (Fig. 2e). Leaf relative water content is important parameter which shows the water stress tolerance in plants. Only the higher concentration of Cd was more resistible and the toxicity was found to be the highest at 5 mM $CdCl_2$ with water decline 49.25% in 30 days old treated leaves (Fig. 2f).

Concentration of Cd in roots, shoots and leaves: During the experimental study maximum uptake of Cd concentration was found in roots than other aerial plant-parts (shoots and leaves) with increasing time interval. The maximum Cd concentration 168.56 mg kg^{-1} at 5 mM Cd was measured significantly ($p \leq 0.05$) high in 30 days old treated roots (Table 1). While the Cd uptake of 19.951 mg kg^{-1} at 5 mM Cd concentration was significantly ($p \leq 0.05$) high in 30 days old treated shoots (Table 1). On 30 days, the maximum uptake of 0.999 mg kg^{-1} Cd soil in leaves at 5 mM Cd concentration was low as compared to Cd accumulation in shoots (Table 2). Thereafter, translocation factor decreased insignificantly TF<1 in shoots with increasing Cd concentration. The TF<1 showed low translocation of Cd into the shoot from root with 10, 20 and 30 days (Table 2).

Proline content: Proline content was found to be enhanced significantly ($p \leq 0.05$) with increasing metal concentration during 10, 20 and 30 days (Fig. 3a). The maximum proline 0.76 µmol g^{-1} was recorded at 5 mM Cd of 30 days ($p \leq 0.05$). The present study suggested the supportive role of proline in metal tolerance in Cd treated *M. oleifera* seedlings.

Polyphenol content: Polyphenol content was found to be enhanced significantly ($p \leq 0.05$) with increasing Cd concentration (Fig. 3b). The maximum polyphenol 76.667 mg g^{-1} was recorded at 5 mM Cd of 30 days ($p \leq 0.05$).

Antioxidant enzymes activity: The significant increase in APX, CAT and GR enzymes activity was observed in Cd treated plant as compared to control. For 10 and 20 days, APX activity was noted highest against 3 mM Cd compared to activity in control leaves. For 30 days, 2 and 3 mM of Cd showed little variation in enzyme activity with 31.35 and 26.51 mM mg^{-1} protein, however APX activity was found higher in treated leaves in comparison to their respective control showing 20.84 mM mg^{-1} protein activity (Fig. 3c). Same results were noted for CAT enzyme significantly ($p \leq 0.05$). The CAT activity was found to be enhanced in leaves of treated seedlings with 10, 20 and 30 days in response to increasing Cd concentration

