Journal of Hazardous Materials 186 (2011) 565–574

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Insights into cadmium induced physiological and ultra-structural disorders in *Juncus effusus* L. and its remediation through exogenous citric acid

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ARTICLE INFO

Article history:
Received 20 February 2010
Received in revised form 9 November 2010
Accepted 9 November 2010
Available online 18 November 2010

Keywords:
Plant growth
Cd accumulation
Antioxidants
Ultra-structural modification
Chloroplast

ABSTRACT

This study appraised cadmium (Cd) toxicity stress in wetland plant *Juncus effusus*, and explored its potential for Cd phytoextraction through chelators (citric acid and EDTA). Cadmium altered morphological and physiological attributes of *J. effusus* as reflected by growth retardation. Citric acid in the presence of 100 μM Cd significantly countered Cd toxicity by improving plant growth. Elevated Cd concentrations reduced translocation factor that was increased under application of both chelators. Citric acid enhanced Cd accumulation, while EDTA reduced its uptake. Cadmium induced oxidative stress modified the antioxidative enzyme activity. Both levels of citric acid (2.5 and 5.0 mM) and lower EDTA concentration (2.5 mM) helped plants to overcome oxidative stress by enhancing their antioxidative enzyme activities. Cadmium damaged the root cells through cytoplasmic shrinkage and metal deposition. Citric acid restored structure and shape of root cells and eliminated plasmolysis; whereas, EDTA exhibited no positive effect on it. Shoot cells remained unaffected under Cd treatment alone or with citric acid except for chloroplast swelling. Only EDTA promoted starch accumulation in chloroplast reflecting its negative impact on cellular structure. It concludes that Cd and EDTA induce structural and morphological damage in *J. effusus*; while, citric acid ameliorates Cd toxicity stress.

1. Introduction

Last few decades have witnessed an enormous apprehension for the environmental pollution contributed through toxic metal accumulation in the soil and water. Industrial mining, burning of fossil fuels and sewage disposal operations are the major sources of metal pollution creating major environmental problems [1]. They contribute to the contamination of soils with excessive amounts of metals like Pb, Zn, Cu, Mn and Cd [2]. Cadmium is a non-essential element whose high water solubility, neurotoxic, mutagenic and carcinogenic nature make it extremely toxic to living organisms [3]. In plants, it inhibits photosynthesis, and diminishes water and nutrient uptake [4,5], resulting in chlorosis, growth retardation, browning of root tips, ultra-structural damage and ultimately to death [6]. Its higher concentration causes oxidative stress to the plants by accelerating the production of reactive oxygen species (ROS) and lipid peroxidation [7]. In order to combat oxidative damage, plants have evolved antioxidative enzyme defense system consisting of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), etc. [8]. These enzymes catalyze the dismutation of highly reactive O^{2-} into non-toxic forms like O_2 and H_2O.

Phytoextraction is an environmental friendly and cheaper remediation technology for metal polluted soils [9]. However, low bioavailability and limited translocation of some metals to the shoots are major obstacles in the phytoextraction process experienced by most of the plant species. A combination of high biomass-producing plant species and chemically assisted phytoextraction would be helpful to overcome this constraint [10]. Synthetic chelators and low molecular weight organic acids (LMWOA) are in common use as they improve the bioavailability of metals in soils. Ethylene diamine tetra acetic acid (EDTA) is among the efficient synthetic chelators that improves metal accumulation in shoots [11]. However, it is unsuitable for practical use because it degrades slowly and persists longer in soil that enhances its leaching risk and toxicity in the environment [12]. On the other hand, LMWOA, are good alternatives to EDTA due to their easy biodegradation as the natural products of root exudates, microbial secretions, and plant and animal residue decomposition in soils [13].

Role of citric acid in reducing Cd toxicity has been established in many plants [14]. It is more efficient as compared to other LMWOA

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doi:10.1016/j.jhazmat.2010.11.037

due to its high efficiency for increasing phytoextraction of toxic metals. Among three LMWOA (citric, oxalic, and tartaric acid), citric acid rendered higher copper uptake in tobacco *Nicotiana tabacum* [15]. Similarly, citric acid exhibited greater capacity for Cd extraction in Indian mustard (*Brassica juncea* L.) than that of malic, oxalic and succinic acids [16]. Compared to malic and oxalic acid, citric acid performed better for reducing Cd toxicity in wheat [17].

Wetland plants have reported high growth rate and great potential for phytoremediation. There are examples of various wetland plants e.g. salix, *Salix phylicifolia* L. and *S. borealis* Fr. [18]; cattail, *Typha latifolia* L.; common reed, *Phragmites australis* L. [19]; and mat rush, *Juncus effusus* L. [20] having ability to accumulate metals in their tissues. *J. effusus* is one of the most common and dominant species of plants in wetlands that produces very high aboveground biomass. It is ecologically important plant that is widely used for construction and restoration of wetlands, wastewater treatment, and biological processing. Its stem is economically important as raw material for woven products including straw mats, seats, hats, baskets, thatching, weaving mats, etc. In spite of great progress in unveiling the phytoremediation mechanisms, still many questions are unrequited e.g. role of chelators and antioxidant enzymes in metal detoxification, translocation and sequestration. In the present study, we investigated the toxic effects of elevated levels of Cd on biomass, antioxidative defense systems and ultra-structure of *J. effusus* L. Furthermore, we compared the efficiency of citric acid and EDTA for phytoextraction of Cd, and tolerance mechanism of plants against this stress.

2. Materials and methods

2.1. Plant material and cultural conditions

Seeds of commercial cultivar (Nonglin-4) of mat rush (*J. effusus* L.) were provided by Prof. W.Q. Shen, University of Nottingham Ningbo, Ningbo, Zhejiang province, China. Until sowing in Zhejiang University, Hangzhou, China, seeds were stored in the dark at 4 °C. Seeds were surface sterilized according to Xu et al. [21] using a solution containing 0.05 g of potassium permanganate ($KMnO_4$) in 80 mL sterile water for 72 h transferred to ethanol (70%) for another 60 min, and washed with sterile distilled water. The seeds were then shifted into 1.0% sodium hypochlorite (NaClO) solution, containing 2 drops of Tween-20 and kept shaking for 20 min prior to three washings with sterile distilled water. The seeds were marinated in mercuric chloride (0.1%) for 10 min, and rinsed again for 5 times with distilled water under aseptic conditions.

These surface-sterilized seeds were grown on hormone-free MS medium [22] containing 2% sucrose and solidified with 8% agar under fluorescent light (100 $\mu mol\ m^{-2}\ s^{-1}$) in a light regime of 16 h/8 h (light/dark) at 19 ± 2 °C. Two weeks old seedlings were precultured for one week in hydroponic solution containing macro and micronutrients including $Ca(NO_3)_2{\cdot}4H_2O$ 2.00 mM, KH_2PO_4 0.10 mM, $MgSO_4{\cdot}7H_2O$ 0.50 mM, KCl 0.10 mM, K_2SO_4 0.70 mM; and H_3BO_3 10.00 μM, $MnSO_4{\cdot}H_2O$ 0.50 μM, $ZnSO_4{\cdot}7H_2O$ 1.0 μM, $CuSO_4{\cdot}5H_2O$ 0.20 μM, $(NH_4)_6\ Mo_7O_{24}{\cdot}4H_2O$ 0.01 μM, Fe–EDTA 100 μM. The pH of nutrient solution was kept at 5.8 by daily checking and adding 0.1 mM NaOH or HCl. After every 3 days, the nutrient solution was replaced with fresh one. Plants were grown in the triplicate in glasshouse under natural light, day/night temperature of 19/20 °C and relative air humidity of 70/85%.

