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Citric acid enhances the phytoextraction of manganese and plant growth by alleviating the ultrastructural damages in *Juncus effusus* L.

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ABSTRACT

Chelate-assisted phytoextraction by high biomass producing plant species enhances the removal of heavy metals from polluted environments. In this regard, *Juncus effusus* a wetland plant has great potential. This study evaluated the effects of elevated levels of manganese (Mn) on the vegetative growth, Mn uptake and antioxidant enzymes in *J. effusus*. We also studied the role of citric acid and EDTA on improving metal accumulation, plant growth and Mn toxicity stress alleviation. Three-week-old plantlets of *J. effusus* were subjected to various treatments in the hydroponics as: Mn (50, 100 and 500 μM) alone, Mn (500 μM) + citric acid (5 mM), and Mn (500 μM) + EDTA (5 mM). After 2 weeks of treatment, higher Mn concentrations significantly reduced the plant biomass and height. Both citric acid and EDTA restored the plant height as it was reduced at the highest Mn level. Only the citric acid (but not EDTA) was able to recover the plant biomass weight, which was also obvious from the microscopic visualization of mesophyll cells. There was a concentration dependent increase in Mn uptake in *J. effusus* plants, and relatively more deposition in roots compared to aerial parts. Although both EDTA and citric acid caused significant increase in Mn accumulation; however, the Mn translocation was enhanced markedly by EDTA. Elevated levels of Mn augmented the oxidative stress, which was evident from changes in the activities of antioxidative enzymes in plant shoots. Raised levels of lipid peroxidation and variable changes in the activities of antioxidant enzymes were recorded under Mn stress. Electron microscopic images revealed several modifications in the plants at cellular and sub-cellular level due to the oxidative damage induced by Mn. Changes in cell shape and size, chloroplast swelling, increased number of plastoglobuli and disruption of thylakoid were noticed. However, these plants showed a high degree of tolerance against Mn toxicity stress, and it removed substantial amounts of Mn from the media. The EDTA best enhanced the Mn uptake and translocation, while citric acid best recovered the plant growth.

1. Introduction

Phytoextraction, the use of plants to transport the metals from soils to the harvestable parts, is an environment-friendly and cheaper technology for the remediation of metal polluted soils [1]. However, most of the plant species experience low bioavailability and limited translocation of some heavy metals to the shoots, and it poses a major constraint in the phytoextraction process. Chelate-induced phytoextraction can be used for enhancing the uptake and translocation of metals in plants [2,3]. Synthetic chelators and low molecular weight organic acids (LMWOA) have the ability to enhance heavy metals bioavailability in soils. Ethylene diamine tetraacetic acid (EDTA) is although an efficient synthetic chelator [3,4]; however, its slow degradation rate and long persistence in soil increase its leaching risk making it unsuitable for practical use. On the other hand, LMWOA e.g. citric acid is good alternatives to EDTA for the phytoextraction of heavy metals [5,6]. The LMWOA are easily biodegradable as the natural products of root exudates, microbial secretions, and plant and animal residues decompose in soils [7].

Manganese (Mn) is an essential micronutrient for plants for various metabolic processes and is involved in redox reactions as a cofactor for different enzymes [8]. However, excessive use of acidic fertilizer results in lowering down the pH of soils, which increases the Mn availability [9] and its toxicity to plants [10]. High levels of Mn in the soil as well as in water can cause oxidative stress by accelerating the production of reactive oxygen species and lipid peroxidation [10]. The antioxidant defense system of plants mainly includes antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antiox-

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idant compounds such as glutathione (GSH), ascorbic acid (AsA), carotenoids and cysteine. The SOD catalyzes the dismutation of highly reactive O^{2-} to O_2 and H_2O_2 that is further decomposed to H_2O and O_2 either by APX of the ascorbate–glutathione cycle or by GPX and CAT localized in the cytoplasm and other cellular compartments. The GR is also a complementary enzyme of the ascorbate–glutathione cycle, maintaining a high GSH/oxidized glutathione (GSSG) ratio for protection against oxidative damage [11].

Wetland plants have great potential for phytoremediation due to their ability to tolerate stressful conditions [12]. Several wetland plants have been reported to accumulate heavy metals in their tissues, such as salix (*Salix phylicifolia* L. and *S. borealis* Fr.) [13], cattail (*Typha latifolia* L.) and common reed (*Phragmites australis* L.) [14]. High tolerance and phytoextraction ability of *Juncus effusus* to Mn has already been documented. Ghaly et al. [15] found higher Mn removal efficiency of *J. effusus*, while Groudeva et al. [16] treated the waters contaminated with crude oil and toxic heavy metals (e.g. Mn) by some wetland plants including *J. effusus*. It has broad tolerance to wet, acidic, nutrient-poor conditions, good reproductive potential, persistent seed bank and clonal growth of tussocks [17]. It produces shoots continuously whole the year, with an extremely high production rates averagely 0.7 kg ash-free dry mass/m^2/year [18], which is among the highest biomass production rates in the plant community. Selection of the crops with characteristics such as, fast growing, deep-rooted, easily propagated, a high biomass production rate and a high accumulation of the target metals are essential for phytoremediation [5,6,19]. *J. effusus* is widely used for establishing and restoring the wetlands, metal accumulation [12], wastewater treatment [20], and microbial activity enhancement [21]. Its stem serves as raw material for the woven products like straw mats, seats, hats, baskets, thatching and weaving mats [22].