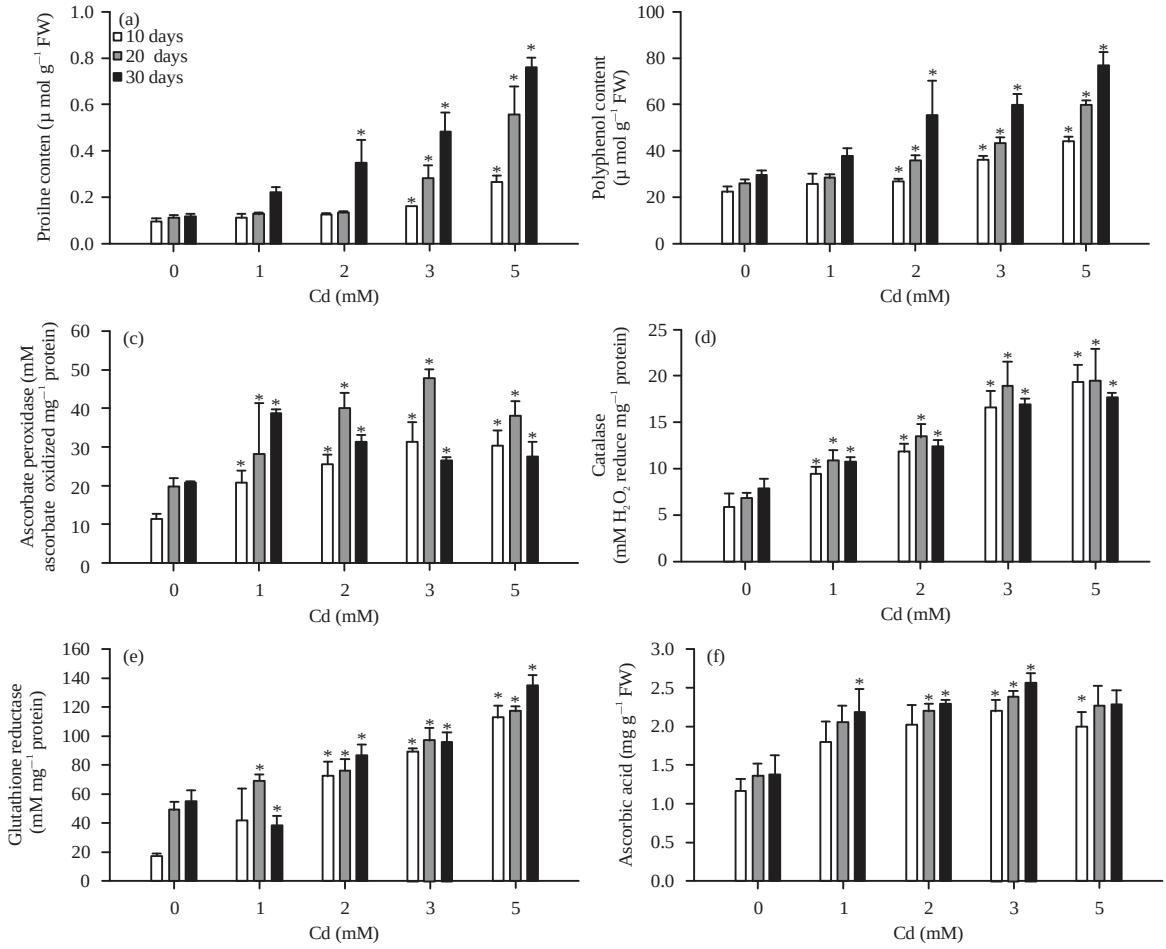

Fig. 3(a-f): Effect of Cd concentrations on (a) proline (b) polyphenol (c) APX enzyme (d) CAT enzyme (e) GR enzyme activity and (f) ascorbic acid in leaves of *M. oleifera* at 10, 20 and 30 days

Values indicate the Mean±SD (n = 5). *Denotes mean value significantly from control (0) at $p \leq 0.05$

(Fig. 3d). Among all stress periods the maximum CAT activity 19.35 mM mg^{-1} protein at 20 days was measured significantly high against 5 mM Cd. Like APX and CAT enzymes GR enzyme activity also showed a significant increase in concentration and time depended manner. The maximum CAT activity 134.980 mM mg^{-1} protein was observed against 5 mM Cd at 30 days (Fig. 3e). The increase in ascorbic acid content was observed during growth period (10, 20 and 30 days) in treated seedlings (Fig. 3f). Leaves of treated seedlings showed significant ($p \leq 0.05$) increase with 2.283, 2.583 and 3.25 mg g^{-1} ascorbic acid content at 3 mM Cd. Whereas, maximum concentration of 5 mM Cd was reported to cause slight decrease in ascorbic acid content at each stress stage.

Soluble protein content: The maximum soluble protein level 15.95 mg g^{-1} at 5 mM Cd was recorded significantly ($p \leq 0.05$) high in treated leaves on 30 days (Fig. 4a). Qualitative analysis of protein profiles is shown in gel images (Fig. 5a-c). Differential expression of protein bands was observed through SDS-PAGE gel electrophoresis. For 10 days, total 15 protein bands ranging from 7-171 KDa molecular weights were expressed in control leaves whereas Cd treated leaves showed 15 bands ranging from 8-186 KDa molecular weights. In 10 days old protein gel profile 6 different polypeptides fractions of 75 KDa, 48 KDa at 2 mM, 75 KDa at 3 mM and 97 KDa, 75 KDa and 48 KDa (black arrows) were observed during gel analysis, which were absent in control protein sample. For 20 days old plants total 13 bands ranging from 15-161 KDa were appeared in control while 1-11 protein bands of 14-161 KDa molecular weights in treated leaves. In 20 days old leaves 3 prominent polypeptides fractions of 97 and 48 KDa were reported at 2 mM concentration, while it was absent in control. This band might be expressed in response to heavy metal stress as metal binding polypeptides to sequester excessive metal ions.

