CHAPTER 5

5 Accumulation, distribution and ultra-structural changes of *Monochoria hastata* plant exposed to Cadmium

5.1 Introduction

Cadmium (Cd), a non-essential element for plant growth and development is considered as one of the most potential environmental phytotoxicants, even at low concentration [1, 2]. Cd is mobile metallic element found in the environment and it accumulates throughout the food chain posing a serious threat to human health [3, 4]. The diverse anthropogenic and industrial pathways by which Cd release into the environment are mainly electroplating, tanneries, oil refineries, iron and steel industry effluents, plastic paints and over use of chemical fertilizers and pesticides [5, 6, 7, 8].

The ability of some plant species to accumulate a high concentration of heavy metals from their immediate environment has been proposed as an environmentally sustainable, easy, cost effective and in situ remediation technology for detoxification of polluted water and soil, making it preferable to other chemical or mechanical techniques [9, 10, 11]. The most common uptake method of heavy metals by plants include the sorption by plant systems, which is a combination of various physical and chemical processes and then subsequently transported to the aerial part of the plant.

Different species of macro and microflora were reported to accumulate high concentration of Cd [12, 13]. In recent years, many aquatic plants, usually those found in polluted water bodies have been suggested for waste water treatment [14, 15, 16, 17, 18, 19]. They have the ability to accumulate unusually high concentration of heavy metals, without impact on their growth and development [19]. Hyperaccumulators plants that accumulate exceptionally high concentrations of toxic metals in their living tissues have encouraged much research worldwide [20, 21, 22, 9, 23]. A number of plants accumulate extraordinarily high concentrations of Cd in their living tissues have been identified as hyperaccumulator for phytoremediation of Cd [24, 25, 26, 27, 28, 29, 30]. Lombi et al. [26] has reported that *T. caerulescens* J. & C. Presl belongs to the Brassicaceae or Cruciferae family, is best known as a Cd hyperaccumulator and can accumulate 14, 000 μg Cd g^{-1} d wt.

Table 5.1 Aquatic plants that accumulate Cd from water environment

Scientific name	Element uptake	Amount uptake mg kg^{-1} (DW)	References
Limnocharis flava (L.) Buchenau	Cd	>1000	[31]
Azolla pinnata R. Br.	Cd	740	[32]
Eichhornia crassipes (Mart.)Solms	Cd	575	[33]
Wolffia globosa (Roxb.) Hartog & Plas	Cd	500	[34]
Echinochloa polystachya(Kunth) Hitchc	Cd	230-300	[35]
Helianthus annuus L.	Cd	40-330	[36]
Rorippa globosa (Turcz. Ex Fisch. & C.A. Mey.) Hayek	Cd	>100	[37]
***Monochoria hastata* L.**	**Cd**	**>100**	**Current study**

According to Baker [38] accumulation and exclusion are two basic strategies by which plants respond to elevated concentrations of heavy metals. Uptake and accumulation of metals at higher concentrations can be cytotoxic in some plant species, causing structural and ultrastructural changes affecting the growth and physiological well-being of the plants [39, 40]. The effect of excessive accumulation of Cd^{2+} on plant species has been extensively studied with biological and ecological consequences that depend on its availability in the soil or water [41]. It can induce severe disturbances and environmental stress in the physiological processes of a plant [42, 43]. The most common Cd toxicity symptoms due to excessive deposition of the metals are an oxidative stress, leaf roll and chlorosis, inhibition of root growth and elongation, reddish veins and petioles, damage to the light- harvesting complex in photosystems and reduction of the chlorophyll biosynthesis. The damage to photosynthesis may be due to decline in photosynthetic pigments and rubisco activity, a decrease in chlorophyll and an increase in lipid peroxidation within these organelles [44]. Besides Cd is a non-redox metal unable to perform single electron transfer reactions, but can generates oxidative stress by impairing the mitochondrial and photosynthetic electron transfer chains [45, 46, 47, 48, 49, 50, 51]. Cd induces

several ROS which can accelerate lipid peroxidation, thus affecting cell membrane fluidity and permeability due to an alteration in the composition of membrane lipids [52]. When plants are exposed to heavy metal stress they induce to develop a defence system through production of antioxidants and antioxidative enzymes to reduce the oxidative damage to biomolecules.

Anatomic and structural changes are known to be some of the effects of Cd in plants [53, 54]. Metal tolerant plants rely on improved mechanisms for metal homeostasis. The cell membrane may play an important role in metal homeostasis, either by preventing or reducing entry into the cell or through efflux mechanisms, selectively effluxing the essential cations. The selective efflux of essential cations thus restricts the entry of toxic ions. A strong body of evidence in the literature has shown the cause of Cd toxicity is its chemical similarity with essential elements, in particular Zn, but also Ca and Fe, deregulating the homeostasis of the latter elements or causing their displacement from proteins [55]. Thus, accumulation of Cd in plant may also cause a decrease of mitotic index, inhibits cell division and cell proliferation. Moreover, Cd can profoundly induces numerous changes in plant growth and a series of physiological processes in plants, such as enzyme activity, respiration, photosynthesis and nutrient assimilation [56, 57, 58].

In order to survive in Cd-contaminated areas and to avoid Cd toxicity, plants have developed intra and extracellular mechanisms for metal detoxification [59, 60, 61]. The detoxification involves the chelation of metal cation by ligands and sequester away from the sites or metabolism into the vacuole or cell wall and reduces the concentration of free Cd^{2+} in the cytosol [51]. The exclusion or reduced uptake of metal is one strategy for avoiding heavy metal build-up. The other strategy is by stimulating the efflux pumping of metals that have entered the cytosol. The most widely studied mechanism of Cd detoxification and tolerance is the chelation of toxic metals that have entered the cytosol by high affinity ligands [62, 63]. Much research has focused either on the various forms of complexes that produced by Cd for detoxification [64, 65].

The techniques which are generally used to investigate the cellular distribution of heavy metals in plant tissues include particle- induced X-ray emission (micro-PIXE) [66, 67], nuclear micro- probe technique (NMP) [68], electron energy loss spectroscopy (EELS) [69] and transmission electron microscopy (TEM) [70]. Most studies of Cd distribution within plants measure only bulk tissue (e.g., root,

shoot, leaf, stem, etc.) concentrations of Cd. The studies that have examined the cellular and/or subcellular distributions of Cd in plant tissues used a variety of histochemical, imaging and physical fractionation methods. The histochemical methods included using Cd specific dyes to locate Cd in fresh tissues [71, 72].

Monochoria hastata (L) is a common wetland species that naturally grows in swamps and paddy field of Assam, India. It is a rapidly growing, high biomass plant with an intensive root system seems to be an ideal plant to clean up water and soil contaminant. The efficacy of *Monochoria hastata* in the absorption and accumulation of As has recently been reported [73]. Similarly, two other wetland species of plant of same genera that of *Monochoria hastata* found to have shown the phytoremediation potential namely Cr, Cd and Cu by *Monochoria vaginalis* [74], and Pb by *Monochoria korsakowi* [75]. However, this is the first study of use of *M. hastata* for phytoremediation of Cd and regarding the cellular and subcellular distribution of Cd. Therefore, present study of accumulation Cd by *M. hastata* assume great significance as it is one of the most widely distributed wetland plant which is consumed as vegetable by majority of population of NE India. Understanding of the distribution, translocation and bioaccumulation of cadmium in *M. hastata* can provide fundamental information on metal toxicity and can throw light on the mechanisms of cadmium uptake. Therefore, the objectives of the present study were to investigate the cadmium uptake, subcellular localization, metal toxicity and ultrastructural damages caused by cadmium in leaves, stems and roots of *M. hastata.* It is hypothesized that plant *M hastata* may be a potential accumulator of Cd with its profuse root system, large biomass and fast growth rate and then it can eventually lead to entrance into the food chain as it is an edible plant.

5.2 Materials and methods

5.2.1 Plant Material

Monochoria hastata (L) Solms Laubach is an emergent aquatic herb in the Pontederiaceae family. The plant inhabits in shallow water, freshwater pools and mudflats in rivers, ditches, canal banks, swampy ground and in rice fields of Assam. An erect, ascending or occasionally creeping, emergent or floating rhizomatous herb with a robust stem, 30 cm or more long, and with petiole up to 90 cm long with a leaf-blade of about 20 x 15 cm (**Figure 5.1**). This plant is eaten as a vegetable. Usually the leaves and stems are cooked but the inflorescence can be eaten raw. The

rhizome can be used as edible and used as cattle feed. The plant has medicinal properties. The plant can also be used as an ornamental plant.

Figure 5.1 *M. hastata* in natural condition

5.2.2 Experimental Design

The *Monochoria hastata* (L) plants were collected from local wetland of Sonitpur district, Assam, India. The collected plants were washed thoroughly in running tap water to avoid any surface contamination, blotted with clean blotting paper for any surface moisture avoiding damage to root and leaf apices. They were then put in polythene vessels for 7 days acclimatization in modified Hoagland's nutrient solution. The modified Hoagland's solution contains (in M): KNO_3 $5X10^{-3}$, Ca $(NO_3).4H_2O$ $5X\ 10^{-3}$, $MgSO_4.7H_2O$ $2X10^{-3,}$ KH_2PO_4 $1x10^{-3}$, FeSO4.7H2O $0.02x10^{-3}$, H_3BO_3 $0.045x10^{-3}$, $MnCl_2.4H_2O$ $0.01x10^{-3,}$ CuSO4.5H2O $0.3x10^{-6}$, $Na_2MoO_4.2H_2O$ $0.1x10^{-6}$, and $ZnSO_4.7H_2O$ $0.8x10^{-6}$ (pH) [76, 77]. Acclimatized plant of *M. hastata* were placed in each experimental tub, which contain 2 L of full strength Hoagland's solution supplemented with and 5, 10 and 15mg/L of Cd solution and placed under natural sunlight. One control group of plants was also prepared where Cd treatment was not provided.

The solutions were analysed before starting the experiments (0 h) and after 2^{nd}, 4^{th}, 6^{th}, $8^{th}$and 10^{th} days to determine the changes in concentration of Cd during the experiments. Metal treatments were supplemented with nutrient solution to avoid any water and nutrient deficiencies. However, plants were analysed only at the termination of the experiments to see the accumulation of Cd in different parts viz. root, shoot and leaf.

5.2.3 Cd solution preparation

All the chemicals used in this phytoremediation studies are of analytical grades (Merck chemicals, Germany) which are used without any further purification. In all experiments, deionized water (Milli-Q Millipore 18.2 MΩ cm^{-1} conductivity) is used for the preparation, dilution and analytical purposes of solutions. The Cd solutions were of prepared with analytical grade $CdCl_2$ solution.

5.2.4 Microwave digestion

It is a commonly used important technique to digest organic material in an acidic environment with an oxygen source (hydrogen peroxide) before measuring the quantity of heavy metals in the solution. This method increases the decomposition of the sample and thus, helps more quantity of heavy metals to become soluble in the solution.

5.2.5 Study of Total Chlorophyll concentration

The method used for estimation of chlorophyll was given by Anderson and Boardman [78]. Total chlorophyll content was determined by using the following formula:

Total chlorophyll= $\{(12.7XA_{645}) + (8.02XA_{663})\}$ X V/1000XW mg chlorophyll/g fresh leaf weight.

Where,

A_{645}= Absorbance at 645 nm wavelength

A_{663}= Absorbance at 663 nm wavelength

V= Final volume of the extract (mL)

W= Fresh weight of the leaf (g)

5.2.6 Element concentrations

For elemental analyses, roots, shoots and leaves were ground, weighed samples digested with HNO_3 (70%) in a high pressure microwave and concentrations were measured by ICP-OES (Optima 2100, Perkin Elmer). Certified National Institute of Standards and Technology plant (peach leaf) standards were carried through the digestions and analyzed as part of the quality assurance/quality control protocol. Reagent blanks and spikes were used where appropriate to ensure accuracy and precision in the analysis. Plant samples were dried at 60°C for 3 d, and then digested at 180°C for 105 min in 5 mL of concentrated nitric acid. Samples were cooled to room temperature, 1 mL of 30% (w/v) hydrogen peroxide was added, the mixture

was heated at 180°C for 20 min, cooled, and deionized water added to a final volume of 12.5 mL.