Earlier studies with *J. effusus* focused mainly on the accumulation and transportation mechanism of different heavy metals [23], however, there is little work on the use of chelators to enhance metal phytoextraction, especially that of Mn in this plant. Furthermore, the role of citric acid in alleviating the Mn toxicity stress through ultrastructural modification at cell and sub-cellular level has rarely been studied. The objectives of this study were to explore the potential of *J. effusus* for the phytoextraction of Mn and to compare the effects of citric acid and EDTA on the uptake and translocation of Mn and plant growth. We studied the toxic effect of elevated levels of Mn alone or in conjunction with citric acid and EDTA on plant biomass and shoot ultrastructure of *J. effusus*. The mechanism of Mn tolerance and antioxidative defense system in *J. effusus* plants were also investigated.

2. Material and methods

2.1. Plant material and cultural conditions

A commercial cultivar of mat rush (*J. effusus* L.), Nonglin-4 was used in this study. Seeds were collected from Ningbo city of Zhejiang province, China, and were kindly supplied by W.Q. Shen, University of Nottingham at Ningbo. All seeds, upon receipt in Zhejiang University, Hangzhou, China, were stored in the dark at 4 °C until use. Seeds were surface-sterilized for 72 h in 80 mL sterile water with 0.05 g $KMnO_4$. Then were transferred in 70% ethanol for 60 min prior to washing three times with sterile water. It was followed by continuous agitation in 1.0% sodium hypochlorite with 2 drops of Tween-20 before rinsing another three times with sterile water [24]. These sterilized seeds were placed in glass growth vessels containing 30 mL hormone-free MS medium [25] solidified with 0.8% agar for germination and growth. Two-week-old seedlings were then pre-cultured for 1 week in a basic nutrient solution containing (in mmol L^{-1}) $Ca(NO_3)_2{\cdot}4H_2O$, 2.00; KH_2PO_4, 0.10; $MgSO_4{\cdot}7H_2O$, 0.50; KCl, 0.10, K_2SO_4, 0.70; and (in μmol L^{-1}) H_3BO_3, 10.00; $MnSO_4{\cdot}H_2O$, 0.50; $ZnSO_4{\cdot}7H_2O$, 1.0; $CuSO_4{\cdot}5H_2O$, 0.20; $(NH_4)_6\ Mo_7O_{24}{\cdot}4H_2O$, 0.01; Fe–ethylene diamine tetraacetic acid (EDTA), 100. The pH of nutrient solution was adjusted to 5.8 daily by 0.1 mM NaOH or HCl. Plants were grown in the glasshouse under natural light, day/night temperature of 19–20 °C and relative air humidity of 70–85%. The nutrient solution was renewed after every 3 days.

2.2. Mn treatments, and sample preparation

After pre-culturing for 2 weeks on the basic medium, well grown and uniform size seedlings were selected for various Mn treatments. Manganese was applied as $MnSO_4$ and the plants were exposed to different Mn concentrations viz. 50, 100 and 500 μM. Based on the previous findings, each of citric acid and EDTA (5 mM) was applied as amendment to the highest level of Mn treatment (500 μM). While control plants were grown on the basic nutrient solution (containing 0.5 μM Mn). Three biological replicates were used. Plants were harvested after 14 days of treatment. At the time of harvest, roots were soaked in 20 mM Na–EDTA for 15 min to remove excess metal ions adhering to the root surfaces. Fresh samples of shoots were immediately frozen in liquid nitrogen and stored at −80 °C for analysis of antioxidants and malondialdehyde (MDA) contents, with three technical replicates.

2.3. Mn determination

For quantification of Mn, the plants were separated into roots and shoots, and dried at 70 °C for 48 h. Dried plant samples (0.1 g) were digested with 5 mL HNO_3 and 1 mL $HClO_4$ in closed Teflon vessels until transparent. The digested material was washed into a 50 mL flask and made to volume using de-ionized water. Manganese concentration in plant samples was determined on the Inductively Coupled Plasma Mass Spectrophotometer (Agilent 7500a). The amount of Mn taken up by plants was expressed as mg kg^{-1} dry weight. Quality control and quality assurance (QA/QC) for Mn in plants were met by using the standard reference material GBW10010 (GSB-1) from Institute of Geophysical and Geochemical of Earth (IGGE), China.

2.4. Determination of biochemical components

The samples were washed with distilled water and ground with a mortar and pestle under the chilled condition in the homogenization buffer specific for each enzyme. The activities of antioxidative enzymes such as SOD and POD and MDA content were simultaneously determined according to Leul and Zhou [26] as the following.

Superoxide dismutase (SOD) activity was assayed by using the photochemical nitro blue tetrazolium (NBT) method. The samples (0.5 g) were homogenized in 5 mL extraction buffer consisting of 50 mM phosphate (pH 7.8). The assay mixture in 3 mL contained 50 mM phosphate buffer, pH 7.8, 26 mM L-methionine, 750 μM NBT, 1 μM EDTA, and 20 μM riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD is defined as being present in the volume of extract that causes inhibition of the photoreduction of NBT by 50%.