2.2. Cadmium treatment and sample preparation

Uniform size seedlings selected after two weeks of pre-culturing were subjected to various concentrations of Cd (as $CdCl_2$) viz. CK, 10, 50 and 100 μM. Treatment of CK represents control plants grown in the basic nutrient media without Cd. Cadmium concentration in non-polluted soil is in the range of 0.04–0.32 mM, whereas, it may reach up to 1.00 mM under high polluted soils conditions [3]. However, application of higher Cd concentrations under hydroponic conditions exhibits unrealistically greater amounts than under natural sludge-amended soil solutions [23] and can lead to misunderstanding for phytoextraction [24]. Therefore, to obtain representative experimental results, studies of Cd uptake by plants need using concentrations that will likely be encountered in naturally occurring soil solutions. In the pre-experiment, we tested mat rush plants also against higher Cd levels (200–500 μM). Since these Cd concentrations severely damaged the plants (data not shown), we opted for lower Cd concentrations (0–100 μM) for this experiment.

Two concentrations (2.5 and 5.0 mM) each of citric acid and EDTA were applied in combination with the highest level of Cd (100 μM). These concentrations were selected on the basis of pre-experiment, in which various concentrations of these chelators were used (1.25, 2.5, 5.0, 7.5 and 10.0 mM) to enhance Cd uptake by *J. effusus*. Concentrations above 5.0 mM of both EDTA and citric acid exhibited negative effect, while lower concentration (1.25 mM) rendered non significant impact on plant growth and Cd uptake. Chelators in this study were used as amendments to enhance phytoextraction of Cd contaminated water; therefore, they were applied only with Cd. Moreover, earlier studies also showed that application of EDTA alone in the growth medium had no significant effect on plant growth, chlorophyll content and antioxidant enzyme activity [25].

Plants were removed after 14 days of treatment, and roots were soaked in 20 mM Na_2–EDTA for 15 min to wash the excess metal ions adhering to root surface. Fresh samples of leaves were frozen immediately in liquid nitrogen and stored at −80 °C to determine antioxidants and malondialdehyde (MDA) contents.

2.3. Determination of Cd and nutrient elements

For the quantification of Cd and nutrient elements, plants were separated into roots and shoots, and dried at 70 °C for 48 h. Oven dried (70 °C) plant samples (0.1 g) were digested with 5 mL HNO_3 and 1 mL $HClO_4$ in closed Teflon vessels by heating until transparent. Digested material was washed into 50 mL flask, and made the volume with de-ionized water. Cadmium and nutrient elements concentration in plant samples was determined with inductively coupled plasma mass spectrophotometer (Agilent 7500A). The Cd, K, Mg and Ca uptake by plants was calculated as $mg\ kg^{-1}$ of plant dry weight. Quality control and quality assurance for Cd analysis of plants were attained through standard reference material GBW10010 (GSB-1) from Institute of Geophysical and Geochemical of Earth (IGGE), China. The analysis showed Cd and nutrient element concentrations in the standard reference material very close to the given value that confirmed the accuracy of the method used.

2.4. Root morphological traits

Roots of the harvested plants were washed carefully using distilled water to remove any contamination. Root morphological traits viz. root diameter, surface area and volume were recorded by root automatism scan apparatus (MIN-MAC, STD1600+) equipped with WinRHIZO software from Regent Instrument Company (USA). Three plants from each replication of all treatments were randomly selected for data collection.

2.5. Biochemical assay

Plant leaf samples were washed with distilled water and ground with a mortar and pestle under chilled condition in the homoge-

Table 1
Effects of Cd, citric acid and EDTA on plant biomass of *Juncus effusus*.

Treatment	Fresh biomass (mg plant^{-1})		Dry biomass (mg plant^{-1})	
	Shoot	Root	Shoot	Root
CK	206.3 ± 11.8 a	225.2 ± 10.2 a	35.8 ± 2.7 a	27.2 ± 2.0 a
Cd 10 μM	196.1 ± 13.0 ab	230.7 ± 13.8 a	36.6 ± 2.5 a	23.6 ± 1.9 b
Cd 50 μM	185.2 ± 10.5 b	213.3 ± 11.5 ab	33.0 ± 2.7 ab	20.2 ± 1.6 c
Cd 100 μM	146.6 ± 13.4 c	187.2 ± 9.7 c	32.8 ± 1.5 b	17.5 ± 1.6 d
Cd 100 μM + CA 2.5 mM	143.8 ± 12.5 c	203.5 ± 12.1 b	27.9 ± 2.4 cd	20.4 ± 1.4 c
Cd 100 μM + CA 5.0 mM	135.7 ± 10.1 c	183.6 ± 10.8 c	25.4 ± 1.7 d	18.4 ± 1.8 cd
Cd 100 μM + EDTA 2.5 mM	141.8 ± 13.6 c	183.4 ± 9.9 c	28.8 ± 2.9 c	17.7 ± 1.6 d
Cd 100 μM + EDTA 5.0 mM	115.4 ± 12.2 d	166.4 ± 10.3 d	23.4 ± 2.7 d	16.9 ± 1.8 d

Values in each column followed by the same letter are not significantly different at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

nization buffer specific for each enzyme. Activities of antioxidative enzymes viz. SOD and POD, and MDA content were simultaneously determined according to Leul and Zhou [26] as in the followings.

Superoxide dismutase (SOD) activity was assayed by photochemical nitroblue tetrazolium (NBT) method. Leaf samples (0.5 g) were homogenized in 5 mL extraction buffer (50 mM phosphate of pH 7.8) under chilled conditions. Photoreduction of NBT (formation of purple formazan) was recorded at 560 nm using 3 mL mixture containing 50 mM phosphate buffer (pH 7.8), 26 mM L-methionine, 750 μM NBT, 1 μM EDTA, and 20 μM riboflavin.

For determination of peroxidase (POD) activity, guaiacol was used as the substrate. The reaction mixture (3 mL) which contained 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2 and enzyme extract was used for measuring POD at 470 nm. Oxidation of guaiacol caused increase in the absorbance that was recorded to estimate POD activity.

Malondialdehyde (MDA) contents were determined as 2-thiobarbituric acid (TBA) reactive metabolites. Plant leaf samples (0.2 g) were homogenized in a solution of 10 mL [0.25% TBA made in 10% trichloroacetic acid (TCA)]. The extract was heated in water bath at 95 °C for 30 min, quickly cooled down on ice and then centrifuged at 5000 × *g* for 10 min. The supernatant was used to measure MDA contents at 532 nm. Another measurement was taken at 600 nm for correcting non-specific turbidity. The MDA contents were represented as μmol g^{-1} fresh biomass using an extinction coefficient of 155 mM cm^{-1}.

Reaction mixture (3 mL) consisting of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA–Na_2, 0.3 mM ascorbic acid, 0.06 mM H_2O_2 and 100 μL enzyme extract was used for measuring ascorbate peroxidase (APX) activity. The activity of enzyme was calculated based on change in absorption at 290 nm 30 s after H_2O_2 addition [27].

Glucothione reductase (GR) was measured by monitoring the decrease in absorbance caused by nicotinamide adenine dinucleotide phosphate (NADPH) oxidation ($E = 6.2$ mM^{-1} cm^{-1}) at 340 nm [28]. The reaction mixture used for determining GR activity contained 25 mM potassium phosphate buffer (pH 7.8, having 0.2 mM EDTA), 0.5 mM glutathione disulfide (GSSG), 0.12 mM NADPH, and enzyme aliquot.