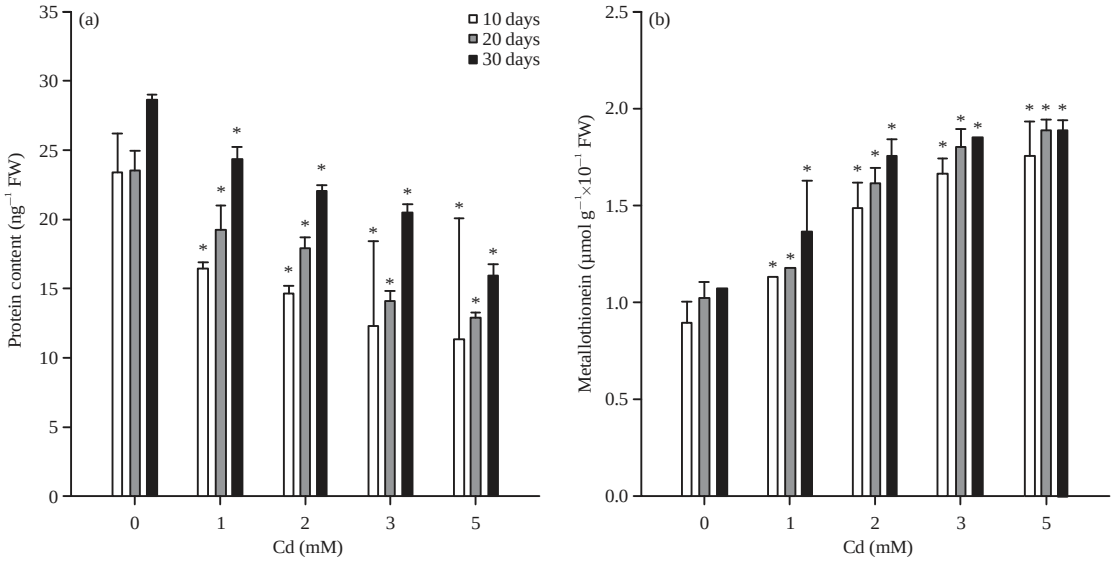

Fig. 4(a-b): Effect of Cd concentrations on (a) soluble proteins and (b) metallothioneins proteins in *M. oleifera* leaves at 10, 20 and 30 days

Values indicate the Mean±SD (n = 5). *Denotes mean value significantly from control (0) at p≤0.05

Table 3: Differential expression of APX and CAT genes in treated *M. oleifera* leaves and roots at different Cd concentrations of 10, 20 and 30 days stress period

Concentration	Expression (log fold changes) of APX gene in Cd treated leaves			Expression (log fold changes) of CAT gene in Cd treated leaves		
Cd (mM)	10 Days	20 Days	30 Days	110 Days	20 Days	30 Days
1	0.097±0.11*	0.169±0.24*	0.649±0.04*	0.275±0.13*	0.289±0.11*	0.622±0.18*
2	0.110±0.08*	0.404±0.24*	0.773±0.14*	0.311±0.11	0.531±0.18*	0.773±0.26*
3	0.203±0.02*	0.509±0.05*	0.764±0.11	0.330±0.11	0.548±0.22	0.689±0.03*
5	0.245±0.02*	0.712±0.09*	1.325±0.23*	0.675±0.23*	0.721±0.28*	0.952±0.3*
In roots						
5 mM	0.128±0.06*	0.367±0.12*	0.547±0.11*	0.098±0.03*	0.399±0.1*	0.812±0.2*

Values indicate the Mean±SD (n = 5). *Denotes mean value significantly from control (0) at p≤0.05

For 30 days old plants, total 10 protein bands ranging from 10-56 KDa molecular weights were reported in control leaves. At 5 mM concentration of Cd only 1 band (black arrow) of 43 KDa molecular weights was expressed. Protein expression of molecular weights 50-60 and 40-55 KDa was seen intense in 10 and 20 days old gel profiles, while in 30 days old plants bands of 40-45 KDa molecular weights were intense.