Peroxidase (POD) activity was measured with guaiacol as the substrate in a total volume of 3 mL. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2 and plant extract. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm. Enzyme activity was calculated in terms of μmol of guaiacol oxidized min^{-1} g^{-1} fresh weight at 25 ± 2 °C.

The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as 2-thiobarbituric acid

(TBA) reactive metabolites. Plant fresh tissues (0.2 g) were homogenized extracted in 10 mL of 0.25% TBA made in 10% trichloroacetic acid (TCA). Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 5000 × *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as μmol g^{-1} fresh weight by using an extinction coefficient of 155 mM cm^{-1}.

The assay for ascorbate peroxidase (APX) activity was carried out in a reaction mixture of 3 mL containing 100 mM phosphate (pH 7.0), 0.1 mM Na–EDTA, 0.3 mM ascorbic acid, 0.06 mM H_2O_2 and 100 μL plant extract. The change in absorption at 290 nm was recorded 30 sec after addition of H_2O_2 [27].

Glutathione reductase (GR) was assayed following the method of Foyer and Halliwell [28] by monitoring the decrease in absorbance at 340 nm caused by NADPH oxidation (E = 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.8, containing 0.2 mM EDTA), 0.5 mM GSSG, 0.12 mM NADPH, and the plant extract aliquot.

2.5. Transmission electron microscopy

Shoot segments (2–3 mm in length) of selected plants treated with Mn (500 μM), Mn (500 μM) + citric acid (5 mM), and Mn (500 μM) + EDTA (5 mM)) were obtained and fixed in 2.5% glutaraldehyde (v/v) in 0.1 M of PBS (sodium phosphate buffer, pH 7.4) at room temperature and washed three times with same PBS. The samples were post fixed in 1% OsO_4 (osmium (VIII) oxide) for 1 h, then washed three times in 0.1 M PBS (pH 7.4) with 10 min interval between each washing. Later, they were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) with 15–20 min interval and finally by absolute acetone for 20 min. The samples were infiltrated and embedded in Spurrs' resin overnight. After heating the specimens at 70 °C for 9 h, ultra-thin sections (80 nm) were prepared and mounted on copper grids for viewing by a transmission electron microscope (JEOL TEM-1230EX) at an accelerating voltage of 60.0 kV. Data for cells and sub-cellular organelles were recorded using software JeDa 801D Morphology Image Analysis Systems. Data for cell and sub-cellular organelles were recorded from at least 30 cell samples and were averaged.

2.6. Data analysis

One-way analysis of variance (ANOVA) was performed for plant growth, biomass and biochemical parameters. Data were analyzed using SAS v.9 software. All results were expressed as mean ± SE from three replications. Least significant difference (LSD) test was applied at 5% level of probability to separate the means with statistically significant difference.

Table 1
Biomass and height of *Juncus effusus* L. plants under the effects of Mn alone and with chelators.

Treatment	Shoot length (cm)	Dry weight (mg $plant^{-1}$)	
		Shoot	Root
CK	16.93 a	35.12 ab	26.61 a
Mn 50 μM	16.15 a	37.84 a	18.44 c
Mn 100 μM	15.51 ab	35.52 ab	19.29 b
Mn 500 μM	13.51 c	33.42 b	18.41 c
Mn 500 μM + 5 mM CA	14.42 b	35.12 ab	21.58 b
Mn 500 μM + 5 mM EDTA	14.11 b	31.44 c	20.38 bc

Each value is the mean of three individual replicates. Means followed by the same letter are not significantly different at $P < 0.05$. CK is the control plants grown in the basic nutrient media. CA = citric acid.

3. Results

3.1. Plant growth attributes

Manganese toxicity to *J. effusus* plants was appraised by its influence on dry biomass and height (Table 1). Although there was a reduction in plant dry biomass and height at the higher levels of Mn, however, the plants did not show any visible phytotoxic symptoms. Only the Mn (500 mM) treatment caused significant decrease in plant height. Shoot weight was changed variably but non-significantly with Mn treatments, and there was a significant linear reduction in root weight at increased Mn levels. Compared to the plants treated with Mn (500 μM) alone, addition of chelators, both citric acid and EDTA significantly increased the shoot length. The EDTA in Mn (500 μM) treated plants significantly reduced the shoot dry weight. However, addition of citric acid along with Mn (500 μM) caused significant increase in root and non-significant in shoot biomass.

3.2. Uptake and translocation of Mn

Manganese contents were examined in the roots and shoots of *J. effusus* plants after 14 days of treatments. There was an increase in Mn contents per plant with increasing Mn concentration in the medium, and it was further enhanced with the addition of chelators, especially with EDTA (Table 2). Relatively higher Mn contents were found in the shoots compared to roots on per plant basis in *J. effusus*. Similarly, there was a concentration-dependent increase in Mn contents in *J. effusus* plants with increasing levels of Mn in the growth medium. However, higher amounts of Mn were accumulated in the roots compared to that in shoots. A significant enhancement in Mn accumulation was recorded under the combined application of Mn (500 μM) with citric acid or EDTA. Translocation factor (TF) value expressed as the ratio of Mn concentration in shoot and root had a consistent reduction with increasing levels of Mn, however, it was improved with the addition of chelators along with Mn (500 μM).