Catalase (CAT) activity was estimated using a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and enzyme extract. The measurements were taken at 240 nm by recording decline in H_2O_2 absorbance during 1 min interval and used for calculating CAT activity ($E = 39.4$ mM^{-1} cm^{-1}) [29].

2.6. Transmission electron microscopy

For the ultra-structural study of cells, root and shoot samples of *J. effusus* plant were collected from the highest treatment of Cd (100 μM) alone, and in combination with citric acid (5.0 mM) and EDTA (5.0 mM). Morphometric data was recorded from at least 30 samples of cell and cellular organelles by software JeDa 801D Morphology Image Analysis Systems and averaged.

Leaf and root segments (approximately 2–3 mm in length) of sampled plants were fixed in 2.5% glutaraldehyde (v/v) in 0.1 M PBS (sodium phosphate buffer, pH 7.4) at room temperature for 24 h prior to three washing with the same PBS. The samples were then fixed in 1% OsO_4 (osmium oxide) for 1 h and washed three times using the same buffer solution. The samples were treated with a series of graded ethanol having concentrations (50, 60, 70, 80, 90, 95, and 100% ethanol) and finally with absolute acetone for 15–20 min interval to dehydrate them completely. After dehydration, these samples were embedded in Spurr's resin for 24 h. The specimens were heated at 70 °C for 9 h for preparation for ultra-thin sections (80 nm) and mounted on copper grids to review under electron microscope (JEOL TEM-1230EX) at 60.0 kV voltage.

2.7. Data analysis

Statistical analysis of plant growth, biomass and biochemical parameters was undertaken through one-way analysis of variance (ANOVA) using SAS v.9 software. Least significant difference (LSD) test was employed at ≤0.05 probability to compare the means with statistically significant difference.

3. Results

3.1. Plant growth attributes

Alterations in plant growth attributes were studied to assess the Cd toxicity stress to *J. effusus* (Tables 1 and 2). Although no visible phytotoxic symptoms were observed in the plants exposed to different Cd levels, the fresh and dry biomass were significantly reduced under higher Cd concentrations. As compared to Cd (100 μM) alone, plant dry weight remained statistically the same under the chelators application except for the higher dose of EDTA that significantly reduced plant dry weight. However, addition of chelators significantly decreased the shoot dry weight at all levels, whereas no significant change was recorded on root dry weight under application of higher citric acid concentration and two EDTA concentrations. Low level of citric acid (2.5 mM) improved root fresh (non-significantly) and dry weight (significantly). Under elevated Cd concentrations, plant height (root and shoot length), and root morphological traits such as volume, surface area and average diameter were also considerably reduced (Table 2). Application of citric acid significantly improved root morphological characters of Cd stressed plants; on the other hand, EDTA caused significant reduction in root morphological traits.

3.2. Bioaccumulation of Cd and other nutrient elements

In the present experiment, Cd accumulating capacity of *J. effusus* plants appeared to be concentration dependent (Table 3). There was

Table 2
Effects of Cd, citric acid and EDTA on shoot and root morphological characteristics of *Juncus effusus*.

Treatment	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	Root diameter (mm plant^{-1})	Root surface area (cm^2 plant^{-1})	Root volume (cm^3 plant^{-1})
CK	17.3 ± 1.5 a	3.52 ± 0.51 a	26.3 ± 2.9 a	6.21 ± 0.53 a	0.79 ± 0.04 a
Cd 10 μM	16.3 ± 1.6 ab	3.38 ± 0.46 a	25.7 ± 2.2 a	6.02 ± 0.70 a	0.69 ± 0.03 b
Cd 50 μM	14.7 ± 1.1 b	2.60 ± 0.37 b	22.4 ± 1.1 b	4.57 ± 0.31 b	0.59 ± 0.05 c
Cd 100 μM	12.6 ± 1.2 c	2.41 ± 0.41 b	19.5 ± 1.7 c	4.01 ± 0.27 c	0.41 ± 0.03 d
Cd 100 μM + CA 2.5 mM	14.5 ± 1.2 b	2.01 ± 0.32 bc	22.3 ± 2.3 b	5.45 ± 0.51 ab	0.50 ± 0.06 cd
Cd 100 μM + CA 5.0 mM	14.0 ± 1.1 b	2.31 ± 0.28 bc	24.5 ± 2.0 ab	6.09 ± 0.47 a	0.55 ± 0.04 c
Cd 100 μM + EDTA 2.5 mM	14.4 ± 1.2 b	2.20 ± 0.41 bc	21.3 ± 2.1 bc	3.50 ± 0.28 d	0.40 ± 0.04 d
Cd 100 μM + EDTA 5.0 mM	12.2 ± 1.1 c	1.68 ± 0.29 c	16.6 ± 2.1 c	4.30 ± 0.31 bc	0.31 ± 0.03 e

Parameter values sharing similar letter(s) in a column have non-significant difference among themselves at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

Table 3
Effects of Cd, citric acid and EDTA on Cd uptake and translocation in *Juncus effusus*.

Treatment	Cd concentration (mg kg^{-1})		TF value
	Shoot	Roots	
CK	0.86 ± 0.07 f	1.36 ± 0.11 g	0.64 a
Cd 10 μM	62.7 ± 12.5 e	103.2 ± 12.3 f	0.60 a
Cd 50 μM	87.9 ± 9.81 e	164.7 ± 16.7 e	0.53 ab
Cd 100 μM	145.0 ± 13.8 c	403.6 ± 21.7 c	0.35 d
Cd 100 μM + CA 2.5 mM	359.9 ± 21.6 b	696.4 ± 32.1 b	0.51 b
Cd 100 μM + CA 5.0 mM	440.6 ± 25.4 a	1018.8 ± 33.6 a	0.43 c
Cd 100 μM + EDTA 2.5 mM	125.4 ± 12.7 d	256.7 ± 18.2 d	0.49 bc
Cd 100 μM + EDTA 5.0 mM	66.0 ± 8.63 e	146.7 ± 14.9 e	0.45 c

Figures in a column bearing same letter(s) are statistically non-significant with each other at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid; TF (translocation factor), the ratio of metal concentrations in shoots and roots of the plant.

a significant increase in Cd contents, in both roots and shoots of the plant, with the increasing Cd concentrations in the media. The water used in hydroponics was tap water supplied by the municipality, so it may contain Cd in traces. Therefore, the plants in CK also had some Cd contents. Addition of citric acid in media, further increased Cd uptake, conversely, ETDA significantly reduced Cd accumulation. *J. effusus* had a tendency to accumulate more Cd in roots as compared to the aerial parts that was obvious from lower value of TF (translocation factor), ratio of metal concentrations in shoot and root. The TF value decreased with the elevated Cd levels in the medium; however, addition of chelators (citric acid and EDTA) improved the TF value in Cd treated plants.

No significant change on the uptake of different nutrient elements was observed under lower level treatment of Cd (10 μM). Higher concentration of Cd (50 and 100 μM) significantly reduced the uptake of various nutrient elements viz. K, Mg, Ca, Fe and Zn by plant shoots as well as roots (Tables 4 and 5, respectively). Addition of EDTA in the nutrient medium significantly decreased the accumulation of these elements in both shoots and roots of *J. effusus* plant. As compared to treatment with Cd (100 μM) alone, amendment with citric acid slightly improved the nutrient concentration in plants; however, Fe in shoots and Zn in roots were increased significantly under higher citric acid treatment.