5.2.7 Bioconcentration factor (BCF)

At the end of experiment after 10 days, plants were harvested. They were separated into shoots, leaf and roots, and were analyzed for metals accumulation, and the bioconcentration factor (BCF) was determined. Also water samples were collected for metals analysis after a definite interval of time.

The bioconcentration factor (BCF) is a useful parameter to evaluate plant's potentiality to accumulate metal, it provides the ability index of a plant to accumulate metals with respect to metal concentration in the substrate and it was calculated on a dry weight basis [79].

BCF = Metal concentration in plant tissue/Initial concentration of metal in substrate.

For plants, the BCF has been used as a measure of the metal accumulation efficiency, whereby value greater than 1 is an indication of plants potential to phytoextraction [80, 81].

Hyperaccumulating plants are defined by the following characteristics: 1) metal concentrations in the aerial portions are >10,000 mg kg^{-1} dry matter for Zn and Mn; >1,000 mg kg^{-1} for Co, Cu, Ni, As, and Se; and >100 mg kg^{-1} for Cd [82, 83], 2) the translocation factor is >1.0 [84].

5.2.8 Translocation Factor (TF)

The translocation factor (TF) describes the movement and distribution of heavy metals in plants.

TF = Metal concentration (shoot +leaves)/Metal concentration (roots)

Plants exhibiting TF and particularly BCF values less than one are unsuitable for phytoextraction [85]. TF>1 indicates that the plant translocate metals effectively from the root to shoot. Plants with both bioconcentration factor and translocation factor greater than one (TF and BCF> 1) have the potential to be used in phytoextraction. Besides, plants with bioconcentration factor greater than one and translocation factor less than one (BCF> 1 and TF< 1) have the potential for phytostabilization [86].

Plant species were categorized according to their TF values into four groups. First group contain hyperaccumulator plants with TF values above ten. Second group contains hypertolerant plants with TF values above one but below ten. Third

group contains tolerant plants with TF values less than one. This groups of plants have adopted an exclusion strategy. This strategy allows them to form metal stable complexes in their root cells, which results in a limited metal translocation to above-ground parts. The fourth group, categorized as excluders, as these plants can grow in heavy-metal polluted soils without accumulating significant quantities [38]. These plant species have TF values < 0.1. Possibly these plants use mechanism that avoids excessive uptake of metals and metal is absorbed and translocated only in nontoxic quantities. Since plants employed in phytoextraction treatments must accumulate more than 1000 mg/kg DW, should exhibit values of TF and BCF larger than unity, and should produce high quantities of biomass [87, 28, 88].

5.2.9 Biological accumulation coefficient (BAC)

The BAC is the concentration of metals in the plant shoots divided by the metal concentration in the soil/water.

BAC = Metal concentration in shoots/ Metal concentration in soil/water

BAC factors >1 indicates that the plant species has the ability to store metals from the soil/water into the shoots (Baker & Brooks; Baker, Reeves & Hajar; Brown, Chaney & Baker; Wei, Chen & Huang as cited in Khan and Uzair [89].

Tolerant plant species tend to restrict water-root and root-shoot transfers, and therefore have much less accumulation in biomass, whereas hyperaccumulators actively take up and translocate metals into above-ground tissues. Plants with high BAC (greater than 1) are suitable for phytoextraction; those with high BCF (higher than 1) and low TF (lower than 1) have potential for phytostabilisation [86].

5.2.10 Procedure for Microscopic Study

Stem and root samples of 5mm length were cut from 2 cm above and 2 cm below the stem–root intersection, respectively. Leaf samples of 5mm length were excised from the middle portion of the third leaf (lower leaf) from the base of the plant. The leaf, stem and root samples were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were analysed by energy-dispersive X-ray microanalysis using a JEOL 1200EX analytical scanning transmission electron microscope (STEM; Jeol Australasia, Brookvale Australia).

5.2.10.1 Scanning Electron Microscopy (SEM) study

Leaf, stem and root samples were also prepared for scanning electron microscopy (SEM). Root, petiole and leaf samples were washed in running tap water and carefully rinsed with distilled water before microscopy observations. For scanning

electron microscopy analysis (SEM) with conventional preparation, small pieces of roots, petioles and leaves (3-4 mm) were immediately fixed in 3% glutaraldehyde in 0.05M phosphate buffer for 90 min, which was followed by secondary fixation in 2% osmium tetroxide in 0.01M sodium cacodylate buffer for 30 min [48]. The samples were dehydrated in an acetone series. SEM photograph were carried for the samples, using SEM model JEOL-JSM-6390 LV attached with energy dispersive X-ray unit, with an accelerating voltage of 20 kV.

The electron microscope was operated at 120 kV in STEM mode with a large spot size and a beam current of approximately 0.5/10 -9A, measured with a Faraday cup at the level of the specimen. Specimens were analysed at room temperature (25^{o}C) in a carbon holder tilted at 25 ∞ towards the detector. The microscope was operated with two liquid nitrogen-cooled anti-contamination traps, which effectively cry pumped the microscope column.

5.2.10.2 Transmission Electron Microscopy (TEM) study

The leaf, shoot and root samples were subsequently fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer (p^H 7.1) for 8 h and post-fixed with OsO_4. The samples were dehydrated in an ethanol series (95%, v/v) and embedded in Spurrs epoxy resin. Ultrathin sections were obtained using an ultramicrotome and stained with uranyl acetate and basic lead citrate for observation using JEOL TEM [90].

5.2.11 Fourier transform infrared analysis

FTIR spectroscopy was used to identify the chemical groups present in root, shoot and leaf. The samples were examined using Perkin-Elmer Spectrum100 FTIR 2000 spectrometer within range 406-7800 cm^{-1}. KBr was used as background material in all the analysis. The root, shoot and leaf powder (0.0035g) were mixed with 0.5 KBr and pressed to form a pellet. FTIR spectra of plant samples before and after adsorption were compared [91].

5.2.12 Statistical Analysis

The experimental results were expressed as mean ± standard deviation (SD) of triplicate determinations for statistical validity. ANOVA was performed for all the data to confirm their validity using SPSS 18.0. A probability of 0.05 or lower was considered as significant. The accumulation of Cd in the roots and shoots increased significantly ($p<0.05$) up to the 10^{th} days of exposure time in *M. hastata.* The data was analyzed for three different levels of significance based on the 'p' values as,

* Significant (p = 0.01 to 0.05),

** Very Significant (p = 0.001 to 0.01) and

*** Extremely Significant (p < 0.001) (Statistical data were given in Annexure I).

5.3 Results

5.3.1 Visual symptoms

Uptake and accumulation of heavy metals by the plant, not only cause structural and ultrastructural changes, but also result in toxicity symptoms and affect metabolic processes. Several visual toxic symptoms such as withering, chlorosis and failing of leaves appeared in *M. hastata*, especially at 15mg/L after 10 days of application of Cd. Nevertheless, the leaves of control plants did not exhibit any symptoms of damage, during the period of experiment. Yoshihara et al. [92] observed that Cd interfered with Fe translocation from roots to shoots which resulted in Fe deficiency. Therefore, deficiency of Fe has been identified as the primary cause of such visual signs of trouble in plants [93, 94].

5.3.2 Total chlorophyll content

The total chlorophyll content of leaves of *M. hastata* treated with Cd was investigated. The results of the analysis revealed that the total chlorophyll content of the plant decreased with increasing concentration of Cd in the medium (**Figure 5.2**). Similarly, Ewais [95] and Han [96] has also reported that the excess of Pb and Cd usually decreased the concentrations of chlorophylls. It has been established that plant exposition to heavy metals has always resulted in a strong reduction of plant growth as a consequence of significant alterations in many metabolic pathways and photosynthetic activities [97, 98]. Moreover, there was obvious differences in total chlorophyll content in the plants among three different Cd treatment levels (P < 0.05) for a definite day, but not for all days (P > 0.05).

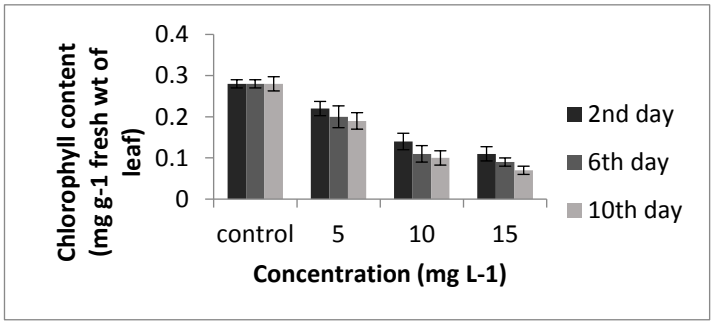


Figure 5.2 Graphical representation of total chlorophyll content in *M. hastata* [Different letters represent statistically significant differences at P< 0.05 from one-way ANOVA (Statistical data in Annexure I, **Table 5.3.2.1**).]

5.3.3 Metal Concentrations in morphological tissues

Accumulation of Cd in different parts of *M. hastata* increases as the metal concentration in solution increases (**Figure 5.3**). A comparison of Cd concentrations in different morphological tissues of *M. hastata* suggested that the roots have significantly higher concentrations of Cd (3981.3mg/kg) in comparison to shoots (583.2mg/kg) and leaves (89.3mg/kg) for 15 mg/L Cd treatment (**Figure 5.3**). The accumulation of Cd in roots and shoots were increased significantly ($p<0.05$) upto the 10^{th} days of exposure time in *M. hastata*. Similar, trend of higher accumulation of Cd in roots than shoots and leaves has also been reported by [99, 100,101, 102]. According to Wang et al. [101] metal-tolerant plants always accumulate higher concentration of Cd in roots than those in above ground parts and could be considered as an important tolerance mechanism. Therefore, higher Cd concentrations in roots of *M. hastata* than those in other tissues could be considered as an important tolerance mechanism of the plant.

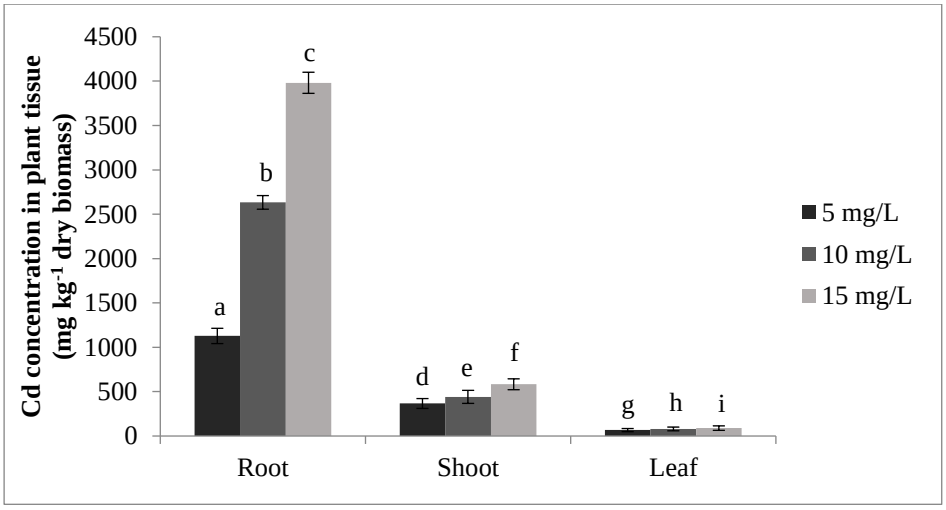


Figure 5.3 Phytoaccumulation of Cd by *M. hastata* [Different letters represent statistically significant differences at $P< 0.05$ from one-way ANOVA (Statistical data in Annexure I, **Table 5.3.3.1-5.3.3.3**).]