Table 2
Manganese uptake and translocation by *Juncus effusus* L. under the effects of Mn alone and with chelators.

Treatment	Mn content (μg $plant^{-1}$)		Mn concentration (mg kg^{-1})		TF value
	Shoot	Root	Shoot	Root	
CK	2.27 e	2.20 d	64.70 e	82.91 f	0.78 b
Mn 50 μM	4.54 d	2.51 d	120.21 d	136.41 e	0.88 a
Mn 100 μM	4.82 d	3.61 c	137.03 d	187.37 d	0.73 b
Mn 500 μM	5.86 c	5.74 b	175.98 c	310.04 c	0.56 d
Mn 500 μM + 5 mM CA	7.84 b	5.72 b	195.26 b	328.97 b	0.59 cd
Mn 500 μM + 5 mM EDTA	9.28 a	9.77 a	295.21 a	452.89 a	0.65 c

Each value is the mean of three individual replicates. Means followed by the same letter are not significantly different at $P < 0.05$. CK is the control plants grown in the basic nutrient media. CA = citric acid. TF (translocation factor) value is the ratio of metal concentrations in shoots and roots of the plant.

Table 3
The MDA contents and antioxidative enzyme activity in *Juncus effusus* L. shoots under the effects of Mn alone and with chelators.

Treatment	MDA content (nmol g^{-1} FW)	SOD activity (U g^{-1} FW)	APX activity (U g^{-1} FW)	POD activity (OD470 g^{-1} FW min^{-1})	GR activity (μmol mg^{-1} protein min^{-1})
CK	33.97 c	231.21 c	165.21 c	18.55 bc	8.87 c
Mn 50 μM	35.68 c	255.91 bc	226.18 a	20.56 ab	21.28 b
Mn 100 μM	42.35 b	288.51 b	188.89 b	10.24 d	23.95 b
Mn 500 μM	47.33 a	316.75 a	181.87 b	7.62 e	31.41 a
Mn 500 μM + 5 mM CA	44.44 ab	307.10 ab	212.09 ab	21.95 a	28.05 ab
Mn 500 μM + 5 mM EDTA	48.59 a	271.80 b	214.28 ab	16.99 c	27.61 ab

Each value is the mean of three individual replicates. Means followed by the same letter are not significantly different at $P < 0.05$. CK is the control plants grown in the basic nutrient media. CA = citric acid.

3.3. Lipid peroxidation and antioxidant enzymes

Lipid peroxidation was measured in the shoots of treated *J. effusus* plants in terms of MDA content (Table 3). Under Mn treatments, elevated levels of lipid peroxides were recorded compared to the control plants. Although a low concentration of Mn (50 μM) had no significant effect on MDA contents, a concomitant increase in lipid peroxides was recorded with increasing levels of Mn (Table 3). There was non-significant effect of adding citric acid and EDTA to Mn (500 μM) treatment on MDA contents.

Activities of antioxidative enzymes in *J. effusus* plant shoots were exhibited variably with different treatments of Mn alone and in combination with citric acid or EDTA (Table 3). The SOD activity showed marked increase in response to Mn stress with a non-significant increase at low Mn but a significant rise at the highest Mn level. Addition of citric acid caused non-significant decrease in the SOD activity; however, it was significantly reduced by the addition of EDTA.

The APX activity significantly increased with the lowest Mn level (50 μM) and then decreased gradually by increasing Mn concentrations, however, still significantly greater compared to untreated plants (Table 3). It was again increased sharply under citric acid or EDTA treatments in combination with Mn (500 μM). Changes in the POD activity of *J. effusus* shoots followed a similar pattern as was observed in case of APX activity. It increased non-significantly at the first treatment and then decreased sharply with increasing Mn concentrations. Compared to the highest level of Mn (500 μM) alone, there was a significant enhancement in POD activity with the addition of citric acid as well EDTA with Mn (500 μM). The GR activity was increased significantly under elevated levels of Mn. Addition of citric acid as well as EDTA to Mn (500 μM) treatment caused non-significant reduction in GR activity compared to Mn (500 μM) alone.

3.4. Morphometric cellular changes

Electron microscopic images of mesophyll cells revealed several changes in the size of cells and sub-cellular organelles of the treated plants (Table 4). Compared to that in control, the plants exposed to the highest concentration of Mn (500 μM) alone and in combination with citric acid or EDTA showed non-significant change in cell size. The cell diameter was reduced significantly with Mn + EDTA over other treatments. Nucleus size was increased with Mn treatment significantly over control, but the increase was non-significant if chelators were added to Mn treatment. Compared to the control plants significant increase in chloroplast size and width was observed in the plants treated with Mn (500 μM) alone and along with EDTA. However, the chloroplast length was increased significantly only in the plants treated with Mn alone. Addition of citric acid to Mn treated plants reduced their chloroplast size although none significantly compared to that in Mn treatment alone.