3.3. Biochemical changes

Lipid peroxidation in *J. effusus* shoots was measured as MDA contents (Table 6). Compared to control plants, there was a non-significant rise in MDA contents under low Cd concentration that significantly increased under higher Cd levels. Addition of chelators along with Cd treatment (100 μM) further enhanced lipid peroxidation, except for low level of citric acid (2.5 mM) that slightly reduced the MDA contents.

Activity of antioxidative enzymes changed variably under different treatments of Cd alone, or in combination with citric acid or EDTA (Table 6). The SOD activity increased slowly and non-significantly under low Cd concentrations, with a significant rise at the highest Cd level. Application of Cd along with low citric acid level further enhanced the SOD activity, whereas, higher citric acid concentrations as well as two levels of EDTA reduced SOD activity. Activities of other antioxidative enzymes, CAT, APX and POD reduced perpetually under increasing Cd concentrations. Compared to the highest level of Cd alone, there was a significant increase in the activities of these enzymes under combined application of Cd with citric acid (2.5, 5.0 mM) or EDTA (2.5 mM). However, higher EDTA concentration significantly reduced the activities of all these enzymes. Under Cd stress, GR activity showed a linear increase with the increasing Cd concentrations in the nutrient solution. In contrast to Cd treatment alone, citric acid or EDTA significantly reduced GR activity that was further decreased with increasing levels of these chelators.

3.4. Shoot ultra-structure

Morphometric observation of electron microscopic images revealed significant reduction in size and diameter of mesophyll cells of plants exposed to combined treatment of Cd and chela-

Table 4
Effects of Cd, citric acid and EDTA on the nutrient concentration in *Juncus effusus* shoots.

Treatment	Mineral nutrient concentration				
	K (mg g^{-1})	Mg (mg g^{-1})	Ca (mg g^{-1})	Fe (μg g^{-1})	Zn (μg g^{-1})
CK	35.7 ± 4.1 a	2.30 ± 0.21 a	3.87 ± 0.29 a	95.2 ± 10.4 a	135.6 ± 12.6 a
Cd 10 μM	37.3 ± 4.6 a	2.41 ± 0.24 a	3.98 ± 0.32 a	102.5 ± 11.4 a	147.0 ± 13.2 a
Cd 50 μM	31.5 ± 3.6 ab	1.92 ± 0.22 b	3.09 ± 0.26 b	93.5 ± 8.71 a	119.4 ± 11.4 b
Cd 100 μM	28.0 ± 3.6 b	1.57 ± 0.18 bc	2.80 ± 0.21 bc	79.9 ± 7.42 b	99.7 ± 10.6 bc
Cd 100 μM + CA 2.5 mM	29.9 ± 2.8 ab	1.66 ± 0.18 bc	2.94 ± 0.23 b	84.3 ± 6.70 ab	111.2 ± 10.2 b
Cd 100 μM + CA 5.0 mM	30.7 ± 2.8 ab	1.79 ± 0.21 b	3.05 ± 0.22 b	95.1 ± 6.83 a	125.0 ± 11.7 b
Cd 100 μM + EDTA 2.5 mM	26.7 ± 2.5 b	1.35 ± 0.16 c	2.61 ± 0.21 c	65.2 ± 6.51 c	85.5 ± 7.82 c
Cd 100 μM + EDTA 5.0 mM	22.5 ± 2.5 c	1.14 ± 0.15 c	2.37 ± 0.22 c	52.6 ± 7.14 c	63.4 ± 6.73 c

Figures in a column bearing same letter(s) are statistically non-significant with each other at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

Table 5
Effects of Cd, citric acid and EDTA on the nutrient concentration in *Juncus effusus* roots.

Treatment	Mineral nutrient concentration				
	K ($mg\,g^{-1}$)	Mg ($mg\,g^{-1}$)	Ca ($mg\,g^{-1}$)	Fe ($\mu g\,g^{-1}$)	Zn ($\mu g\,g^{-1}$)
CK	23.2 ± 2.2 a	1.76 ± 0.14 a	4.11 ± 0.36 a	160.3 ± 15.3 a	325.1 ± 21.9 a
Cd 10 μM	20.9 ± 1.9 ab	1.67 ± 0.18 a	4.02 ± 0.37 a	155.2 ± 16.4 a	318.0 ± 24.4 a
Cd 50 μM	17.0 ± 1.5 b	1.29 ± 0.13 b	3.54 ± 0.32 ab	135.7 ± 12.5 ab	282.7 ± 21.7 ab
Cd 100 μM	15.3 ± 1.7 b	0.98 ± 0.16 c	2.70 ± 0.26 b	119.8 ± 12.2 b	268.1 ± 23.6 b
Cd 100 μM + CA 2.5 mM	16.2 ± 1.8 b	1.05 ± 0.16 bc	2.79 ± 0.28 b	136.2 ± 13.1 ab	294.7 ± 24.3 ab
Cd 100 μM + CA 5.0 mM	17.4 ± 1.6 b	1.09 ± 0.15 bc	2.86 ± 0.25 b	140.3 ± 13.7 ab	302.1 ± 25.8 a
Cd 100 μM + EDTA 2.5 mM	13.5 ± 1.5 c	0.87 ± 0.13 c	2.55 ± 0.21 b	110.2 ± 11.3 bc	216.9 ± 18.7 c
Cd 100 μM + EDTA 5.0 mM	11.9 ± 1.3 c	0.68 ± 0.14 c	2.40 ± 0.22 c	98.32 ± 11.4 c	194.3 ± 15.6 cd

Figures in a column bearing same letter(s) are statistically non-significant with each other at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

Table 6
Effects of Cd, citric acid and EDTA on MDA contents and antioxidative enzyme activities in *Juncus effusus* leaves.

Treatment	MDA ($nmol\,g^{-1}$ FW)	SOD activity ($U\,g^{-1}$ FW)	CAT activity ($\mu mol\,mg^{-1}$ protein min^{-1})	APX activity ($U\,g^{-1}$ FW)	POD activity ($OD470\,g^{-1}$ FW min^{-1})	GR ($\mu mol\,mg^{-1}$ protein min^{-1})
CK	31.0 ± 3.1 e	224.4 ± 12.6 d	2.66 ± 0.51 a	164.4 ± 5.7 c	18.5 ± 1.3 ab	9.1 ± 1.2 cd
Cd 10 μM	36.1 ± 3.0 d	239.6 ± 13.8 c	1.34 ± 0.23 bc	154.3 ± 6.5 c	15.4 ± 1.8 bc	10.6 ± 1.4 c
Cd 50 μM	41.2 ± 3.8 cd	292.2 ± 10.2 b	0.98 ± 0.24 c	151.7 ± 6.2 cd	13.4 ± 1.7 cd	13.4 ± 1.5 b
Cd 100 μM	47.3 ± 5.2 c	324.4 ± 14.8 a	0.87 ± 0.13 c	126.8 ± 5.8 d	11.6 ± 1.1 d	15.5 ± 1.0 a
Cd 100 μM + CA 2.5 mM	44.5 ± 4.4 c	331.5 ± 15.5 a	1.20 ± 0.42 bc	213.2 ± 12.2 b	16.6 ± 1.3 b	14.2 ± 1.2 ab
Cd 100 μM + CA 5.0 mM	54.6 ± 6.7 bc	311.1 ± 14.4 ab	1.41 ± 0.61 b	253.4 ± 14.7 a	20.3 ± 1.6 a	8.01 ± 1.1 cd
Cd 100 μM + EDTA 2.5 mM	55.3 ± 5.7 b	318.1 ± 15.7 a	1.44 ± 0.47 b	165.1 ± 9.6 c	14.5 ± 1.0 c	12.4 ± 1.3 bc
Cd 100 μM + EDTA 5.0 mM	68.3 ± 7.3 a	281.4 ± 16.1 b	0.63 ± 0.21 c	126.2 ± 7.1 d	8.57 ± 1.1 e	7.12 ± 1.3 d

Values of each parameter in a column with different letter(s) have significant difference among themselves at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

tors (citric acid and EDTA) over the control (Table 7). Chloroplast swelling (increased size, length and width) was also obvious in plants treated either with Cd alone or with EDTA. Nucleus size and diameter of *J. effusus* plants remained statistically unaffected under all these treatments.