In the present study, the BCF values evaluated for all three concentrations of Cd was found to be greater than 100: for 5mg/L BCF was 312.36; in 10 mg/L BCF was 315.46 and in 15 mg/L BCF was 310.25 (**Table 5.2**). To evaluate heavy metal accumulation efficiency in plants BCF has been used as a key indicator [103]. This results suggest that *M. hastata* has the ability to remove Cd effectively from solution and plant may potentially be useful for removal of Cd from Cd containing wastewater. In the present study the calculated BCF values of the *M. hastata* was

more than 300 but below 800 and is considered as moderate accumulator of Cd according to classification made by Zayed et al. [79].

The mean translocation factors (TF) for *M. hastata* were, 0.38 for 5 mg/L; 0.19 for 10 mg/L and 0.16 for 15 mg/L respectively. TF of Cd for all concentration is less than the critical value (1.0), which indicates that *M. hastata* is not very effective to transfer Cd from roots to shoots and is categorized as tolerant plants based on the classification made by Baker et al. [38]. Tolerant plants are capable of accumulating Cd concentration above 0.01% of shoot dry weight, without causing toxicity symptoms [28, 104].

Table 5.2 BCF values (dry weight basis), root to stem and stem to leaf TF values, and BAC values of *M.hastata* (n = 3)

Concentration (mg/L)	Root mg/kg	Shoot mg/kg	Leaf mg/kg	BCF	TF	BAC
5	1128±86.08	366.8±55.34	67±17.53	312.36±0.08	0.38±0.005	73.36±0.91
10	2633.3±76.59	441.3±73.49	80±21.33	315.46±0.56	0.19±0.03	44.13±0.02
15	3981.3±119	583.2±61.21	89.3±25.12	310.25±0.17	0.16±0.03	38.88±0.11

(The standard deviation has been obtained for n=3, "n" stands for the number of experiment replicates)

The BAC values for Cd was 41.36 in 5 mg/L; 21.13 in 10 mg/L and 20.21 in 15 mg/L Cd treatment. The highest value for BAC was found in 5 mg/L Cd treatment. The average BAC values in this study show that all the values are >1, indicating that *M. hastata* has phytoextraction potential since the values are all greater than one.

These plants have adopted an exclusion strategy to prevent metal translocation from the root to above-ground tissues. Therefore, according to Baker [38], the *M. hastata* may be considered as metal excluder since its shoot/root ratios lower than 1.

Unlike a hyperaccumulator which rapidly and efficiently translocate Cd to the above ground parts via the xylem [105] *M. hastata* act as moderate accumulator, which retain in root cells most of the Cd taken up from the aqueous solution. However, in contrary to this report a number of studies have indicated that in many Cd-hyperaccumulating plants and in some non-hyperaccumulators, mostly from Compositae family [106] and *T. caerulescens* are capable of transferring high levels of Cd to their shoots from soil or hydroponic solution and store a small amount in their roots [107, 108].

M. hastata is a heavy metal-tolerant species with high BCF and low TF which can be used for phytostabilization of metal contaminated site. Phytostabilization can be used to minimize migration of contaminants in soils [109].

5.3.4 Scanning Electron Microscopic (SEM) studies

To understand the Cd detoxification mechanism in *M. hastata*, it is necessary to map the Cd transport pathway from roots to leaf as well as sites where Cd particles are localized. The SEM technique has been used in several studies to investigate the internal distribution of metals in plant tissues [110, 111]. Thus, leaf, stem and roots of treated plant were examined by SEM equipped with energy dispersive X-ray spectrometer (EDX). The electron microscopy study also indicated different toxic effects of Cd on the cellular structure in leaf, shoot and root of *M. hastata*.

One of the most evident symptoms of Cd toxicity is the stomatal closure which was confirmed by measuring the dimeter of the stomata of the abaxial side of the leaves with the help of SEM. In leaves of the plants treated with Cd especially for 10 days, most of the stomata were found closed as compared to the control plants indicating the significant effects of Cd on the leaf stomata. In our study, in 15mg/L Cd treated *M. hastata* leaf showed closing of stomata (**Figure 5.4A**), whereas in the control it appeared normal (**Figure 5.4B**). The SEM analysis results of leaf epidermis structure revealed that size of stomata aperture in control (9.88µm) (**Figure 5.4C**), is decreased in Cd loaded plant leaf (5.56 µm) (**Figure 5.4D**). SEM micrographs of the abaxial side of the leaf confirmed closure of stomata and a difference of 4.32 µm of stomatal aperture was found between the control and treated plant leaves [112]. EDX spectra of the leaf of the control plant (**Figure 5.4E**) showed presence of no Cd whereas Cd was confirmed in the treated plant besides other elements (**Figure 5.4F**). The closure of the stomata may be a strategy of the plant to prevent water loss through transpiration as the translocation of water and solute get disturbed in the presence of Cd [113]. Cd induced changes in the leaf epidermis structure involved a reduction in the size of the guard cells results in decrease the stomatal opening. Similarly, Sandalio et al. [48] observed stomatal closure in the abaxial side of pea leaves when plant were treated with 50 µM Cd, while in control plants most stomata were open.

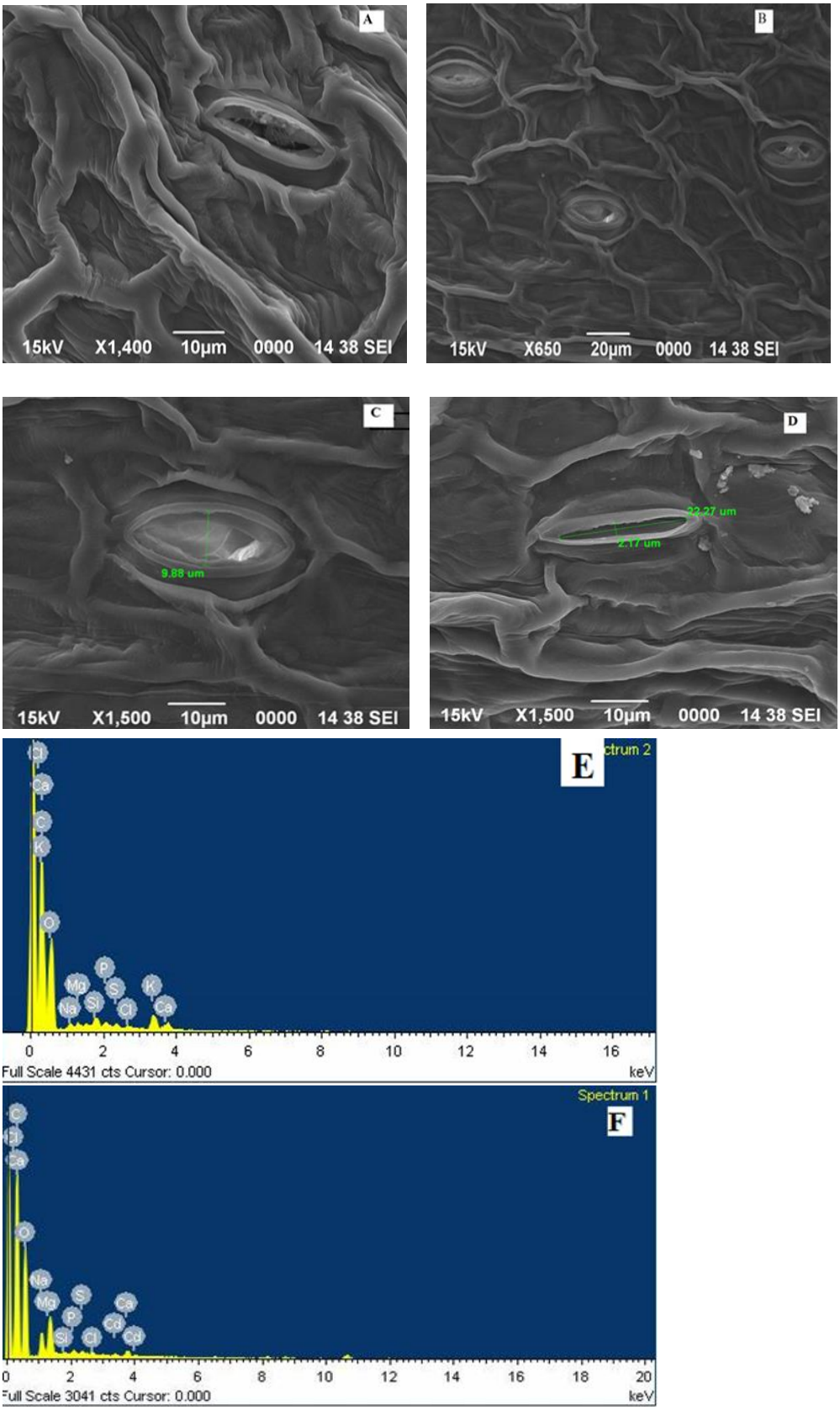


Figure 5.4 SEM- EDX micrographs of leaf surface of *M. hastata* (A–F) (A) leaf epidermis of plant grown in 15mg/L Cd solution (1400X); (B) leaf epidermis of control plant (650X); (C) magnified view of stomata showing diameter in control leaf (1500X); (D) showing closing of stomata (1500X); (E) EDX spectra of control plant leaf; (E) magnified view of plate; (F) EDX spectra of Cd treated plant, showing the presence of Cd ions along with other ions

The SEM micrographs of plant treated with Cd showed changes of the vascular cells of the shoot samples (**Figure 5.5A**). Exposure to 15mg/L Cd for 10 days resulted in a loss of cell shape, decrease in the intercellular spaces, and shrinkage of vascular bundle in *M. hastata* (**Figure 5.5A**) as compared to the control (**Figure 5.5B**). Similarly the SEM micrographs of leaf and root of *M. hastata* also showed similar results. EDX spectra of the Control (**Figure 5.5C**) showed no Cd whereas Cd treated shoot (**Figure 5.5D**) clearly indicated that Cd was present in vascular cells of the shoot samples and electron-dense granules appeared bright when observed. The accumulated Cd was localized by SEM as bright depositions when observed at 250 eV.