3.5. Ultrastructural modifications

Transmission electron micrographs (TEM) of the shoot mesophyll cells of the control and the plants treated with Mn (500 μM) alone and in combination with citric acid or EDTA are shown in Fig. 1. In the control plants, typical mature cells with well defined cell wall, containing nucleus and chloroplast were present (Fig. 1A). The cell wall was thick and compact, and the intercellular spaces were nearly absent Moreover, lens-shaped chloroplasts with thylakoid membrane system forming well-organized grana in heterogeneous stroma and a few dense plastogobuli were also seen in the control treatment cells (Figs. 1A and 2A). Mesophyll cells of plants treated with Mn (500 μM), and Mn (500 μM) with citric acid or EDTA showed some structural modifications. Cell wall became thin, contracted and deformed (Fig. 1B–D) that resulted alteration in cell shape.

3.6. Damage to chloroplast

Ultrastructural images revealed that chloroplast was the obvious site of damage caused by Mn treatments. Although differential modifications in chloroplasts were noticeable, the disorganization of chloroplasts was more common i.e. swelling of chloroplasts and appearance of large number of plastoglobuli (Figs. 2 and 3[B–D]). Uneven swelling and structural alteration of chloroplast as well as increased amount of plastoglobuli were observed in the plants exposed to Mn (500 μM) alone (Figs. 2 and 3B). Swollen chloroplasts and loose thylakoid membranes were also observed in the plants under the combined treatment of citric acid and Mn, however

Table 4
The size of cell and sub-cellular organelles of *Juncus effusus* L. shoots under the effects of Mn alone and with chelators.

Morphometric parameter (μm)	CK	Mn 500 μM	Mn 500 μM + CA 5 mM	Mn 500 μM + EDTA 5 mM
Cell size	50.66 ab	48.98 ab	55.76 a	44.12 b
Cell diameter	16.17 a	15.83 a	13.86 ab	11.90 b
Nucleus size	10.96 b	14.49 a	11.29 ab	12.84 ab
Chloroplast size	12.84 b	16.04 a	14.45 ab	18.11 a
Chloroplast length	5.19 b	6.91 a	5.87 b	6.25 ab
Chloroplast width	3.30 b	4.87 a	4.14 ab	4.91 a

Each value is the mean of three individual replicates. Means followed by the same letter are not significantly different at $P < 0.05$. CK is the control plants grown in the basic nutrient media. CA = citric acid.