Mesophyll cells of the control plant had typical mature cells with a definite cell wall, containing nucleus and chloroplast (Fig. 1a). Chloroplast was lens-shaped that possessed well-organized grana and thylakoid membrane system with a few dense plastogobuli inside stroma (Fig. 2a). Mesophyll cells of Cd treated plants showed no change in cellular shape but there was a substantial increase in the number of plastogobuli inside chloroplast (Figs. 1a and 2b). Application of chelators along with Cd altered cellular and chloroplast shape and structure. These cells were circular in shape with swollen chloroplast and disintegrated thylakoid membrane system (Fig. 1c and d). Higher magnification disclosed loose thylakoid membrane system under treatment of Cd alone or with chelators (Fig. 2b–d). Starch granules appeared only in the chloroplast of plant cells exposed to Cd application with EDTA (Fig. 2d).

3.5. Root ultra-structure

Data collected from micrographs of root meristematic cells demonstrated no significant change in the size and diameter of cell under Cd treatment alone or with citric acid except for treatment of Cd with EDTA that significantly reduced the cell size (Table 7). Compared to control plant cell, Cd treatment alone or with EDTA significantly enhanced the nucleus size. Ultra-structural observations revealed that root cells of untreated plants contained a granular cytoplasm densely filled with numerous sub-cellular organelles. Cells possessed smooth, clean and continuous cell membranes and thick cell walls. Large central vacuole, well-shaped nucleus, and a few mitochondria and plastids were observable in the cytoplasm (Fig. 3a).

Numerous ultra-structural alterations were noticed in root meristematic cells of the plants exposed to high concentrations of Cd (100 μM) alone or in combination with citric acid or EDTA. A considerable amount of Cd was present in the form of crystals and electron dense granules in vacuoles and attached to cell wall along

Table 7
Effects of Cd, citric acid and EDTA on root and shoot cells and sub-cellular organelles of *Juncus effusus*.

Morphometric parameters (μm)	CK	Cd 100 μM	Cd 100 μM + CA 5.0 mM	Cd 100 μM + EDTA 5.0 mM
Shoot cell and sub-cellular organelles				
Cell size	57.1 ± 7.82 a	52.1 ± 6.05 ab	42.1 ± 7.16 b	43.8 ± 6.75 b
Cell diameter	15.7 ± 3.01 a	12.5 ± 3.15 ab	9.53 ± 2.77 b	10.6 ± 2.08 b
Nucleus size	12.3 ± 2.83 a	13.4 ± 3.17 a	10.6 ± 2.08 a	13.9 ± 3.11 a
Chloroplast size	14.2 ± 2.15 b	16.0 ± 2.63 ab	14.4 ± 2.17 b	18.3 ± 3.19 a
Chloroplast length	5.73 ± 0.72 b	6.67 ± 0.68 a	5.17 ± 0.71 b	6.35 ± 0.58 ab
Chloroplast width	4.10 ± 0.21 ab	4.16 ± 0.19 ab	4.09 ± 0.18 b	4.44 ± 0.25 a
Cell wall	0.37 ± 0.04 a	0.31 ± 0.06 ab	0.28 ± 0.05 b	0.24 ± 0.06 b
Root cell and sub-cellular organelles				
Cell size	49.6 ± 2.73 a	52.7 ± 5.11 a	46.8 ± 6.68 ab	41.1 ± 4.81 b
Cell diameter	13.3 ± 2.13 a	13.5 ± 2.16 a	12.2 ± 3.01 a	12.8 ± 2.47 a
Nucleus size	9.66 ± 1.06 b	12.1 ± 1.35 a	10.4 ± 1.52 ab	11.5 ± 1.15 a

Parameter values in each row with similar letter(s) are statistically non-significant with each other at $P < 0.05$.
CK, control plants grown in the basic nutrient media; CA, citric acid.

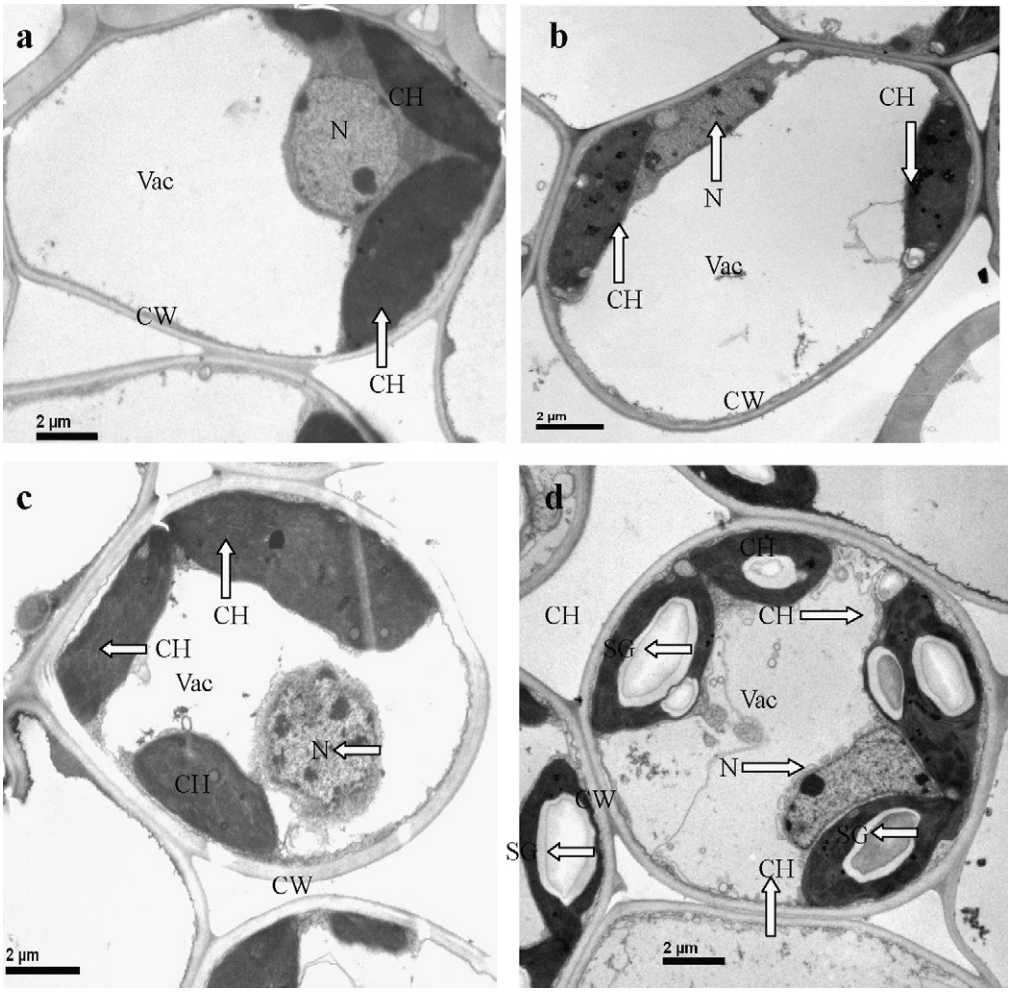

Fig. 1. A whole mesophyll cell of mat rush (*Juncus effusus*) shoot: (a) control; (b) exposed to (100 μM) Cd alone; (c) Cd (100 μM) + citric acid (5.0 mM); (d) Cd (100 μM) + EDTA (5.0 mM). CH, chloroplast, CW, cell wall; N, nucleus; Mt, mitochondria; SG, starch grains; Vac, vacuole.

with shrinkage of cell membrane (Fig. 3b–d). Swelling of nucleus, disruption of nuclear membrane and increased number of nucleoli were some of the other obvious changes observed in the nucleus of Cd treated cells (Fig. 4b–d). Application of citric acid to Cd stressed plants improved the root cell shape and cell wall, also the detrimental cytoplasmic shrinkage was negligible in these cells (Fig. 3c). However, no significant improvement was noticeable under the treatment of Cd with EDTA (Fig. 3d).