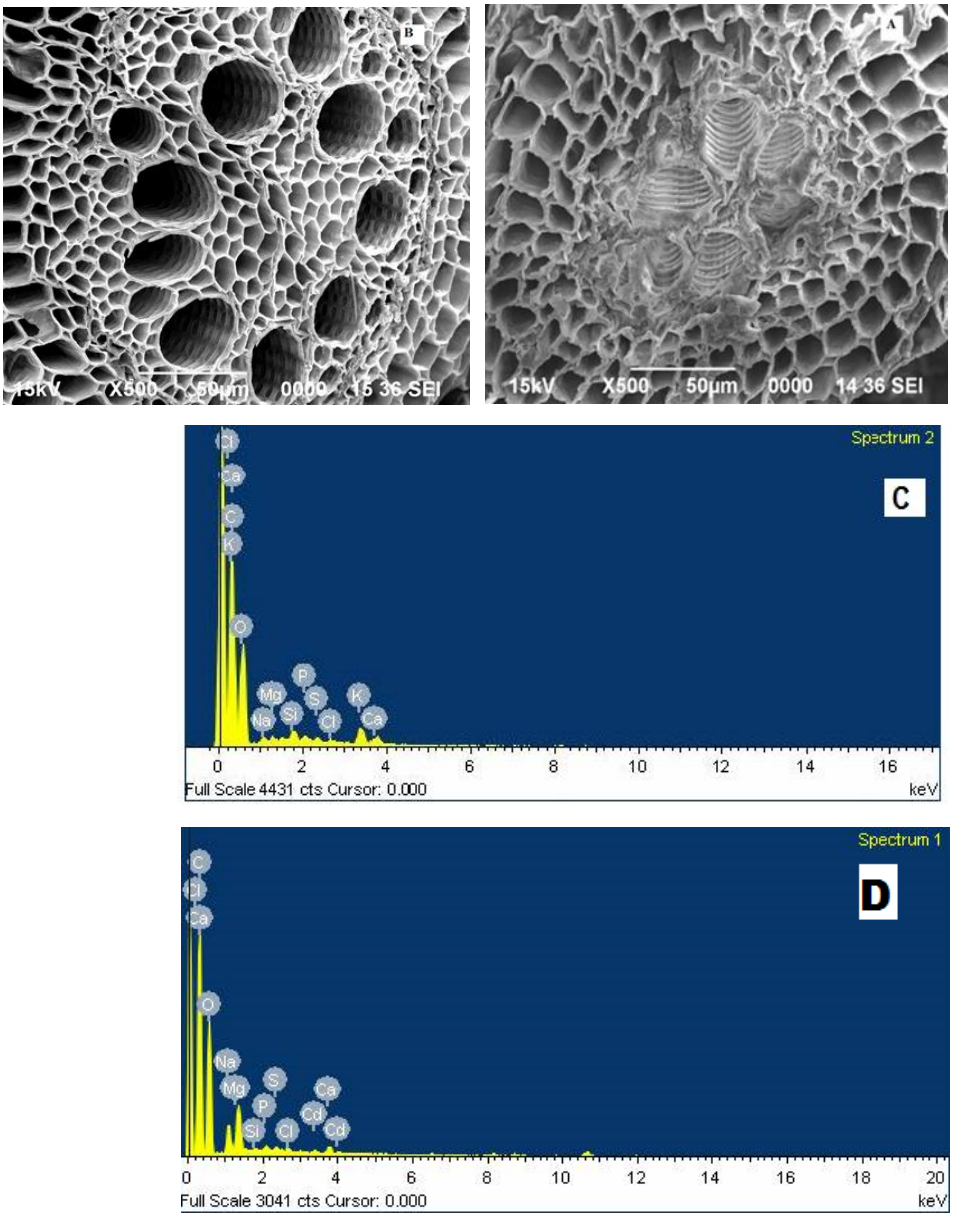
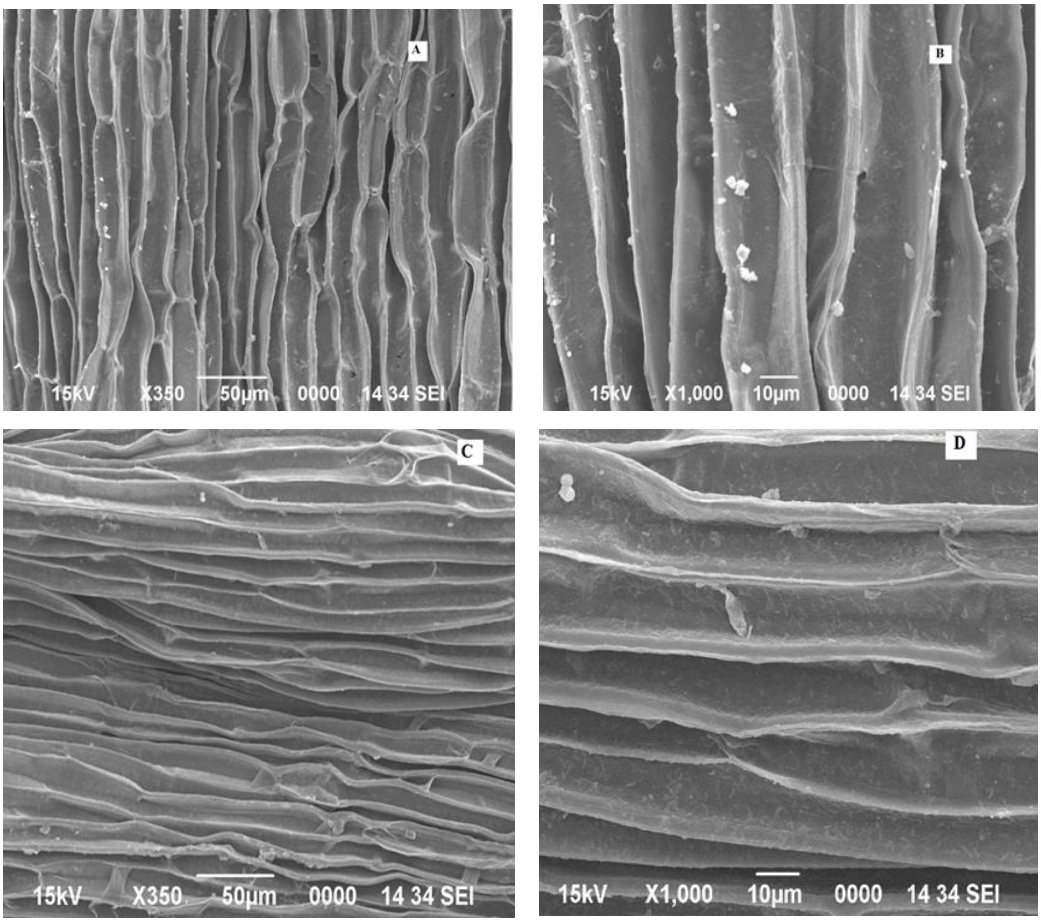
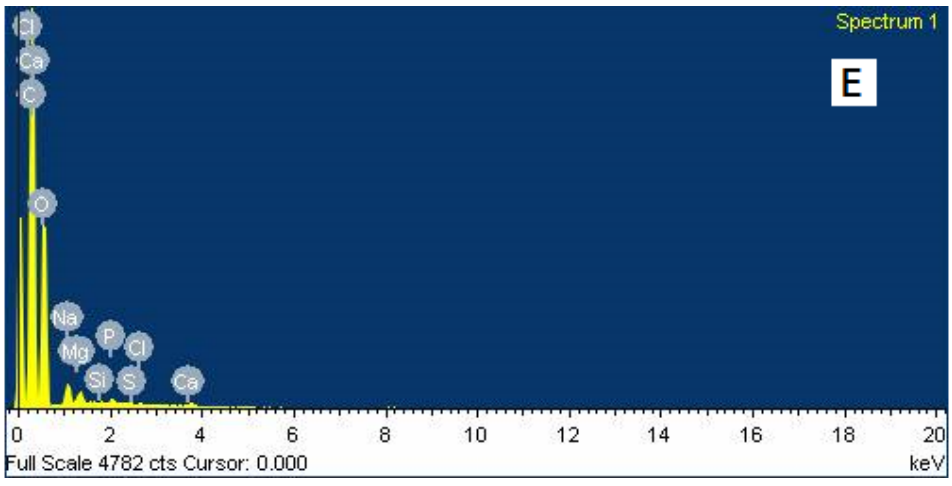


Figure 5.5 SEM- EDX micrographs (A) Shoot of *M. hastata* exposed to 15 mg/L Cd for 10 days ; (B) the control shoot ; (C) EDX spectra of control shoot; (D) EDX spectra of Cd treated shoot

Metal localization within and around roots of *M. hastata* growing in a hydroponic metal solution was studied by energy dispersive X-ray microanalysis of cross section of roots. From **Figure 5.6**, it was seen that some morphological differences seen between the control (**Figure 5.6A, B**) and Cd loaded root surface of the specimen samples (**Figure 5.6C, D**). In the control, surface appears to be smoother than that the metal-loaded samples and some amount of surface shrinking was also apparent in case of metal loaded. The EDX spectra of the root surfaces of the control (**Figure 5.6E**) and Cd-loaded root (**Figure 5.6F**) surfaces are shown.





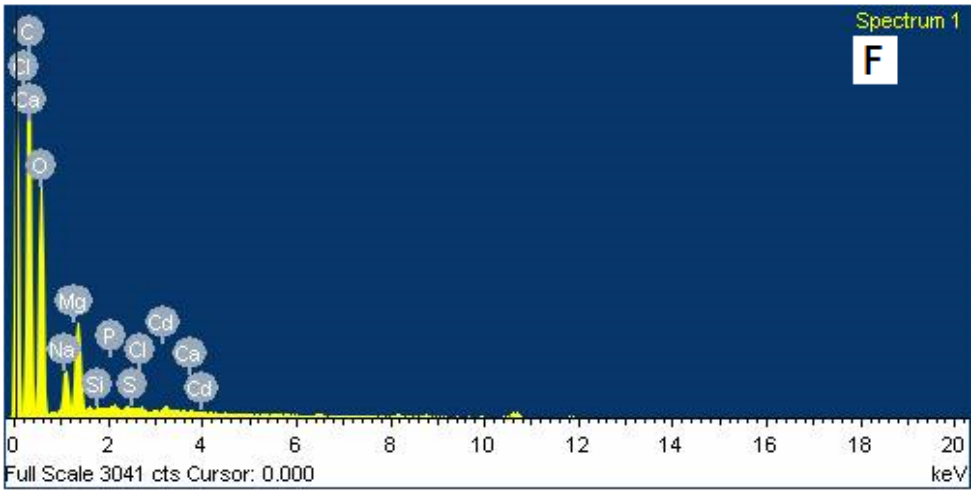


Figure 5.6(A, B) SEM micrographs Control root; (C, D) Cd treated *M. hastata* root, showing some surface shrinking after metal binding; (E) Energy-dispersive X- ray spectra of control root; (F) EDX spectra of treated root confirmed the presence of Cd

5.3.5 Transmission electron microscopic (TEM) studies

The ultrastructural investigation of the root and leaves cells of *M. hastata* exposed to three different concentrations of Cd (5 mg/L, 10 mg/L and 15 mg/L) for 10 days was carried out.

In a section of the leaf of the control plant, multiple chloroplasts were observed within the leaf cells, however, did not exhibit any abnormalities on the ultrastructural level of their organization (**Figure 5.7A**). TEM images of the control plant chloroplast showed that grana of thylakoids and their connecting lamellae are clearly visible (**Figure 5.7A**). Under the normal condition (**Figure 5.7A**), chloroplasts were closely distributed around the plasma membrane of *M. hastata*, and their structures were integral and in ellipse with a typical arrangement of grana and stroma thylakoids. The stroma of the thylakoids of chloroplast was also well-developed, and the lamellar structure of thylakoids in the chloroplast was clear and integral (**Figure 5.7B**)

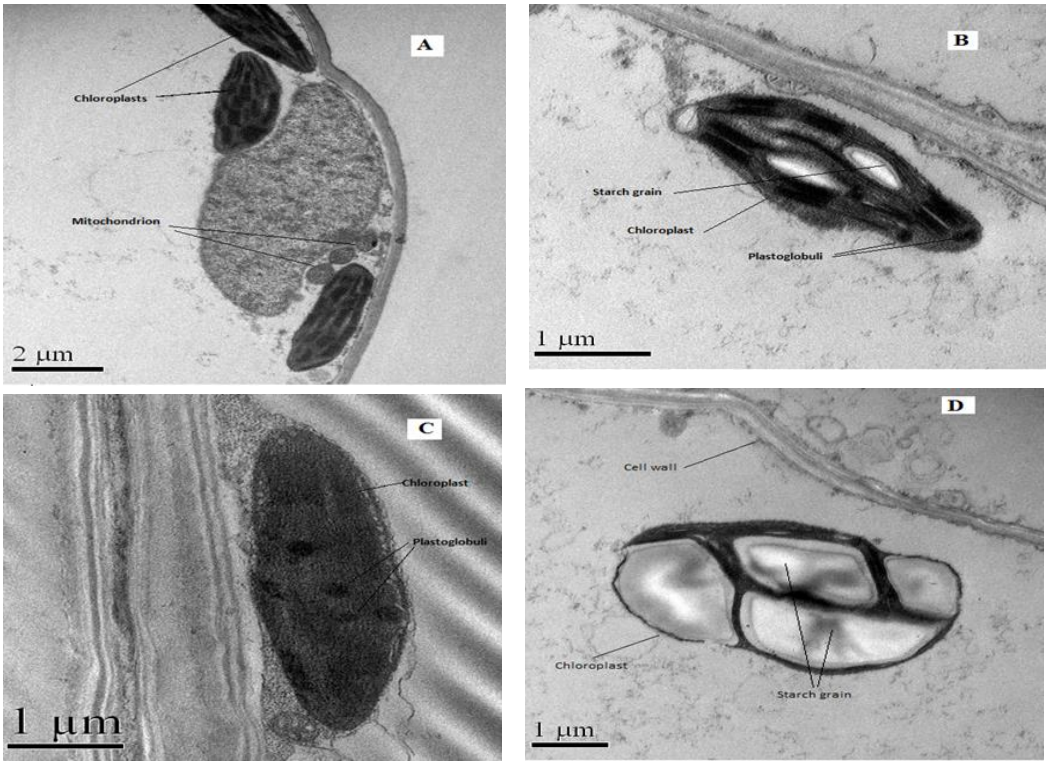


Figure 5.7 TEM Micrograph of chloroplast in the leaf cells of *M.hastata* (A) Control leaf showing numerous chloroplasts; (B) showing one chloroplast; (C) Cd treated with 15 mg/L for 10 days; (D) to show the effect of Cd on thylakoid inside the chloroplast