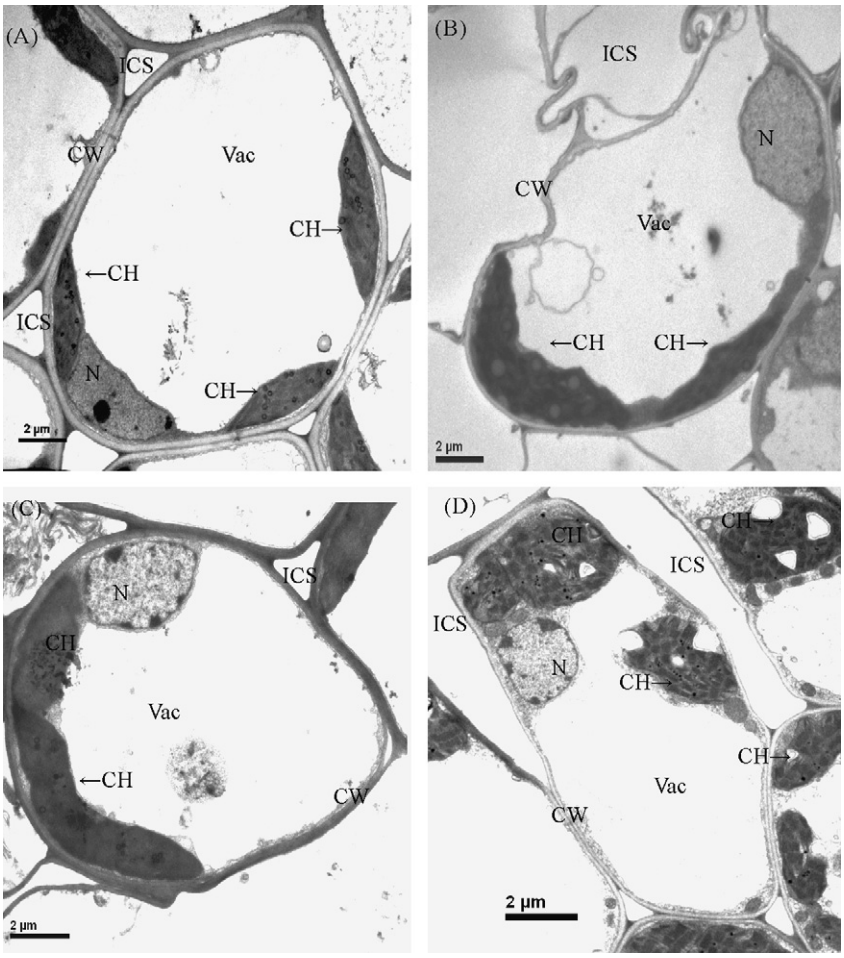

Fig. 1. A whole mesophyll cell of mat rush (*Juncus effusus*) under: (A) control; (B) 500 μM Mn alone; (C) 500 μM Mn + 5 mM citric acid; (D) 500 μM Mn + 5 mM EDTA. Reduction in cell wall thickness and chloroplast swelling is obvious in cells exposed to Mn (500 μM) alone. A large number of plastoglobuli is clearly visible in the chloroplast of plants treated with Mn + citric acid, and their size further increased when exposed to Mn + EDTA. Chloroplast swelling and scattering of thylakoids in the stroma are also visible in Mn treated plant cells. CH, chloroplast; CW, cell wall; ICS, inter-cellular spaces; N, nucleus; Vac, vacuole. Bars A–D = 2 μm.

amount of plastoglobuli was relatively lower. No starch accumulation was noticed in the chloroplast of plants treated either with Mn alone or in combination with citric acid (Figs. 2 and 3B and C). Plants exposed to Mn with EDTA showed chloroplast swelling, thylakoid disintegration, and the appearance of increased numbers of plastogobuli and starch grains (Figs. 2 and 3D). Higher magnification revealed that thylakoid system forming grana was present in chloroplasts of all the plants irrespective of the treatment, however, the arrangement and amount of thylakoid system was disrupted.

4. Discussion

Plant biomass and height serve as indicators of metal toxicity [29]. Manganese toxicity is well known in plants, and the knowledge of its toxic effects on plant biometry may contribute to better understand the toxicity mechanism and plant responses [30]. Current investigations on *J. effusus* divulged that higher level of Mn (500 μM) reduced the plant biomass and shoot length. Growth inhibition could be a consequence of metal interference with a number of metabolic processes such as proteins synthesis [31], enzyme activities [32], impairment of cell and sub-cellular organelles, and reduction of photosynthesis [33]. Interestingly, both the chelators viz. citric acid and EDTA impoverished the toxic effects of Mn on shoot length. The reduced toxicity of Mn due to chelation can be explained by free ion activity model (FIAM) introduced by Morel [34], which suggests that metal toxicity is related to uptake of specific metal species at the organism-solution interface. According to FIAM, the ability of plant to take up metals has a direct relationship with their bioavailability [35]. Markich et al. [36] also reported that biological response of *V. angasi* exposed to Mn was directly proportional to the activity of Mn^{2+}. Tandy et al. [37] suggested that the decrease in Cu and Zn toxicity symptoms in the presence of chelator was the result of reduction in the free metal concentration in solution, therefore agreeing with FIAM.

Our findings revealed that although both EDTA and citric acid caused significant increase in Mn accumulation; however, the Mn translocation was enhanced markedly by EDTA. The chelator-boosted Mn translocation from roots to the shoots was obvious from the increased TF value. This was mainly due the activation of ATPase in the plasma membrane producing charges that are responsible for the translocation of both essential and non-essential metals [38]. The EDTA caused significant reduction in shoot biomass; whereas, addition of citric acid in the media with Mn had growth restoring effect on plant. The EDTA-enhanced phytoextraction of metals is an intensively studied method; however, its poor biodegradability [39] results increased risk of leaching [40] and impairment of plant growth. Muhammad et al. [41] described that EDTA significantly reduced plant height and biomass of *Typha angustifolia* L., whereas citric acid caused significant increases in root dry weight. Evangelou et al. [42] also found similar results from EDTA, which caused plant growth reduction, while citric acid had no adverse effects on plant growth. Therefore, EDTA being recalcitrant to biodegrada-