4. Discussion

Study of plant mechanisms for accumulating metals and countering their toxicity stress helps to improve our comprehension of the processes of metal nutrition and detoxification. Enhanced tissue tolerance, metal sequestration and antioxidative response, collectively determine the ability of plants to trounce destructive effects of Cd [7]. In the present study, there was an overall reduction in plant growth attributes under Cd stress. However, reduction in shoot fresh weight was not similar to the reduction in its dry weight that increased significantly at low Cd concentration in the nutrient media. This indicates that water loss due to Cd stress caused membrane damage and subsequent reduction in fresh biomass. Reduced nutrient uptake by plant might be responsible for growth retardation in EDTA treated plants. Growth inhibition observed in this study could be a consequence of metal interference in plant metabolism [6], modification in antioxidative enzymes activities [4,30], alteration in cell size and chloroplast impairment [31].

In the pre-experiment, we tested five concentrations of EDTA (1.25, 2.5, 5.0, 7.5 and 10.0 mM), and found that increasing EDTA concentrations caused gradual increase in toxicity to the plant growth after 2.5 mM (data not given). The current study has also shown toxicity of EDTA at 5.0 mM concentration visible through reduction in plant growth. This is supported by the work of other researchers, who found EDTA toxicity at higher concentrations in the medium. In a study by Azhar et al. [32], a range of EDTA concentrations from 0.5 mM to 5.0 mM was used in combination with Pb. They reported that gradual increase in EDTA concentrations first reduced Pb toxicity up to the level of 1.5 mM, and then its ameliorating ability started decreasing at higher levels. Positive effect of EDTA was also revealed by Xu et al. [33], who used 1.0–4.0 mM EDTA and found that application of 1.0 and 2.0 mM EDTA recovered sorghum roots from Pb toxicity; however, higher EDTA concentration (4.0 mM) inhibited root elongation.

Elevated Cd concentrations adversely affected the root morphological characters such as surface area, volume and diameter. Since roots are in direct contact with nutrient medium, their morphology influences the uptake of water, minerals and metals; therefore, it is important to study root morphological alteration under Cd induced stress. In the present investigation, higher Cd concentration alone or with EDTA suppressed the growth of root morphological traits. However, application of citric acid improved the root morphology of Cd stressed plants, consequently increasing Cd uptake. In addition, roots are primary sites for metal access, and their uptake

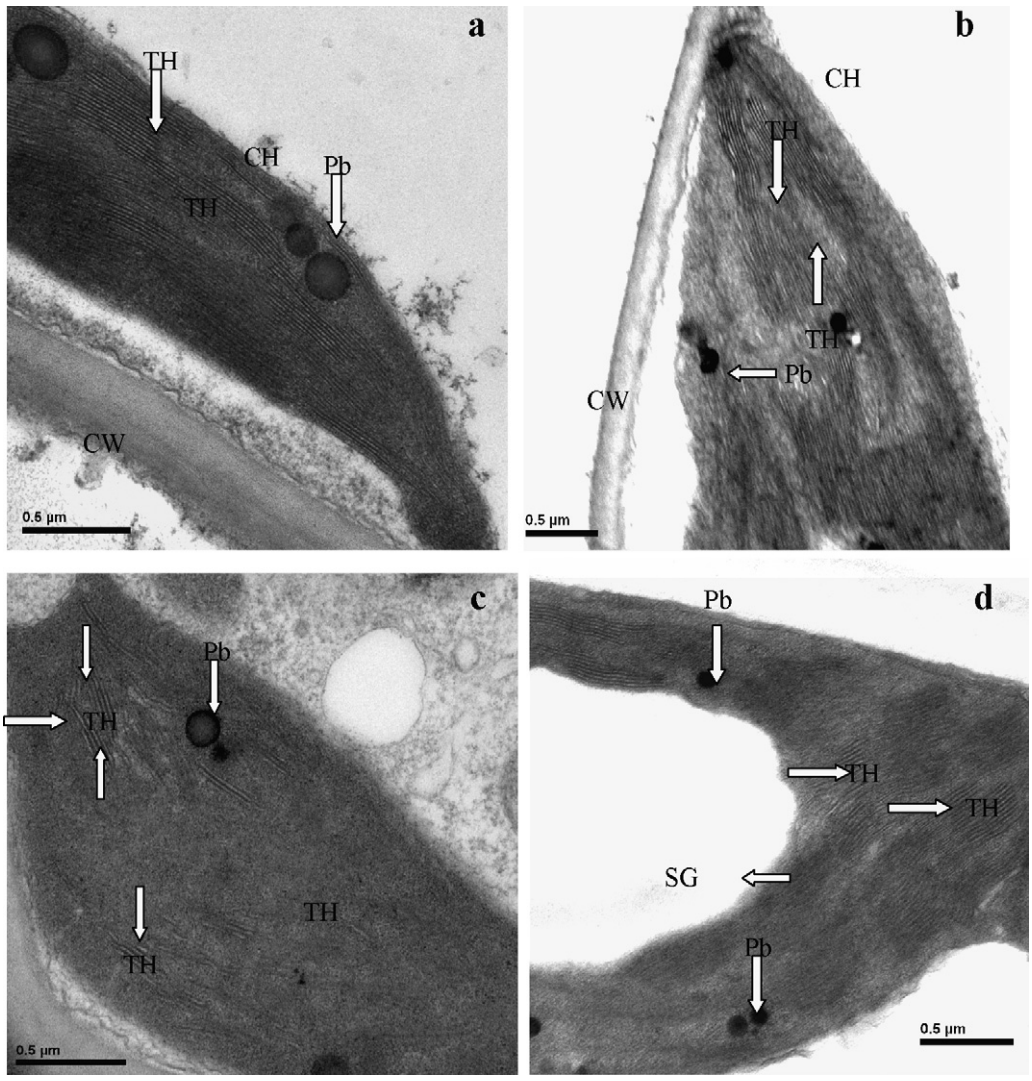

Fig. 2. Magnified view of chloroplast and thylakoid membrane of mat rush (*Juncus effusus*) shoot: (a) control; (b) exposed to (100 μM) Cd alone; (c) Cd (100 μM) + citric acid (5.0 mM); (d) Cd (100 μM) + EDTA (5.0 mM). CH, chloroplast; CW, cell wall; SG, starch grains; Pb, plastoglobuli, TH, thylakoid membrane.

correlates with the concentration in the soil or in the medium [34].