In the plant cell, the whole process of photosynthesis is completed in the chloroplasts [114]. Therefore, the normal performing of plant photosynthesis depends on the integrity of chloroplast ultrastructure. The changes in the shapes of the chloroplasts are evident due to Cd stress after the treatment of *M. hastata* with 15 mg/L Cd solution. The chloroplasts were not spherical, and the thylakoid array structure became smaller and was damaged heavily (**Figure 5.7C**). The thylakoid arrays disassembled and chloroplasts were deformed and swollen (**Figure 5.7C**). One or two grains of starch were also evident within the chloroplasts (**Figure 5.7D).** The swollen shape of chloroplast might be due to an increase in the stroma volume, as illustrated in (**Figure 5.7D**).

Cd can injure the photosynthetic system by replacing Mg in the chlorophyll structure [115], by inhibiting photoactivation of photosystem II [116]. Moreover, it can also affect the chlorophyll biosynthesis [117] the organization and assembly of light-harvesting complexes [118, 119, 120, 121].

TEM results revealed that Cd was also accumulated in the mitochondria. The mitochondria are important targets for toxic effects of heavy metals. In the control

plant, multiple mitochondria within the root cells were intact, most cristae in the mitochondrion double deck membranes were evenly distributed except for small portion (**Figure 5.8A**). The mitochondria were oval with well-developed cristae (**Figure 5.8A**). However, *M. hastata*, treated with Cd solution, separation appeared between the cytoplasm and the cell wall. The most of the cristae were disappearing from mitochondria resulting in reduction in number, except for the small portions that were kept intact (**Figure 5.8B)**. TEM Micrograph also showed electron dense deposition within the mitochondria of the root mesophyll cell (**Figure 5.8C)**.

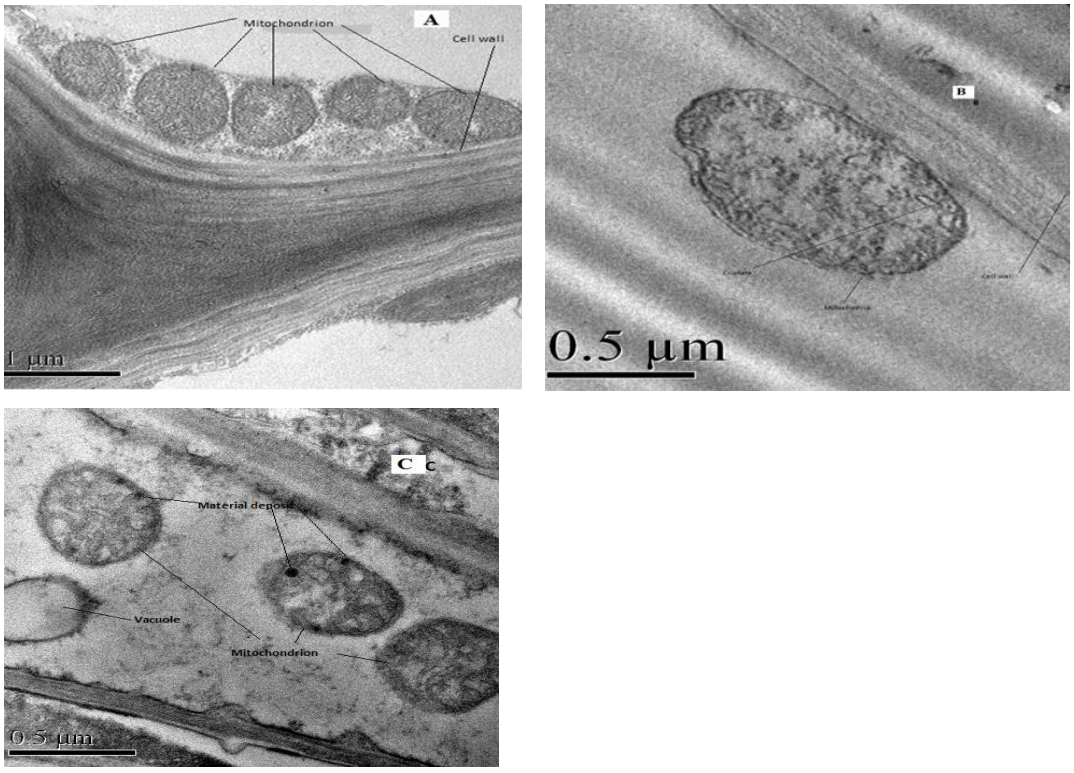


Figure 5.8 TEM Micrograph of the root mesophyll cell mitochondrion (A) control *M. hastata* plant; (B) micrograph of Cd treated mitochondria; (C) showing electron dense deposition within the mitochondria

The structure of nucleus and of nucleolus was found intact in both control (**Figure 5.9A**) and Cd treated (15 mg/L) root cells of *M. hastata* which is evident from (**Figure 5.9B**). However, compared to nucleus, the mitochondria are more easily destroyed by excessive use of Cd in aqueous solution.

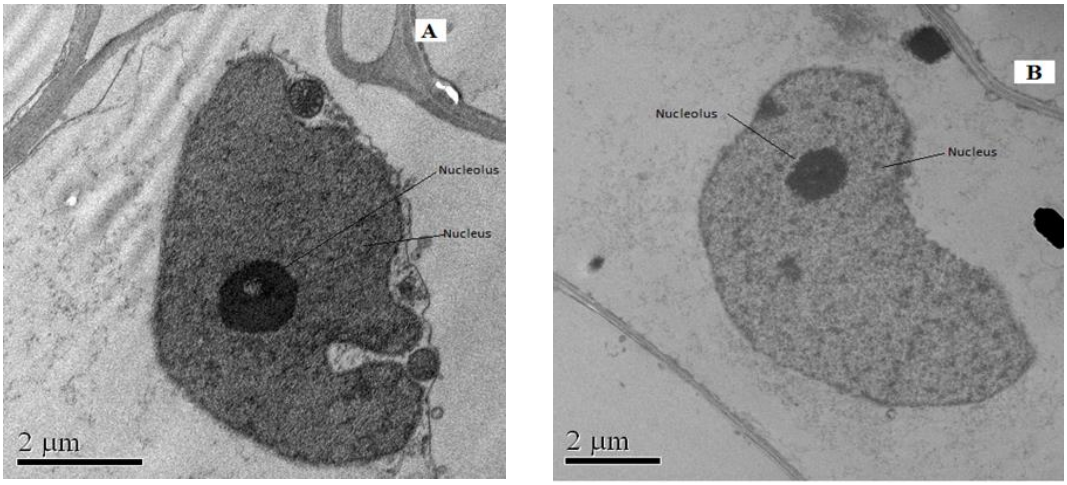


Figure 5.9 TEM of *M. hastata* root mesophyll cells nucleus (A) control; (B) 15/L Cd treated

In the present study the root cortical cells of *M. hastata* exposed to 15 mg/L of Cd concentrations exhibited various ultrastructural changes when compared with control root cells (**Figure 5.10A**). In roots of *M. hastata* treated with Cd solution, a large amount of electron dense materials were observed between the cell wall and plasmalemma (**Figure 5.10B**). TEM observations of the sulfide fixed root sections showed significant amounts of black electron dense depositions along the cell walls of Cd-treated *M. hastata* (**Figure 5.10B**). These electron dense granules containing Cd are observed in the cell walls where they are enclosed by plasmalemma. Damaged membrane systems and serious plasmolysis with separations of the plasma membrane from the cell wall were noted in most root cells of the *M hastata* tissues exposed to 15 mg/L of Cd. The cell wall of *M. hastata* can effectively biosorbed Cd which is considered as one of the protection mechanism to alleviate the toxic effect and protect the damaged induced by the metals. Accumulation of Cd at the cell wall is one way of reducing the levels of toxic metal in the cytosol and a potentially important mechanism for heavy metal detoxification and tolerance [122, 123, 124,125, 126]. According to Neumann et al. [127], cell wall play a role in metal tolerance when the cell wall volume is high compared to the cytosol and vacuole.

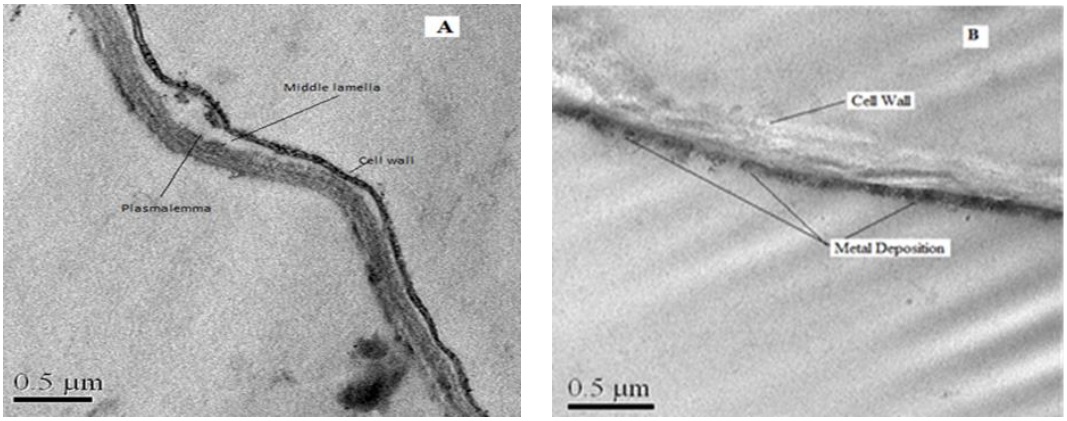


Figure 5.10 TEM micrograph of *M. hastata* root cortical cell (A) control cell showing no metal deposition along with the cell wall; (B) 15 mg/L of Cd treated showing Cd^{2+} deposition along the surface and in the cell wall

In the vacuoles of root cells of *M. hastata*, several small vacuoles were found in mature root cells of *M. hastata* control plant (**Figure 5.11A**). However, plant treated with Cd, electron dense granules were aggregated and formed into larger precipitates with circular or amorphic shape, which were encircled by the membrane (**Figure 5.11B**). Elemental distribution maps showed that Cd was predominantly localized in the vacuoles of root cells. However, due to significant metal accumulation in the Cd-treated plants, the cross sections of roots showed precipitation and an increase in the number of vacuoles in TEM micrographs of roots (**Figure 5.11B**). The involvement of vacuole in sequestration of Cd by many plant species has been a subject of study in many researchers and several families of transporters participate in this process [128]. Upon entering into the cytosol, Cd binds with PCs and are transported from there in the form of metal-phytochelatin complexes to vacuole as their final target [62, 129, 130, 131].