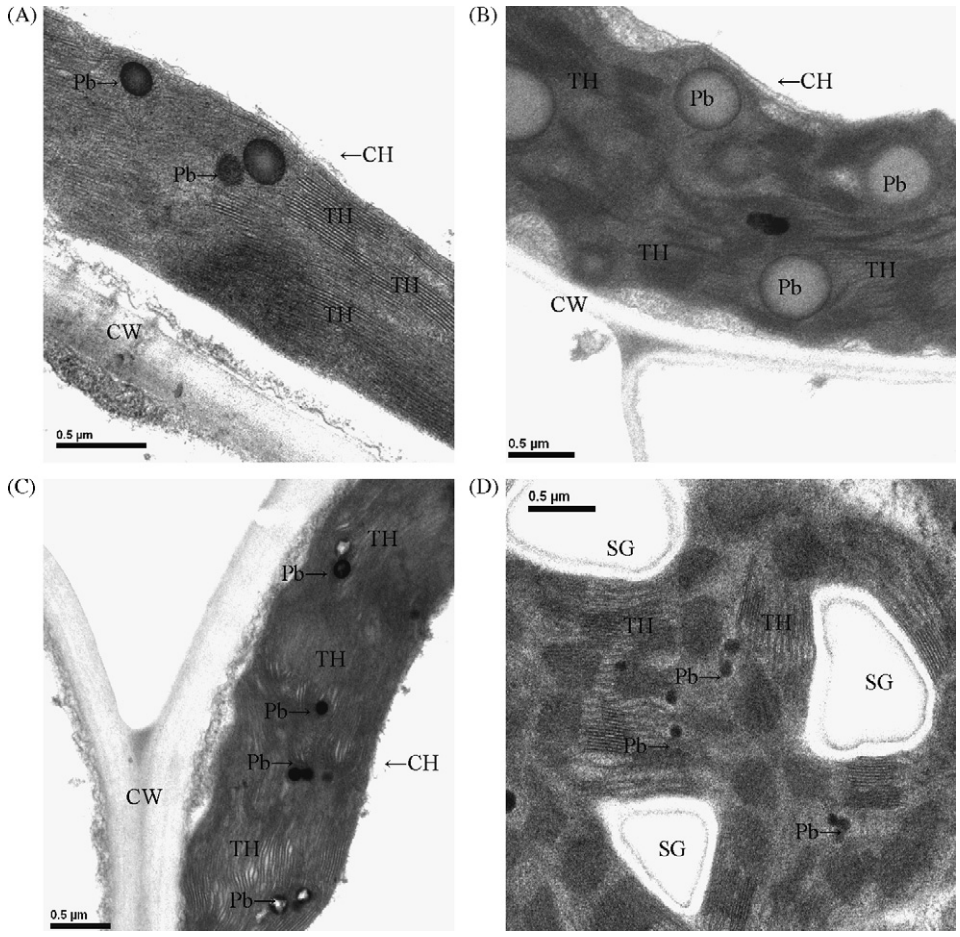

Fig. 3. Magnified view of chloroplast and thylakoid membranes of mat rush (*Juncus effusus*) under: (A) control; (B) 500 μM Mn alone; (C) 500 μM Mn + 5 mM citric acid; (D) 500 μM Mn + 5 mM EDTA. CH, chloroplast; CW, cell wall; SG, starch grains; Pb, plastoglobuli; TH, thylakoid membranes. Bars A–D = 0.5 μm.

tion as well as showing obvious phytotoxicity cannot be a preferred choice for phytoremediation.

Metal uptake in plants is positively related with metal concentration in the growing media [43], and it was also true for Mn in the present study. However, the decreased value of TF under increasing levels of Mn showed that plants stored higher amounts of the metal in their roots. The lowering of Mn transport in *J. effusus* suggests a reduction in the mobility of Mn from roots that contributes to the metal stress tolerance [29,44] or avoiding metal stress [45]. Different factors like decrease in pH, reduction of MnO_2 and Mn complex formation affect the Mn availability. Hocking et al. [46] reported an increased availability of Mn in the rhizosphere due to the presence of LMWOA. Citric acid and other LMWOA provide protons and electrons for reduction of metals in the rhizosphere coupled with oxidation of organic acid [47]. Reduction of Mn(IV) to Mn(II) increases the solubility of MnO_2 in soil, and consequently enhances its accumulation by the plants [48]. Wetland plants species including *J. effusus* growing in metal contaminated soils have strong tendency to store metals in their roots [12]. Such plants remove metals through immobilization in the roots and oxygenated rhizosphere [14]. These plants are suitable as phyto-stabilizers for re-vegetation of waterlogged mine tailings and metal-contaminated lands [12].

In acidic soils, Mn toxicity is one of the important abiotic stresses [49] that affects different physiological and biochemical processes associated with plant growth and development by producing reactive oxygen species [10,33]. These toxic species enhance lipid peroxidation, protein denaturation, DNA mutation, and destruction of membranes. The Mn concentration-dependent increase in the lipid peroxidation in *J. effusus* plants in the present study indicated that Mn caused oxidative stress. Elevated MDA contents were also reported in *Cucumis sativus* with Mn [10], and in soybean under Cd stress [50].

Antioxidant enzymes and certain metabolites play an important role in adaptation and ultimate survival of plants during stress. Oxidative stress modifies the activities of antioxidative enzymes, which reflects a general strategy required to overcome this stress [50]. Increased activity of SOD under Mn stressed plants was observed that was a function of Mn concentration in the plant tissues. The SOD is an essential component of plants antioxidative defense system as it dismutates O^{2-} to H_2O_2 and O_2. Similarly, POD and APX are important enzymes and cause break down of H_2O_2 [51]. A decrease in the activity of these enzymes under higher Mn concentration, suggested a possible delay in the removal of H_2O_2 and toxic peroxides mediated by POD and APX. Enhanced activities of some antioxidative enzymes under combined application of Mn and chelators indicate an increased resistance to Mn-induced oxidative burst in *J. effusus* plants. We observed a significant increase in the activity of GR in response to Mn stress. The GR is also an important element of antioxidative system in a plant cell that catalyzes the reduction of GSSG to GSH in a NADPH-dependent reaction [11]. Our results showed that chelators in the presence of high concentration of Mn lowered down the GR activity, but increased the activities of APX and POD.