Metallothioneins proteins: In present study the level of metallothioneins protein at different Cd concentration was checked. Due to heavy metal exposure metallothioneins were induced in high rate in treated leaves as compared to control leaves (Fig. 4b). The metal concentration and stress time depended secretion of metallothioneins protein was observed in the present study (Fig. 4b). According to given results, the level of metallothioneins increased significantly ($p \leq 0.05$) as metal concentration increased in seedlings. Control leaves showed low amount of metallothioneins as compared to all the metal treated leaves. Among all time periods the maximum metallothioneins level 95.99 µmol $g^{-1} \times 10^{-3}$ was recorded highest in response to 5 mM Cd at 30 days.

CAT and APX expression: The candidate genes selected for the present study were ROS detoxifying pathways: APX and CAT. The present study indicated that Cd stress induced the transcription of APX and CAT genes at all sampling times in both roots and leaves. In Cd treated drumstick roots, the transcript level increased continuously as there is increase in days (Table 3). Among all stress periods high APX expression 0.547 fold was reported at 30 days. Similar expression of the CAT was recorded in treated roots at 10, 20 and 30 days and in response to Cd tolerance (Table 3). The maximum expression of CAT gene was measured at 30 days with 0.812 fold against 5 mM Cd.

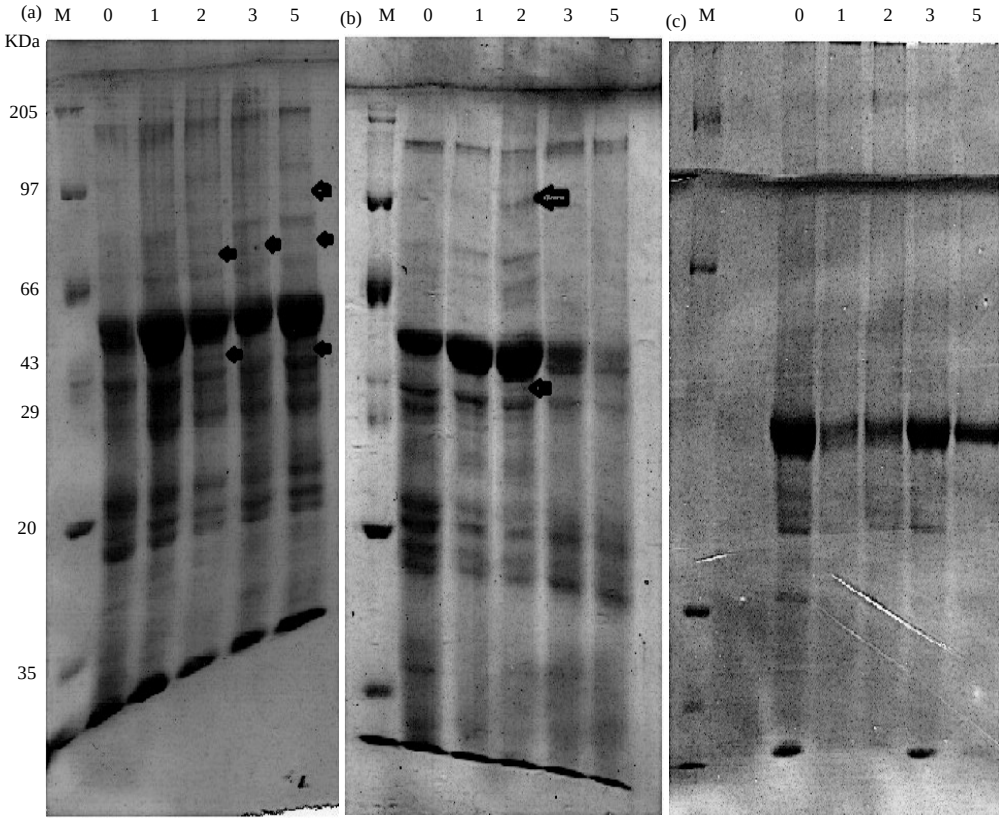

Fig. 5(a-c): Electrophoretic gel pictures of protein expression in *M. oleifera* leaves in response to different Cd concentrations. In figure (a) Protein bands in10 days old leaves, (b) Protein bands in 20 days old leaves and (c) Protein bands in 30 days old leaves) M- broad range protein marker 3.5-205 KDa, (0)-control, 1, 2, 3 and 5 mM Cd concentration