Cadmium uptake in *J. effusus* plants was also found to be a function of Cd concentration, and its uptake increased by elevating Cd concentration in the media or with the addition of citric acid. This could be due to release of strong organic ligands (property of wetland plants) into the environment around plant roots [35] that might have played a role in controlling metal bioavailability in the plant–water interface [36]. Like many other plants [6,7], *J. effusus* showed a propensity to retain higher amount of Cd in their roots compared to aerial parts. This was obvious from low value of translocation factor (TF) that is used to measure the effectiveness of plants for the translocation of Cd from roots to shoots. This lower TF value of *J. effusus* for Cd could be a strategy of plants to avoid metal stress and environmental risk [20].

Exogenous citric acid significantly increased Cd concentrations in roots and aboveground parts of *J. effusus*. Conversely, EDTA in higher Cd treatment significantly decreased Cd accumulation, although there was increase in TF value. Enhanced Cd uptake as well as TF value by adding chelators in the media activates ATPases in the plasma membrane producing changes on transport of ions through the membrane [37]. This showed that chelators (EDTA and citric acid) can modify charges of plasma membrane to facilitate Cd uptake through symplasmic [38] or apoplasmic pathway [39]. Reduced Cd uptake by *J. effusus* in the present study under the combined application of EDTA and Cd is in line with the findings of Schor-Fumbarov et al. [40] who reported significant reduction in Cd uptake by *Nymphaea aurora* plant in the presence of EDTA (equivalent to 1.8 mM). Chen et al. [14] observed that 1.0 mM and 3.0 mM citric acid reduced the Cd toxicity to radish by stimulating its translocation from roots to shoots and converting it into more easily transported forms. Higher Cd concentration negatively affected the nutrient uptake; application of EDTA further reduced the nutrients accumulation. The reduced concentration of nutrient elements in plants would have been either due to ion leakage as a result of EDTA toxicity or increased nutrient chelation by EDTA causing their lower uptake [25].

In our previous study, we found that EDTA amendments increased Mn uptake by *J. effusus* [20]. However, reduction in Cd uptake by EDTA amendments in this study, could be due to difference in role of EDTA in phytochelation of essential (Mn) and non essential toxic metal (Cd) [41]. The negative effect of EDTA on Cd accumulation could be the result of EDTA toxicity or the breaking of phytochelatin-metal bonds or in *J. effusus* the Cd binding groups may compete with EDTA for uptake.

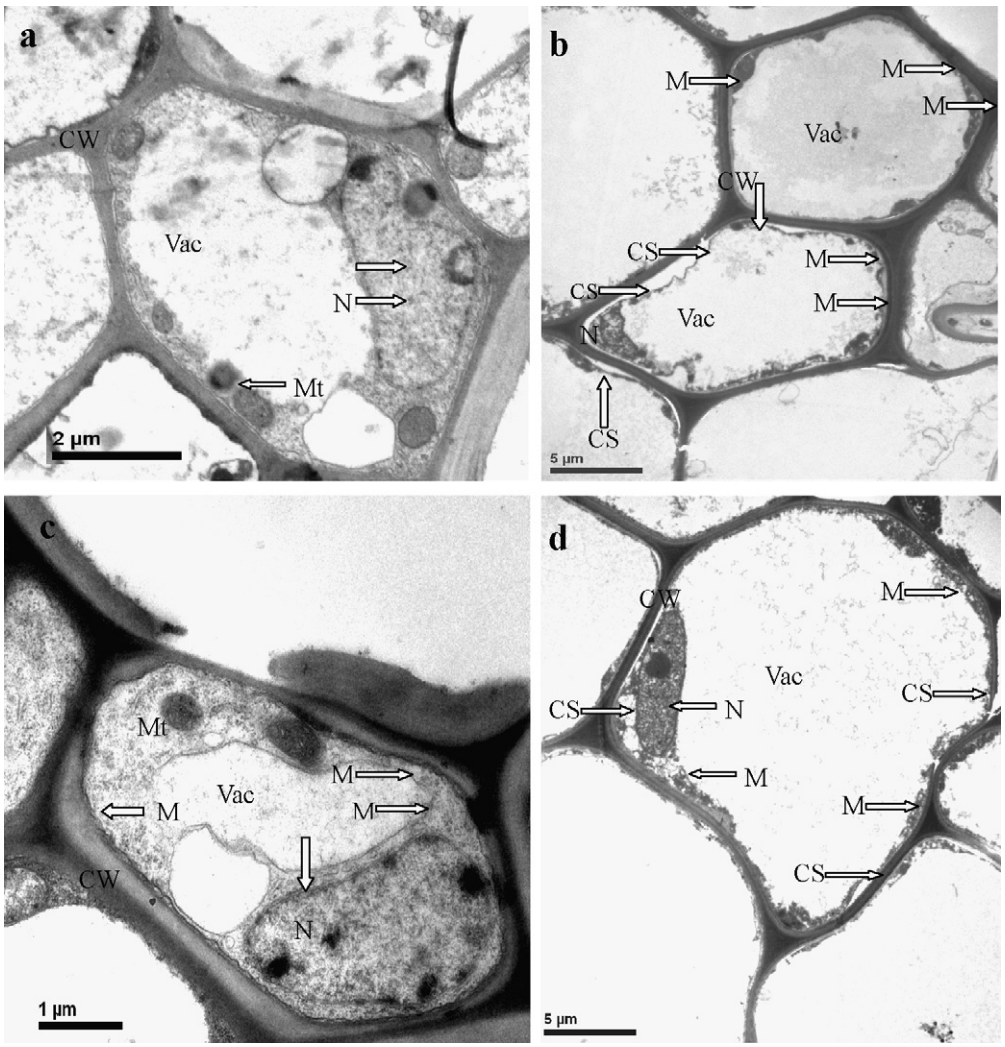

Fig. 3. View of whole cell of mat rush (*Juncus effusus*) root: (a) control; (b) exposed to (100 μM) Cd alone; (c) Cd (100 μM) + citric acid (5.0 mM); (d) Cd (100 μM) + EDTA (5.0 mM). CS, cytoplasmic shrinkage; CW, cell wall; Mt, mitochondria; M, metal deposition; N, nucleus; Nu, nucleolus; Vac, vacuole.

Lipid peroxidation is the oxidative deterioration of lipids containing any number of carbon–carbon double bond producing MDA as the final product, therefore, rise in MDA concentration is generally considered as an indicator of oxidative damage in metal stressed plants [42,43]. We observed concentration-dependent increase in the levels of lipid peroxidation in plant shoot tissues that indicated Cd induced oxidative stress in *J. effusus*. Earlier studies also confirmed our findings of increased MDA contents under Cd stress in soybean [4], *Sedum alfredii* Hance [7] and sunflower [44]. The mechanism of Cd-induced oxidative stress may differ from other metals, and instead of ROS production, it inhibits or stimulates the activity of antioxidative enzymes [45]. There are contrasting reports about Cd effects on antioxidative enzymes e.g. decreased activity of SOD, CAT, APX and GR in *Helianthus annuus* leaves [44], and increased SOD and POD in soybean plants [4]. In the present study, increased activity of some antioxidative enzymes (SOD and GR) in plants under Cd stressed conditions was a function of Cd concentration in plant tissues. Whereas, activity of other antioxidative enzymes CAT, APX and POD declined significantly with increasing Cd levels in the media. Reduction in activity of antioxidative enzymes is a general response to Cd stress due to inhibition of enzyme synthesis or a change in the assemblage of enzyme subunits [46]. Accumulation of H_2O_2 along with growth reduction could be another possible reason for reduction in antioxidative enzyme activity [47]. Compared to the application of Cd alone (100 μM), addition of citric acid (2.5 and 5.0 mM) and EDTA (2.5 mM) had an opposite effect on antioxidative enzymes. There was reduction in SOD and GR activity, whereas increased activities of CAT, APX and POD, except for higher EDTA concentrations. Modification in the activity of antioxidative enzymes suggests that chelators, especially citric acid might have played a role in defying Cd-induced oxidative stress in *J. effusus* plants.