Electron micrographs reflected that ultrastructural alterations in shoot cells were mainly concentrated in the membranes, cell wall and chloroplasts (Figs. 1 and 2). Swelling of chloroplasts, increased number of plastoglobuli, and disruption of thylakoid membrane

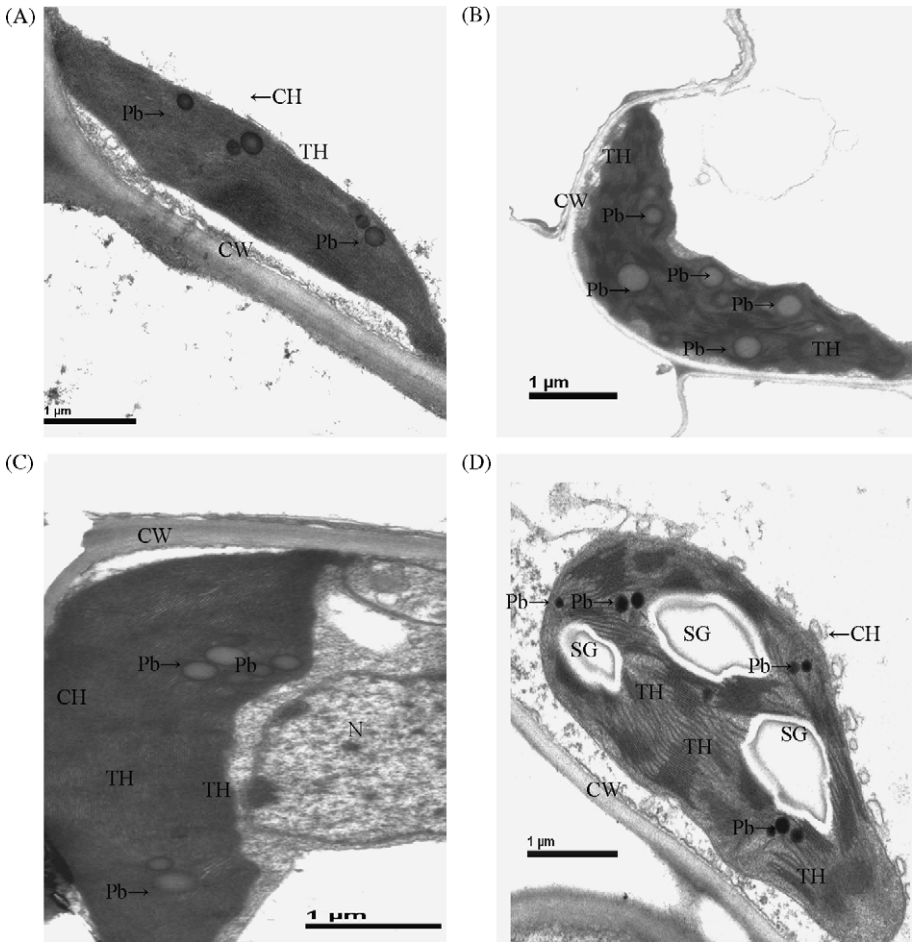

Fig. 2. Chloroplast and thylakoid membrane of mat rush (*Juncus effusus*) under: (A) control; (B) 500 μM Mn alone; (C) 500 μM Mn + 5 mM citric acid; (D) 500 μM Mn + 5 mM EDTA. Chloroplast swelling and increased number of plastoglobuli are evident in plant cells exposed to Mn alone as well as with citric acid or EDTA. Starch granules are viewed only in the chloroplast of plants treated with Mn + EDTA. CH, chloroplast; CW, cell wall; SG, starch grains; Pb, plastoglobuli; TH, thylakoid membranes. Bars A–D = 1 μm.

systems were seen under elevated levels of Mn alone as well as in combination with EDTA. A reduction in the number of plastoglobuli in the chloroplast of plants treated with citric acid and Mn indicated that citric acid might have played a role in alleviation of toxic effects of Mn in *J. effusus* plants. Thylakoid swelling along with lipid droplets is a general symptom of different stresses [52]. Formation of plastoglobuli is linked with the break down of thylakoids [53], and it indicates disturbed lipid metabolism [54]. Damage to chloroplast was the result of Mn-induced oxidative stress. Chloroplast is highly susceptible to oxidative stress caused by elevated oxygen levels, electron flux, and the presence of metal ions in their microenvironment [55]. Heavy metal stress was reported to change the morphology of chloroplast, increase the number and size of starch grains as well as plastoglobuli in plants [56]. The positive role of citric acid in the alleviation of Mn toxicity was, therefore, obvious from the reduced number of plastoglobuli in the chloroplast of citric acid treated plants.

5. Conclusion

J. effusus plants tolerated higher Mn concentration stress without visible symptoms of phytotoxicity. Substantial amounts of Mn were accumulated by plants with more storage in the roots. Retention of toxic metal in the roots and less translocation to the aerial parts can be regarded as a positive feature in wetland plants. These plants may have the ability to increase antioxidant protection for combating negative consequences of Mn-induced oxidative stress. Decline in some antioxidant activities could be responsible for the growth reduction after plant exposure to Mn toxicity stress. Chelators increase the accumulation of Mn in plant, especially the uptake and translocation of Mn is enhanced markedly. Ultrastructural examination of shoot cells is helpful in understanding the mechanism of Mn stress tolerance. The study explores the prospects of using citric acid for enhancing the accumulation of Mn, and restoring the plant growth under Mn stress.

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