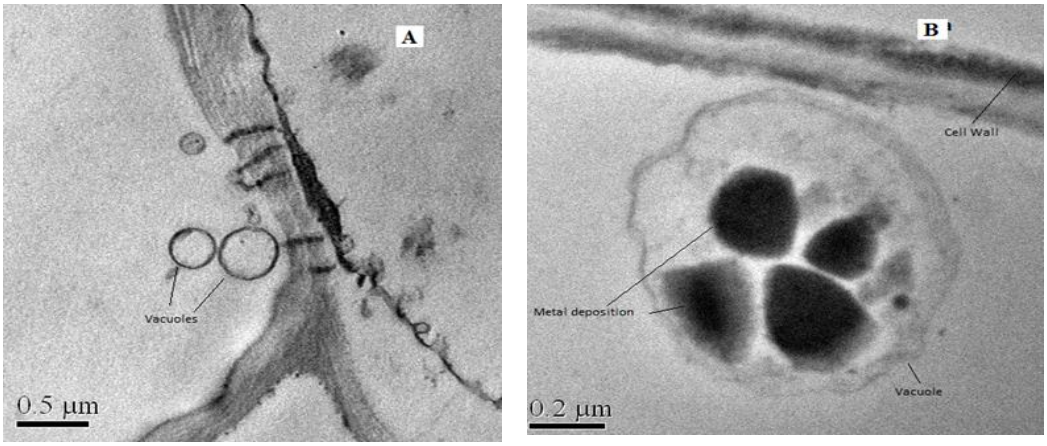


Figure 5.11 TEM micrograph of vacuoles of root cells (A) control showing no deposition within the vacuole; (B) Metal accumulation within the vacuole

5.3.6 Fourier Transformed Infrared Analysis (FTIR)

The FTIR technique was used to identify the different functional groups of *M. hastata* is involved in binding the Cd ions. It is seen that there is a significant shift of the absorption peaks of functional biomass groups of plants after adsorption of Cd ions from the aqueous solution. The shifts in the absorption peaks generally indicate the existence of Cd ions binding process taking place in the plant biomass. FTIR indicates that carboxylic, alcohol/ phenolic, amide, amino and thiol groups are responsible for the binding of the metal ions.

FTIR is a potential technique to elucidate the different functional group responsible for binding the metal ions during the biosorption experiments. It is one of the most widely used techniques (method) to identify the chemical constituents and functional groups responsible for binding the metal ions. Results of FT-IR spectroscopic studies have revealed the existence of various chemical constituents in leaves, roots and shoots of *M. hastata*. The absorption bands and the corresponding wave number (cm^{-1}) of dominant peaks obtained for absorption spectra were defined in **Figure 5.12- Figure 5.17**

The absorption band at 3431.65 cm^{-1}(control leaf) assigned to the O-H stretching frequency, which shifted to 3421.78 cm^{-1}(Cd ion loaded). The characteristics peaks appeared at 3431.65 cm^{-1} in control leaf (CL), 3419.42 cm^{-1} in control shoot (CS) and 3412.73 cm^{-1} in control root (CR) may be due to bonded –OH stretching vibration shifted to 3421.78 cm^{-1}in treated leaf (TL), 3430.32 cm^{-1}treated shoot (TS) and 3436.73cm^{-1} treated root (TR) respectively due to complexation of Cd ion.

The medium strong peaks of O-H of carboxylic acid at 2921.81(CL), 2921.62 cm^{-1}(CR), 2921.51 cm^{-1}(CS) were also shifted to 2925.61 cm^{-1} (TL), 2926.74 cm^{-1}(TR) and 2923.78 cm^{-1}(TS) after coordination of Cd^{2+} ions with carboxylic acid group. The C-H asymmetrical stretching methylene group appears at 2921cm^{-1} (CL), 2921.51 cm^{-1} (CS) and 2921.61 cm^{-1} (CR) have been shifted to 2925.61cm^{-1}(TL), 2923.78 cm^{-1}(TS), and 2926.74cm^{-1} (TR) respectively.

Very strong absorption bands observed at 1640.36 cm^{-1}(CL), 1645.90 cm^{-1}(CS), 1638.05 cm^{-1} (CR) may be due to the presence of bonded C=O stretching frequencies of amide, have been shifted to 1642.15 cm^{-1} (TL), 1627.21 cm^{-1} (TS) and 1633.72 cm^{-1} (TR) . This is in accordance with earlier observations [14]. A

weak absorption bands appeared at 1384.45 cm^{-1} in (CL), 1384.61 cm^{-1}(CS) and 1382.91 cm^{-1}(CR) is due to C-H asymmetrical/symmetrical stretching of methyl/methylated group have also been shifted to 1384.97cm^{-1}(TL), 1318.79 cm^{-1}(TS) and 1384.28 cm^{-1}(TR) could be due to bindings of Cd ion. Again the band at 1384.45 cm^{-1}(control leaf) has been assigned to CH_3 symmetric stretching shifted to 1384.97 cm^{-1} (TL) after complexation with Cd ions.

The C-N stretching frequencies appeared at 1061.58 cm^{-1} (CL), 1063.04 cm^{-1} (CS) and 1033.44 cm^{-1} (CR) which shifted to1060.01 cm^{-1}(TL), 1067.63 cm^{-1} (TS) and 1032.27 cm^{-1}(TR) could be due to loading of Cd ions. A change in peak position in the spectrum of the Cd loaded biomass indicates the involvement of amide group in the biosorption process. The band observed at 781.94 cm^{-1} (CL) which could be assigned due to (C=O), is not practically shifted in treated leaf (781.81cm^{-1}) when plant was exposed to Cd ions. In addition, the peak at 781.88 cm^{-1} (CS) assigned to the (S-S) shifted to781.58 cm^{-1} due to involvement of thiol group in binding with Cd ions during bio absorption process. Another shift of the peak at 621.19 cm^{-1} (CL) to 625.19 cm^{-1} (TL) also suggest the involvement of CH_2- group in binding with Cd ions.

The next absorption peak at 520.01 cm^{-1} (control leaf) may be due to the presence of CH_3-CH_2 asymmetric/symmetric group, were shifted to somewhat lower frequencies and appeared at 518.41 cm^{-1}(with Cd loaded) could be due to complexation. The above change in the spectra may be attributed to the interaction of Cd ions with the plant biomass. A shift of band position at 469.86 cm^{-1} to 477.23 cm^{-1} (TR) (with Cd loaded) metal exposed to *M. hastata* possibly for Thiol. In addition, the peak at assigned to the S-H stretching vibration, 471.95cm^{-1}(CS) and 469.86cm^{-1} (CR) have been shifted to 424.44 cm^{-1} (TS) and 477.23cm^{-1}(TR) respectively after complexation with Cd ions.

Table 5.3 Difference between adsorption bands (cm^{-1}) of *M.hastata* leaf, shoot and root before and after adsorption of Cd^{2+} ion on it

Leaf

IR Absorption bands (cm^{-1}) before adsorption	IR Absorption bands (cm^{-1}) after adsorption	Differences	Assignment
3431.65	3421.78	9.87	Bonded O-H stretching or N-H stretching vibration
2921.81	2925.61	- 3.8	Methyl C-H asymmetry/symmetry stretching
1640.36	1642.15	- 1.79	C=O stretch
1384.45	1384.97	- 0.52	CH3 symmetric stretching
1061.58	1060.01	1.57	Interaction of nitrogen from amino group
621.19	625.19	- 4	Thiol and Sulfhydral groups
520.01	518.41	1.6	Thiol and Sulfhydral groups

Shoot

IR Absorption bands (cm^{-1}) before adsorption	IR Absorption bands (cm^{-1}) after adsorption	Differences	Assignment
3419.42	3430.32	-10.9	Bonded O-H stretching or N-H stretching vibration
2921.51	2923.78	-2.27	Methyl C-H asymmetry/symmetry stretching
1634.14	1627.21	6.93	C=O stretch
1415.97	1415.78	0.19	C-H asymmetry
1247.99	1251.40	-03.41	Aromatic C-H band
1065.04	1067.65	- 02.61	Interaction of nitrogen from amino group
781.88	781.58	0.3	Thiol and Sulfhydral groups
619.71	624.53	- 04.82	Thiol and Sulfhydral groups
544.72	562.22	- 17.5	Thiol and Sulfhydral groups

Root

IR Absorption bands (cm^{-1}) before adsorption	IR Absorption bands (cm^{-1}) after adsorption	Differences	Assignment
3412.73	3436.73	- 24	Bonded O-H stretching or N-H stretching vibration
2921.62	2926.74	- 5.12	Methyl C-H asymmetry/symmetry stretching
1638.05	1633.72	4.33	C=O stretch
1453.33	1462.22	- 8.89	C-H asymmetry
1382.91	1384.28	- 1.37	CH_3 symmetric stretching
1243.45	1248.88	- 5.43	Aromatic C-H band
1033.44	1032.27	1.17	Interaction of nitrogen from amino group
779.15	777.77	1.38	Thiol and Sulfhydral groups
694.58	693.33	1.25	Thiol and Sulfhydral groups
469.86	477.23	- 7.37	Thiol and Sulfhydral groups

FTIR analysis confirms the presence of hydroxyl, amide, thiol, sulfahydral and amino groups present in *T. natans* biomass that might interact with arsenic during the absorption process [14].

The infrared spectra of protein are characterised by a set of absorption regions of known structures. Studies in the amide region are amide I and amide II. The amide I band are principally from the C=O stretching vibration of the peptide group. The amide II band is mainly N-H bonding with coordination from C-N stretching vibrations.

A characteristic broad features in the range of 3300-2500 cm^{-1} that overlaps the C-H stretching region and with a secondary absorption close to 2600 cm^{-1} is observed for a hydrogen bonded O-H of most carboxylic acids. Other bonds that are associated with the C-O and O-H components tend to be less pronounced and stretching may be overlapped with other finger print absorptions of the molecules. These are located in the range of 1320-1210 cm^{-1} (C-O stretch) and 960-850 cm^{-1} (hydrogen bonded with O-H out-of-plane bonding).