Due to complementary relation between the structure and function of plants, the effect of Cd on cellular organization is important for understanding the physiological alterations. Electron microscopy helps to assess damage at the tissue and ultrastructural levels providing basis for macroscopic examination [48]. In the present investigation, plant root cell showed modifications like detrimental plasmolytic shrinkage, and increased number of nucleoli, etc. under the treatment of Cd alone or in combination with EDTA. However, cell and nucleus structure improved while, cytoplasmic shrinkage was nearly absent under application of Cd with citric acid. This demonstrates the capacity of citric acid for recuperating root structure that was also evident from the study of root morphological traits. Alteration in physiological processes such as, absorption, transportation and cellular localization of Cd directly count for its toxicity. In the present study, increased Cd accumulation near the cell wall of Cd treated plant cells played a

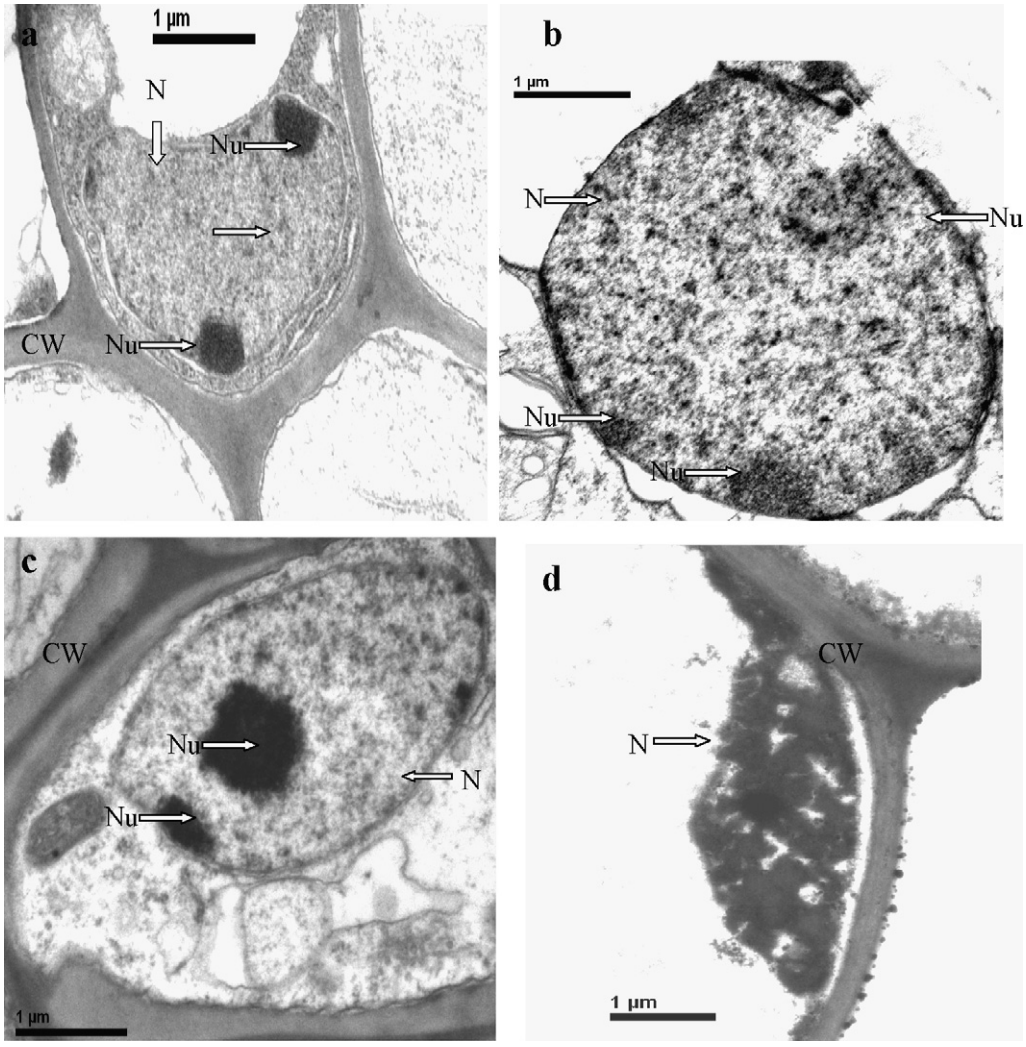

Fig. 4. Nucleus of root tip cells of *Juncus effusus*: (a) control; (b) exposed to (100 μM) Cd alone; (c) Cd (100 μM) + citric acid (5.0 mM); (d) Cd (100 μM) + EDTA (5.0 mM). CW, cell wall; N, nucleus; Nu, nucleolus.

significant role in Cd tolerance by preventing the circulation of free Cd ions in the cytosol [3]. Similarly, increased number of nuclei under Cd stress initiated synthesis of new proteins responsible for Cd tolerance in *J. effusus* [6].

Chloroplasts are highly susceptible to oxidative stress caused by elevated oxygen concentration, electron flux, and presence of metal ions in their microenvironment [49]. Cadmium induced stress increased the number of plastogobuli and disrupted thylakoid membrane inside the chloroplast. Application of chelators (citric acid and EDTA) caused chloroplast swelling and impairment of thylakoid systems. This imbalanced synthesis of thylakoid membranes can cause premature cell senescence in plants [7]. Starch accumulation in the chloroplast was noticed only under the combined application of EDTA and Cd that is a general sign of stress in plants. Increased nutrient deficiency or disturbed vein loading system [31] due to high Cd translocation into shoot may lead to the starch accumulation in the chloroplast. Chloroplast impairment and starch accumulation in Cd treated plants have been reported in earlier studies [31]. Jin et al. [7] investigated the effects of different Cd concentrations on two ecotypes of *Sedum alfredii* Hance and observed that Cd concentrations (10–40 μM) disrupted the thylakoid membranes system inside chloroplast in non Cd-hyper accumulating ecotype. Whereas, in Cd-hyper accumulating ecotype plants, only higher concentrations of Cd (400 μM) caused significant disintegration of chloroplast showing higher amount of plastogobuli, chloroplasts swelling and loose thylakoid membranes.

5. Conclusion

In this experiment, we found that *J. effusus* plants had the ability to survive under Cd stress without showing any visible symptoms of phytotoxicity even at higher Cd levels. Declined activities of some antioxidant enzymes might be responsible for the growth diminution after exposition of plants to the highest Cd concentration. Citric acid application increased the activities of these antioxidant enzymes helping plants to overcome oxidative stress. Accumulation and sequestration of more Cd in roots might also have played a role in avoiding Cd toxicity stress. Growth retardation under joint application of EDTA and Cd reduced the Cd accumulation. Higher Cd concentrations caused ultra-structural alteration in root and shoot cells. Citric acid application significantly enhanced Cd accumulation in *J. effusus* by recovering plant roots from Cd-induced ultra-structural damage. These findings enabled us understanding the tolerance mechanisms of *J. effusus* against Cd stress.

Acknowledgements

This work was sponsored by National Natural Science Foundation of China (30871652 and 31071698), Zhejiang Provincial Nat-

ural Science Foundation (R307095), Special Program for Doctoral Discipline of the China Ministry of Education (20090101110102), China Postdoctoral Science Foundation (20090461401), and the 111 Project from China Ministry of Education and the State Administration of Foreign Experts Affairs (B06014).

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