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MAT RUSH (*JUNCUS EFFUSUS* L.) TROUNCES MANGANESE TOXICITY THROUGH ULTRA-MORPHOLOGICAL MODIFICATIONS AND MANGANESE RESTRICTION IN ROOTS

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Abstract

This study appraised phyto-remediation efficiency and tolerance mechanism of *Juncus effusus* as was evidenced by ultra-structural modification in its roots under manganese (Mn) toxicity. Three-week-old *J. effusus* plants were treated with different concentrations of Mn (50, 100 and 500 µM) in hydroponics. Although higher Mn levels caused modifications in growth, biomass, height and root morphological traits, *J. effusus* tolerated Mn toxicity without showing any obvious phyto-toxic symptoms even under the highest level of Mn (500 µM). With incremental Mn levels in the growth media, the plants showed a steady increase in Mn uptake, while translocation factor (TF) for Mn declined. This illustrated the tendency of *J. effusus* plants to avoid Mn-induced stress by restricting maximum Mn in root tissues. Electron microscopy of root tip cells elucidated plant tolerance mechanism to Mn toxicity. Modification in cellular shape and size, and increased number of vacuoles and mitochondria appeared to play a major role in induction of tolerance against Mn toxicity, and ultimate survival of plant.

Key words: Plant biomass; Manganese toxicity; Root tip cells; Ultra-structure.

Introduction

Manganese (Mn), an essential micronutrient, is imperative for plant growth and metabolism due to its role in redox reactions as a cofactor for different enzymes (Millaleo *et al*., 2010). However, excessive crop irrigation practices and application of acidic fertilizers in many parts of the world have lowered the soil pH levels leading to increased Mn availability in soils (Huang *et al*., 2014) and subsequent toxicity to plants (Gangwar *et al*., 2011). Increased Mn concentration inside plant tissues can alter the metabolic processes by oxidative damage and nutrient imbalance (Shi *et al*., 2006).

Plants growing under Mn polluted environments can endure Mn toxicity stress through two major pathways i.e., constitutive (in sensitive plants) or adaptive (in tolerant plants) (Mou *et al*., 2011). Cell wall plasma membrane of tolerant plants is the main site of metal tolerance, where maximum metal accumulation takes place. In order to tolerate Mn toxicity, Mn ions are sequestered within the cells i.e., vacuole, close to cell wall or in leaf trichomes to protect active cellular organelles (Papadakis *et al.,* 2007). Some plants produce metal lothionein, which can protect plants from Mn-induced toxicity and oxidative burst by capturing Mn ions (Foley & Singh, 1994). The plants possessing characteristics such as rapid growth, deep rooting system and increased biomass production are generally considered important for plants to adapt to environmental stresses (Römkens *et al*., 2002).

Many wetland plants including *Juncus effusus*, cattail (*Typha latifolia* L.) and *Cyperus malaccensis* etc., demonstrate great tolerance to metal toxicity along with potential for phytoremediation (Deng *et al*., 2004). Although, heavy metal hyper-accumulation and metal stress tolerance in plants are genetically independent traits; the application of hyper-accumulators with ability to tolerate metal-induced stress is considered more useful (Maestri *et al*., 2010; Zulfqar *et al*., 2012). Due to its potential to produce high above ground biomass and adaptation to acidic soils (Grime *et al*., 1990), *J. effusus* could be an ideal candidate for phytoremediation of Mn polluted soils. It has been widely used for construction and restoration of wetlands, phytoremediation of heavy metals (Rahman *et al*., 2014), wastewater treatment, and stimulation of microbial activity (Kuehn *et al*., 2000).

In previous studies, we explored the phytoextraction potential of *J. effusus* against excessive cadmium and lead concentration in growth media (Najeeb *et al*., 2009, 2011, 2014). The present experiment was conducted to investigate the tolerance mechanism of plant roots against Mn toxicity stress. For this objective, we assessed the toxic effects of Mn, on plant growth, biomass and root morphology, and studied the role of ultra-structural modifications in plant roots to defy Mn toxicity stress.

Material and Methods

Plant material and cultural conditions: Seeds of Nonglin-4 (a commercial cultivar of *J. effusus* L.) were kindly supplied by Dr. W.Q. Shen, University of Nottingham Ningbo, Ningbo, Zhejiang, China, and were stored in the dark at 4°C until use at Zhejiang University, Hangzhou, China. Solution of $KMnO_4$ (0.05 g) in 80 mL sterile water was used for sterilizing these seeds for 72 hours. The seeds were treated with 70% ethanol for 1 hour, washed 3 times with sterile water and then continuously shaken in 1.0% sodium hypochlorite having 2 drops of Tween-20 before final rinsing with sterile water (Xu *et al*., 2009).