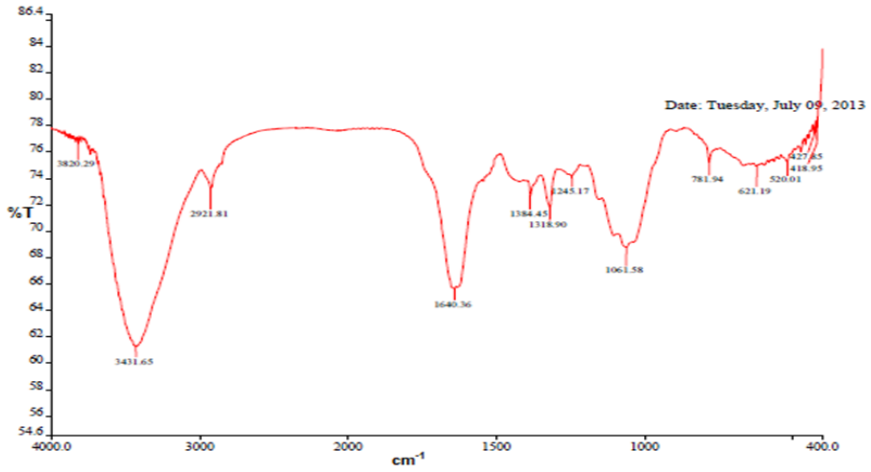


Figure 5.12 FTIR spectra of control leaf biomass of *M. hastata*

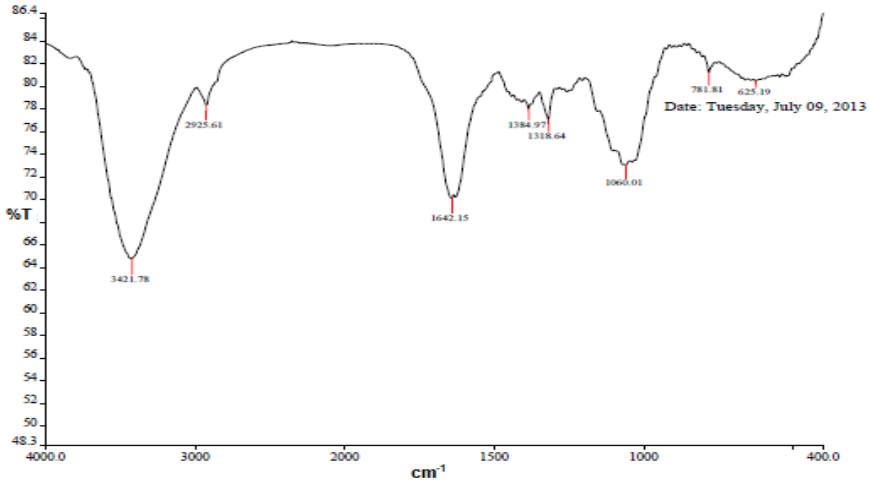


Figure 5.13 FTIR spectra of Cd treated leaf biomass of *M. hastata*

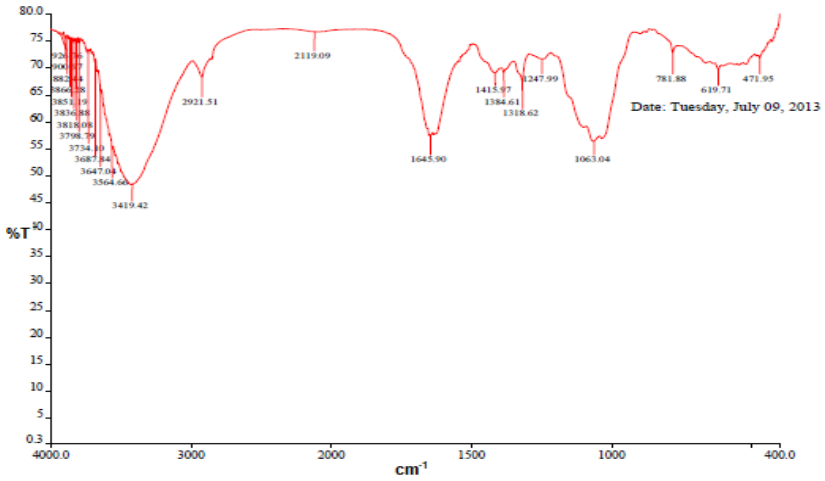


Figure 5.14 FTIR sprectra of control shoot biomass of *M. hastata*

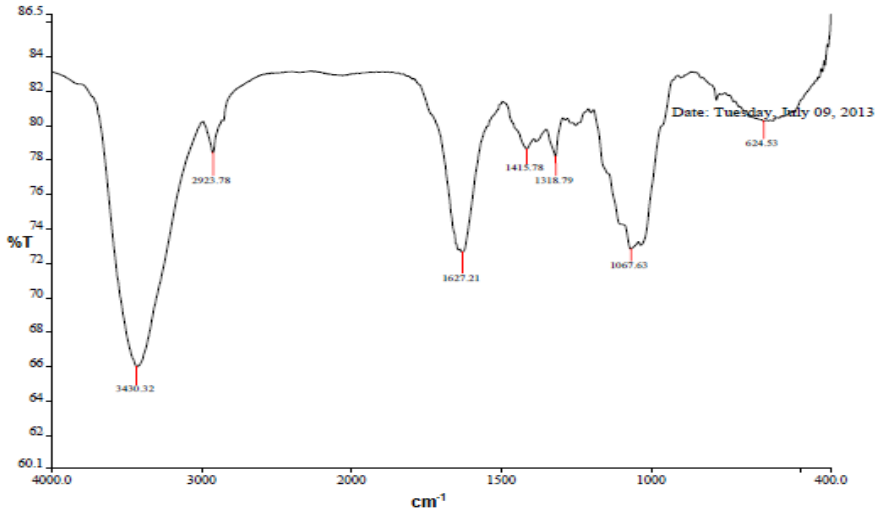


Figure 5.15 FTIR sprectra of Cd treated shoot of *M. hastata*

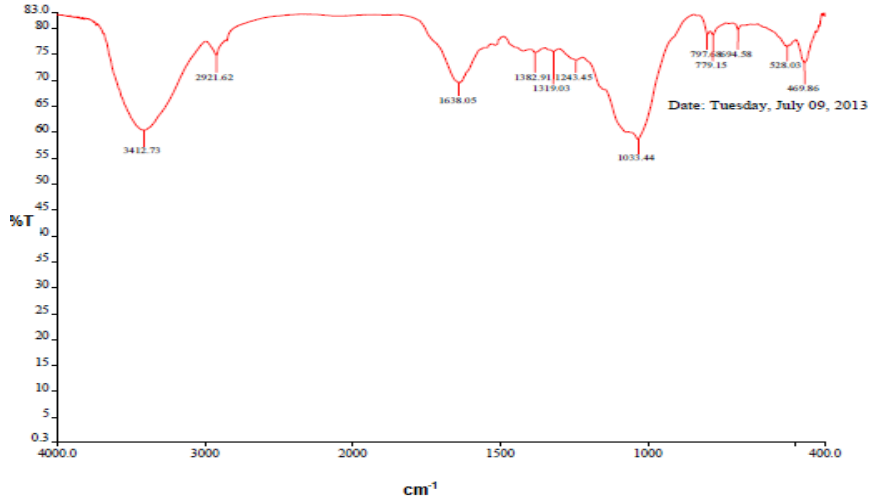


Figure 5.16 FTIR sprectra of control root biomass of *M. hastata*

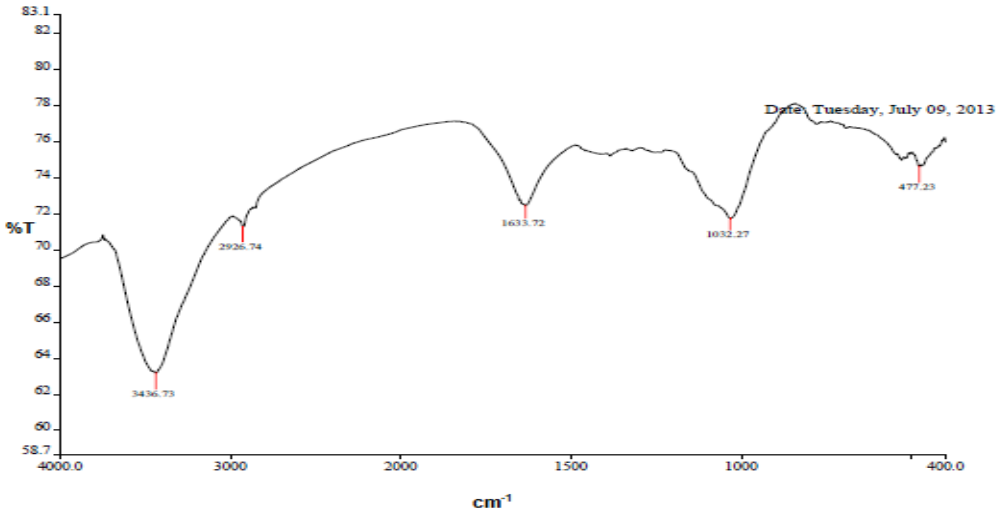


Figure 5.17 FTIR spectra of Cd treated root biomass of *M. hastata*

5.4 Discussion

5.4.1 Visual symptoms

Chlorosis of leaves is one of the most prevalent morphological changes and one of the first observable symptoms of heavy metal toxicity which is a result of decreased rate of chlorophyll biosynthesis and content. Evidence of chlorosis, leaf roll, leaf yellowing and failing of leaves were the main easily visible symptoms of Cd toxicity in *M. hastata* after 10 days of metal application, especially at 15mg/L Cd level. Leaf chlorosis is one of the visual sign of trouble due to exposition of plant to high concentration of Cd which is associated with the Fe deficiency [132, 133, 92]. Inhibition of Fe uptake has been reported in a number of plant species if the concentration of Cd in the medium is more [134]. Similarly, Carrier et al. [121] asserted that Cd-induced Fe deficiency in the shoots of plant, may be the cause for the development of chlorosis [135, 136]. However, there is disagreeing reports refuting such assertion and according to Carrier et al. [121] chlorosis is not attributable to mineral deficiency in *Brassica*. On the other hand Root et al. [137] and Chaffei et al. [138] recommended that chlorosis induced by Cd may be due to alterations in the Fe/Zn ratio, rather than to Fe deficiency, since plants treated with Cd showed a greater concentration of this micronutrient.

5.4.2 Total chlorophyll content

Decrease in chlorophyll content is one of most common responses to Cd stress in plants. Chlorophyll content reduction associated with application of excess of Cd to the *M. hastata* was observed in the present study. The damage to photosynthesis occurs mainly from an impaired efficiency of the Rubisco activity, a decrease in chlorophyll and an increase in lipid peroxidation within these organelles [44]. Cadmium inhibited net photosynthesis in green algae, corn, soybean, and pigeon pea [139, 140, 141]. In *Brassica napus* the effect of cadmium on chlorophyll content showed that the presence of Cd decreased the content of chlorophyll and increased non-photochemical quenching [142]. Inhibition of photosynthesis due to both long-term and short-term Cd exposure was demonstrated by a large number of studies in many species, such as oilseed rape (*Brassica napus).* [143] sunflower (*Helianthus annuus*) [144] *Thlaspi caerulescens* [145] maize, pea, barley [146] mungbean

(*Vigna radiate*) [147]. The primary sites of action of Cd are photosynthetic pigments, especially the biosynthesis of chlorophyll [148] and carotenoids [149].

5.4.3 Metal concentration in Morphological tissues

Analysis of Cd concentrations in plant organs showed that Cd concentrations in root were significantly higher than those in shoot. The bioaccumulation factors (shoot/soil concentration ratio) are more important than shoot concentrations, when considering the potential of phytoremediation of a given species [23]. Cd has no biological function for plants, although some plants accumulate high amounts of the metal in their tissues. In case of Cd, level 100 mg kg^{-1} (0.01%) of Cd in the dry leaf tissue was chosen as being extraordinarily high accumulation and is used as a threshold value for the metal [21, 150]. This concentration was chosen as being exceptionally higher than that normally found in plants.Once Cd enters the roots through the cortical tissue of a plant it follow the symplastic or apoplastic pathways before entering the xylem which is further translocated to the shoot and leaf [151, 152, 153]. In ICP-OES analysis illustrated that, the lesser translocation of Cd from roots to leaf occurred (**Figure 5.3**) and the distribution of Cd in the plant decreases in the order root > stem > leaf. This suggests that Cd is not readily translocated to the above ground part. The foliar concentration in the present investigation is slightly less than threshold values of hyperaccumulation of Cd 100 μg/g dry. Again according to Zayed et al. [79] BCFs less than 300 represent a poor accumulator of heavy metals, whereas BCF values between 300 and 800 indicate moderate accumulation and BCFs greater than 800 represent a good accumulator. Therefore, *M. hastata* is not a Cd hyperaccumulator but a moderate accumulator according to this classification. The ability of *M. hastata* to take up high concentrations of Cd from solution indicates that this plant species has the ability to remove Cd effectively. Therefore, *M. hastata* may potentially be useful for removal of Cd from the Cd polluted wastewater. Metal accumulation potential and BCF can be varying among different groups of aquatic macrophytes and some aquatic plant species have been shown to exhibit higher accumulation of Cd. For example, Nakada et al. [154] found high BCF values for Cd (1700) in *Elodea nuttallii* and Sela et al. [155] reported a very high BCF values for Cd (24,000) in the roots of *Azolla filiculoides*. Other studies reported CFs in roots of 600 and 1600, respectively [156, 157]. However, in comparison, some other aquatic plant species were proven to have lower accumulation of Cd, and very low BCF values were observed. For examples,

Miller et al. [158] reported that the BCF of 2.7 for Cd in soft-water pipe wort and while Brix et al. [159] found that the BCF of 6 for Cd accumulation in *Zosterna marina* grown in contaminated sites. Some earlier reports also support the trend of higher accumulation of Cd in roots than in above ground parts [151]. But the extent of Cd transport into above ground parts of plant differs widely with the plant species.According to Ramos et al. [160] in spite of the different mobility of metal in plants, the metal content is generally greater in roots than in the above ground tissue. Generally, accumulation beyond threshold value in the above ground parts of plant are quite unusual, and represent a distinct form of plant response, implying some characteristic.In a study on soybean it was found that more than 98% of the accumulated Cd was retained by roots and only 2% was transported to shoots [161]. However, *T. caerulescens* is capable of accumulating high levels of Cd in shoots from soil or hydroponic solution [107, 108]. The low concentration of metals in the shoot of aquatic macrophytes may be due to the slow mobility of metal transport from root to shoot and also the formation of complex compounds with COOH groups that may inhibit the translocation of metal to shoot [162].