The expression trend of CAT and APX genes in leaves can be described that the drumstick plant started its antioxidant defence mechanisms immediately after Cd stress and upregulated the antioxidant genes to synthesize the CAT and APX enzymes that can reduce reactive oxygen species and oxidative damage in plant cells (Table 3). With increasing Cd concentration APX gene expression showed elevation at 10 and 20 days old leaves, whereas, 30 days old leaves showed insignificant fall in gene expression at 3 mM Cd, although APX transcript level enhanced at 5 mM significantly ($p \leq 0.05$). The highest expression with 1.325 transcript level was reported at 5 mM Cd of 30 days ($p \leq 0.05$). Like APX gene, the up regulated expression of CAT gene also showed metal concentration as well as time interval depended amplification (Table 3). Among all concentrations and growth days the highest CAT transcript level of 0.952 fold was analysed at 5 mM Cd of 30 days ($p \leq 0.05$).

DISCUSSION

The most common effect of Cd toxicity in plants is stunted growth due to low water potential, leaf chlorosis, hampered nutrient uptake and alteration in the activity of many key enzymes of various metabolic pathways[37]. The present study revealed that the effect of Cd toxicity in roots and the other aerial parts varied depending on Cd concentrations. In *M. oleifera* root and shoot growth was found to be affected with high retard at 3 and 5 mM Cd in comparison to 1 and 2 mM Cd. Similar results of decreasing root and shoot length by Cd was also reported by John *et al.*[38] in *B. juncea* though no plant drooping was observed even at high 5 mM concentration during growth period (Fig. 1). Reduction in fresh and dry weight and water potential of *M. oleifera* leaves was affected by the Cd contaminated soil. Biomass and water content reduction in *M. oleifera* leaves was found similar to reduction in *B. napus*, *R. sativus*[39] and *B. juncea*[40] grown in Cd contaminated soil. The comparative study suggests that

the roots of *M. oleifera* are more competent passage to Cd flow when compared with the aerial plant parts. For each Cd treatment its concentration was always higher in roots as compared to shoots and leaves at all stress periods. Higher accumulation of Cd in root than aerial plant parts and TF<1 suggest that Cd transport may possibly depend on binding to the extracellular matrix[41]. Immobilization of Cd by negatively charged polysaccharide molecules (pectin) within the cell wall[42,43], accumulation of Cd substance in plasma membranes[44,45], Cd sequestration across the cell wall of rhizodermal vacuoles or casparian strip[46] are the various physical barriers in roots which may prevent Cd ion transport into aerial parts[47]. The concentration of accumulated metal in roots of *M. oleifera* was compatible with the study of Fowotade and Abdallah[48] and Zhu *et al.*[49]. The study entitled "Estimation of trace metals in *M. oleifera"* has revealed about 0.259 mg kg^{-1} concentration of Cd was adsorbed by plant roots in certain metal contaminated place of Nigeria. This work coincides with the study carried out by Dong *et al.*[50], who confirmed the maximum Cd accumulation in roots of treated *T. aestivum* at high Cd treatment with different stress periods.

Moringa oleifera plant-parts are widely used for regional, edible or fodder purpose by herbivores and common people, so high uptake of Cd metal in plant roots and low translocation factor not only reduces threat of environment pollution but also control entering Cd into the food chain.

The present study suggested that proline level was enhanced in Cd treated plants. Proline, an imino acid is well known to get more elevated under environmental abiotic stress, to detoxify reactive oxygen species, to regulate osmotic potential and enzyme activity at the cellular level in bacteria and higher plants as sunflower, *V. radiata*, *B. juncea*, *T. aestivum* against metal stress[51,52]. Increased accumulation of proline may cause improved tolerance to heavy metal stress in plants53. This report showed that increasing concentration of Cd stress (1, 2, 3 and 5 mM) caused a continuous increase in polyphenol level as defence mechanism against metal[54]. Polyphenols can also act as a major part of antioxidants against the detrimental effect of reactive oxygen species and free radicals in plants[55].