After sterilization, the seeds were grown in glass growth vessels containing 30 mL hormone-free MS (Murashige & Skoog, 1962) medium solidified with 0.8% agar. Two-week-old seedlings were pre-cultured for one 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 medium was kept to 5.8 by regular addition of 0.1 mol L^{-1}NaOH or HCl. Plants were grown under glasshouse conditions with 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.

Manganese treatment and sample preparation: After two weeks of pre-culturing on the basic media, healthy and uniform seedlings were selected for various Mn treatments. Manganese was applied as $MnSO_4$, and the plants were exposed to different Mn concentrations, i.e. 50, 100 and 500 µM, while the control plants were grown on the basic nutrient solution listed above (Containing 0.5 µM Mn). The plants were harvested after 14 days of treatment. At the time of harvest, the roots were soaked in 20 mM Na_2–EDTA for 15 min to remove excess metal ions from root surfaces.

Growth inhibition: Relative growth rate (RGR) of *Juncus effusus* plants were estimated on dry biomass basis and calculated using the equation of Maine *et al.* (2001):

$$RGR = (W1 - W2)/(T1 - T2)$$

W1 and W2 (mg) are plant dry biomasses at time T1 and T2 (day), respectively.
Whereas, growth rate inhibition (GRI) was calculated using the equation of Drost *et al*. (2007).

$$GRI = \frac{1 - PGR \text{ of Mn} - \text{treat plants}}{PGR \text{ of control}} \times 100$$

Manganese determination: The harvested plants were separated into roots and shoots, dried at 70ºC for 48 hours, and the samples were used for determination of Mn contents. These dried plant samples (0.1 g) were digested with 5 mL HNO_3 and 1 mL $HClO_4$ in closed Teflon vessels until transparent. De-ionized water was used for washing of digested material in a 50 mL flask. The Mn content in plant samples was analyzed by Inductively Coupled Plasma Mass Spectrophotometer (Agilent 7500a). The Mn contents in plants were expressed as mg kg^{-1} dry weight.

Transmission electron microscopy: Root tip segments (approximately 2-3 mm in length) of the selected plants were washed with 0.1 M PBS (Sodium Phosphate Buffer, pH 7.4) and fixed in 2.5% glutaraldehyde (v/v) in PBS at room temperature. Samples were post-fixed in 1% OsO_4 (osmium (VIII) oxide) for 1 hour, and washed again with the same buffer solution for 3 times. These samples were dehydrated through a series of ethanol concentrations (50, 60, 70, 80, 90, 95, and 100%) and then final with absolute acetone each for 15-20 min. The samples were cleaned and embedded in Spurr's resin for 24 hours. The specimens were heated at 70°C for 9 hours and cut into ultra-thin (80 nm) sections for mounting on copper grids to observe under transmission electron microscope (JEOL TEM-1230EX) at an accelerating voltage of 60.0 kV.

Data analysis: One-way analysis of variance (ANOVA) was performed for plant growth, biomass, and Mn accumulation. Data were analyzed using SAS (version 9.0) software. All results were expressed as mean ± SE from three replications. Least significant difference test (LSD) was applied at 5% level of probability to separate the significant means.

Results

Biomass production and metal uptake by plant: Toxic effects of Mn on *J. effusus* were evaluated by its influence on plant biomass, growth rate, height, and root morphology as summarized in Tables (1 & 2). Plants exposed to different Mn levels demonstrated no visible symptoms of phyto-toxicity; instead these plants displayed slight increase in shoot dry biomass at the lowest Mn treatment (50 µM). Higher Mn concentrations (100 and 500 µM) in the growth media, on the other hand, significantly reduced shoot and root dry weight of *J. effusus*. Rise in the shoot and root dry weight ratio (S/R) under increasing Mn concentrations suggested that Mn toxicity had relatively higher inhibitory effects on roots. Lower Mn concentration (50 µM) had a positive effect on plant growth attributes that was obvious from the negative value of growth inhibition rate; however, the growth rate exhibited downward trend under higher Mn level. Similarly, plants showed no significant reduction in shoot length and root morphological traits (volume, surface area, and diameter) lower Mn concentration, while increasing Mn concentrations significantly reduced these traits.

Manganese uptake and translocation: Manganese contents in the roots and shoots of *J. effusus* plants were examined 14 days after treatments. There was a concentration-dependent rise in Mn contents in *J. effusus* tissues under increasing Mn levels in the growth medium (Table 3). With increasing Mn concentration in growth media, there was a consistent reduction in translocation factor (TF) of Mn indicating potential of *J. effusus* plants to deposit comparatively higher Mn ions in root than shoot tissues.