Tissue concentration has been used as a criterion for identifying hyperaccumulators; however, the translocation factor may give a better idea regarding the metal accumulation capacity of the plants [20]. Plants with a higher translocation factor can be considered as a better candidate for phytoremediation.

TF values are less than one is categorized as tolerant plants according to Baker et al. [38]. Tolerant plants have adopted an exclusion strategy to prevent metal translocation from the root to above-ground tissues. Formation of stable metal complexes in the root cells is thought to be the mechanism of prevention of metal translocation from the root to shoot in this exclusion strategy. For instance, Quartacci et al. [163] observed that in some plants the first step of defense against Cd toxicity is preferential exclusion of Cd from active tissues and organs. They showed that 98% of total Cd was retained in the roots of *Phaseolus vulgaris* and only 2% was translocated to the shoot; presumably, much of the Cd in the root was in the apoplast or the vacuoles. Chandra et al. [162] suggested that the low concentration of metals in the shoot of aquatic macrophytes may be due to the slow mobility of metal transport from root to shoot and also the formation of complex compounds with COOH groups that may inhibit the translocation of metal to shoot.

The concentration of Cd in the roots being higher than in the aerial part but some amount of Cd was also found in leaves and stems, demonstrating that this metallic element was not totally immobilized in the plant. Some plants are potential to absorb Cd from environment but the amount absorb depends on both the activity of the root and its interaction with the surrounding solution.

5.4.4 Scanning Electron Microscopic studies (SEM)

The analysis by SEM of the abaxial side of the leaves showed stomatal closure in plants treated with 15 mg/L of Cd while in control most stomatal were open. The decrease in stomatal aperture in the present study may be due to rapid and preferential absorption of Cd by subsidiary cells followed by changes in membrane permeability causing decrease in cell turgor as reported in Cd treatment *Phyllanthus amarus* [90]. Indeed, deformation of stomata and decrease of its diameter in Cd exposure caused to reduce photosynthesis. Size of stomata ostiole was also reduced by Cd concentrations. Bondada et al. [164] reported that closed stomata of the leaf results in a slower rate of diffusion due to greater diffusion gradient of water vapour. The decrease in size of stomatal aperture in the leaves is in the line with the hypothesis that metals induce water stress [113]. Stomatal opening, transpiration and photosynthesis have been reported to be affected by Cd in nutrient solution [133]. The reason of decreasing the stomata opening seem is increased cell wall thickness and reduction of turgor pressure. A linear relationship between photosynthesis and inhibition of transpiration was observed in clover, lucerne, and soybean that suggest Cd inhibited stomatal opening [165]. Cadmium also causes stomatal closure in higher plants [166] and an overall inhibition of photosynthesis [140, 141, 167].

In response to Cd-induced stress, plants have previously been shown to induce water loss prevention mechanisms [120] that include stomatal closure [54] and a reduction in stomatal length [168]. Stomatal closure is caused by a decrease in guard cell turgor induced by ABA and the efflux of K^+ and associated anions, such as Cl^- and/or malate, which are triggered by an increase in cytoplasmic Ca^{2+} concentration [169]. However, the stomatal closure could be caused by a direct interaction of toxic Cd on guard cells. The decrease in stomatal aperture may be due to rapid and preferential absorption of metals by subsidiary cells followed by

changes in membrane permeability causing decrease in cell turgor as reported in Cd treatment *Phyllanthus amarus* [90, 121, 123, 170] inhibition of membrane proteins like H-ATPase [124] and changes in lipid composition linked to peroxidation of unsaturated fatty acids induced by free radicals [125,126, 163] are some of the mechanisms probably involved.

The SEM micrographs showed changes of the vascular cells of the shoot samples. Exposure to 15 mg/L $CdCl_2$ resulted in a cell shape, decreases in the intercellular spaces and shrinkage of the vascular bundles in *M. hastata*

The SEM micrographs showed changes of the vascular cells of the leaf samples. Exposure to 15 mg/L $CdCl_2$ resulted in a change in cell shape, decreases in the intercellular spaces and shrinkage of the vascular bundles in *M. hastata.*

5.4.5 Transmission Electron Microscopic studies (TEM)

Chloroplasts from *M. hastata* plants grown at 15mg/L $CdCl_2$ exhibited alteration in the organelles as well as disorganization of thylakoids and stroma when compared to those of control plants, which exhibited chloroplasts with an ellipsoidal form and a typical arrangement of the granum and stroma. In the plant cell, the whole process of photosynthesis is completed in the chloroplasts [114]. Therefore, the normal performing of plant photosynthesis depends on the integrity of chloroplast ultrastructure. The changes in the shapes of the chloroplasts are evident due to Cd stress after the treatment of *M. hastata* with Cd solution. Our experimental results of 15 mg/L Cd solution demonstrated that after *M. hastata* treated with Cd, chloroplasts were swollen and not in spherical shape. The membrane of chloroplast was damaged (**Figure 5.7C**). A large number of reports has shown that Cd inhibit photosynthesis in green algae, corn, soya been and pigeon [139, 140, 141]. An increase in the stroma volume and starch accumulation due to Cd stress has already been reported [171]. The swollen shape of chloroplast might be due to increase in the stroma volume, as illustrated in figure (**Figure 5.7D**). Moreover, an increase level of starch accumulation within the chloroplast was exhibited. Literature survey showed that Cd is an effective inhibitor of photosynthesis [172, 167, 173] and damages the photosynthetic apparatus, in particular the light harvesting complex II [119] and photosystems I and II [174]. Alkantara et al. [175] investigated the

inhibition of root Fe(III) reductase induced by Cd leads to Fe(II) deficiency which seriously affects photosynthesis.

The mitochondrial inner membrane (IM) serves as the site for ATP production by hosting the oxidative phosphorylation complex machinery most notably on the crista membranes. Therefore, reduction in surface area may hampers not only production of ATP but also many other chemical reaction. Data from transmission electron spectroscopy (TEM) revealed that Cd was accumulated in the electron-dense precipitates in the mitochondrion membranes of root cells treated with 15 mg/L Cd without apparent changes in their local environment (**Figure 5.8C**). Reduction in the number of cristae in the mitochondria is one of most common features to Cd stress in plants [176]. Disruption of the crista structure has been implicated and an accumulation of electron dense materials were observed in membranes of mitochondria (**Figure 5.8C**). Cd inhibits the photoactivation of photosystem 2 (PS2) and may stimulate the generation of ROS in the mitochondrial electron transfer chain [45]. In *Allium cepa* Cd treatment induces abnormalities, such as extensive vacuolization, damage to mitochondrial cristae [176].

In the present study the compartmentalization and sequestration of Cd into the cell wall of *M. hastata* is one of the molecular mechanism where cell wall is involved for detoxification and tolerance and thereby reduces the solution concentration of free metal ions [177]. This involves the chelation of metal ions by specific ligands with high-affinity for binding. The cell wall acts as a cation exchanger, holding variable quantities of metal and providing for some metal exclusion [178]. Earlier studies showed that the plasma membrane is the site for the accumulation of a number of heavy metals including Cd [179].

The subcellular localization studies showed that at the cellular level, cell wall can effectively biosorbed Cd and act as a suitable storage reservoir for excessively accumulated metals [180, 53]. But it is likely that metal tolerance could rely on the ability of a plant to store accumulated excess metals in organs or subcellular compartments where no sensitive metabolic activities take place [181]. The incorporation or accumulation of Cd in the cell wall depends on how efficiently cell wall can bind to potentially toxic Cd and transported through the apoplastic route thereby reducing the cytoplasmic Cd. In this regards it is established that the plant cell walls primarily composed of polypose, lignin and protein, providing carboxyl,

hydroxyl, amino and aldehyde groups. Therefore, they can bind Cd ions and thereby inhibiting its diffusion into cell [182].

TEM study revealed that Cd was localized as electron-dense precipitates in the vacuoles of root cells of *M. hastata* treated with Cd. Storage and localization of Cd as electron-dense precipitates in the vacuoles of root cells of *M. hastata* treated with Cd may play an important role for Cd detoxification by maintaining low levels of free Cd in cytosol [183, 184]. In fact, plants have a range of intracellular ligands potentially involved in metal accumulation and detoxification. Phytochelatins (PCs) and metallothioneins are two major sulfur-containing classes of metal chelating ligands that have been identified in plants and these may play a significant role in metal tolerance [63, 178, 185, 186, 187].

In fact subcellular localization studies revealed that in vacuole, cadmium is released from the Cd-phytochelatins complexes, which is then returned to the cytoplasm and the metal may become bound to an organic acid in the vacuole [127, 188]. Haydon and Cobbett [189] reported that organic molecules mainly malate and citrate, are also quite effective as metal binding ligands within the acidic vacuolar environment. Evidence for plant vacuoles as the site of Cd sequestration appears to be quite conclusive and prevents the free circulation of Cd ions in the cytosol [133]. Therefore vascular compartmentalization of Cd in the present study may be a part of this detoxification mechanism which involves the chelation of the metal ions by specific ligands protecting the cytosol from free Cd ions and the toxic metals are sequestered away from sites or metabolism in the cytoplasm, especially into the vacuole [21, 190, 178, 191].

5.4.6 Fourier Transform Infrared (FTIR) Analysis

In the present study binding of Cd ions with functional groups has been confirmed from shift of characteristic FTIR bands position from its actual position of individual functional groups present in the biomass *M. hastata*. The presence of characteristic functional groups of *M. hastata* biomass such as carboxylic acids, hydroxyl group, amine, amide, sulphur derivatives, polysacchakarides, carbohydrates which are responsible for binding with Cd ions was confirmed by FTIR technique. The negatively charged groups such as carboxyl, hydroxyl, amino and aldehyde groups on the cell wall bind Cd ions and thereby inhibiting its diffusion into cell might does

make plant more tolerant to the effect of Cd restrict their transportation across cytomembrane.

5.5 Conclusion

The shoot/root ratios of Cd is less than one and it has quite considerable extent of BCF value suggesting that *M. hastata* is moderate accumulator. Uptake and accumulation of heavy metals by the plant, not only cause structural and ultrastructural changes, but also result in toxicity symptoms and affect metabolic process. TEM microanalysis showed that Cd is accumulated in the vacuoles and cell wall and thereby prevent it entering plant cells.

The FTIR technique was used to identify the different functional groups of *M. hastata* involved in binding the cadmium ions. Analysis of Cd concentrations in plant organs showed that Cd concentrations in roots were higher than in the aerial part. Therefore, plant growing in water bodies polluted with Cd should be strictly avoided using rhizome and above ground parts as vegetables and animal feeds.

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