In plants, toxic metals induce oxidative stress by generating reactive oxygen species (ROS) via hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), hydroxyl radicals (OH^-) and singlet oxygen (O_2^-)[56]. In response to the increased ROS, the antioxidant defence system plays an important role against oxidative stress. The enzymatic antioxidant system involves the sequential and simultaneous action of a number of enzymes including APX, CAT and GR for the removal of reactive oxygen species[17]. Under exposure of heavy metals, increase in enzymatic activity of antioxidants and non-enzymatic metabolites was considered as high adaptation of metal tolerance in plants. Similarly, the increase in APX, CAT, GR activity and ascorbic acid content resulted as competent scavenging of Cd induced reactive oxygen species in *M. oleifera* seedlings exposed to Cd metal. Elevated activity of APX and GR suggested the smooth functioning of ascorbate-glutathione cycle by maintaining the glutathione level[57] and efficient tolerance to Cd metal.

This study of soluble protein content coincides with the findings of John *et al.*[38], who found decrease in soluble protein content in Cd stressed *B. juncea.* The decrease in protein content at higher Cd concentrations may be because of enhanced protein degradation process as a result of increased protease activity under metal toxicity. This proves that there is a relation between increased Cd concentrations and plant physiology.

According to Parlikar and Mokashi[58] *M. oleifera* protein has been described as cationic dimeric coagulant molecule, which binds to negatively charged molecules and neutralizes them. In these results, protein molecules showed metal induced differential expression with the presence of additional prominent bands, this may be supportive for metal detoxification or metal binding and sequestration across plasma membrane.

Metallothioneins are non-enzymatic cysteine rich low molecular weight (3-20 KDa) proteins, which induce in high presence of toxic metal ions accumulated in plant. They bind with metal ions and reduce the toxicity by debasing the ratio of the metal ions peroxides into cells[59]. The expression of metallothioneins under heavy metal ions build metal homeostasis, consequently these proteins are regarded as effective biomarkers of heavy metals toxicity, detoxification and tolerance in plants and microorganisms[60]. In addition, excessive accumulation of heavy metal ions into cells may lead to cell damage and reduction in metallothioneins or induction of metallothioneins supporting defence in response to metal ions[61]. The present results containing different levels of metallothioneins against Cd in treated drumstick leaves substantiate Reddy *et al.*[62] study. The similar increasing metallothionein levels have been reported in *Brassica juncea* exposed to Cd and Ni[31].

In the present study, the increased expression of APX and CAT genes was observed in both roots and leaves upon Cd treatment. High expression level of CAT and APX supports that up regulation of these defence genes was among the most efficient defence responses[17]. The results are in

agreement with Kumari *et al.*[33], who reported Cd induced increased APX gene expression and enzyme activity as antioxidant defence mechanisms in wheat. On the other hand, the up regulated activity of CAT enzyme has been reported in *Ulva fasciata*[63], rice cultivars-Taichung Native 1[64] upon Cd stress.

CONCLUSION

Moringa oleifera plant accumulated maximum Cd concentration in roots than shoots and leaves. On the basis of the result obtained for high uptake of Cd and constant biomass production, *M. oleifera* can be classified for phytoextraction of Cd contaminated soil from various sites. Morphological and biochemical responses of plants could be served as potential indicators of metal concentration and its effect in soil. To diminish the Cd induced oxidative effect and free radicals level of proline, polyphenol, ascorbic acid and antioxidative enzymes such as CAT, APX and GR were elevated in *M. oleifera*. Moreover, upregulated transcription of CAT and APX genes in roots and leaves enhance metal tolerance in drumstick plant against heavy metals.

SIGNIFICANCE STATEMENTS

This study discovers that *M. oleifera* plant work as a good metal accumulating plant which can be widely use to remediate the metal contaminated soil in industrial and agricultural areas. This study will help the researcher to uncover the critical areas of environmental contamination clean up using *M. oleifera* as phytoremediation plant that has not been yet explored by other researchers. Thus a new theory on Cd uptake and its phytoextraction may be arrived at.

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Aditya Shastri, honourable Vice Chancellor and Prof. Vinay Sharma, Head of Department Bioscience and Biotechnology, Banasthali University, Rajasthan (India) for providing the lab facilities during the study. Authors are also thankful to Dr. Geeta Yadav, Associate Professor Department of English Banasthali University for language corrections. The present study was supported by Department of Science and Technology (DST) 'CURIE' for providing instrumental facility, atomic absorption spectrophotometer used in metal analysis.

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