Ultra-structure modification in root tip cells: Electron micrographs of root tip cells of *J. effusus* depicted modification in the size and shape of Mn-treated (500 µM) plants. Compared with control, the plants exposed to elevated levels of Mn exhibited a slight reduction in overall cellular size. In control plants, the root tip cells were oval shape with a compact thick cell wall, smooth and continuous cell membranes. These cells contained a large central vacuole, one round nucleus, and few mitochondria and plastids (Figs. 1A & 2A). The plastids had well-defined lamellar membrane system and a small number of round plastoglobuli (Fig. 2A & Fig. 3A). In root meristematic cells of Mn-treated plants, considerably high Mn contents were present inside the vacuole and adjacent to cell wall forming crystals or micro granules. These cells contained higher number of mitochondria and numbers of nucleoli (Figs. 1B & 2B). In addition, swelling of nucleus, shrinkage of plasma membrane and disruption of nuclear membrane was also observed in these cells (Fig. 2B). The plastids of Mn-treated plant cells became globular containing a fused and merged lamellar membrane (Fig. 3B).

Table 1. Plant dry biomass weight of *Juncus effusus* L. as influenced by elevated Mn toxicity.

Treatment	Dry biomass weight (mg)		S/R	RGR	GRI
	Shoot	Root			
Control	34.16 ± 2.44a	25.59 ± 2.16a	1.31 ± 0.12 c	0.89 ± 0.12 bc	-3.88 ± 0.14 d
Mn 50 µM	36.89 ± 2.83 a	24.44 ± 2.34a	1.55 ± 0.16 b	1.04 ± 0.08 a	-16.62 ± 1.2c
Mn 100 µM	32.78 ± 2.15 b	18.91 ± 1.85b	1.73 ± 0.16 a	0.77 ± 0.06 c	13.18 ± 1.1 b
Mn 500 µM	30.52 ± 2.37b	16.91 ± 1.52c	1.81 ± 0.17 a	0.65 ± 0.06 d	26.54 ± 1.8 a

Mean values in the same column sharing similar letter(s) are statistically alike at $p<0.05$
S/R = Shoot to root dry weight ratio, RGR = Relative growth rate of plant, GRI = Growth rate inhibition

Table 2. Root morphological characteristics of *Juncus effusus* L. as influenced by elevated Mn toxicity.

Treatment	Shoot length (cm)	Root length (cm)	Root diameter (mm)	Root surface area (cm^2)	Root volume (cm^3)
Control	16.84 ± 1.43 a	2.87 ± 0.23a	26.12 ± 2.72a	4.86 ± 0.31 a	0.67 ± 0.11a
Mn 50 µM	16.21 ± 1.23a	2.74 ± 0.21a	28.29 ± 2.31 a	4.24 ± 0.33b	0.65 ± 0.09a
Mn 100 µM	15.41 ± 1.46ab	2.31 ± 0.19b	24.24 ± 2.15ab	4.12 ± 0.34 b	0.54 ± 0.08 b
Mn 500 µM	14.02 ± 1.15b	1.34 ± 0.14c	19.78 ± 2.32b	2.74 ± 0.28 c	0.47 ± 0.05 c

*Mean values in the same column sharing similar letter(s) are statistically alike at $p<0.05$

Table 3. Manganese uptake and translocation in *Juncus effusus* L. under elevated Mn toxicity.

Treatment	Mn concentration (mg kg^{-1})		Mn concentration (mg $plant^{-1}$DW)		TF value
	Shoot	Root	Shoot	Root	
Control	62.41 ± 5.17d	79.12 ± 7.43 d	3.86 ± 0.41b	4.89 ± 0.28d	0.79 ± 0.0.8b
Mn 50 µM	118.84 ± 8.74c	131.55 ± 10.18 c	7.41 ± 0.54ab	8.20 ± 0.41c	0.90 ± 0.07a
Mn 100 µM	141.43 ± 10.42 b	190.75 ± 14.86 b	7.31 ± 0.71ab	9.86 ± 0.74bc	0.74 ± 0.08 b
Mn 500 µM	181.48 ± 15.16 a	312.43 ± 21.72 a	8.61 ± 0.47a	14.82 ± 1.04a	0.58 ± 0.06 c

Mean values in the same column sharing similar letter(s) are statistically alike at $p<0.05$
TF = Translocation factor

Discussion

Investigating metal toxicity effects on plant growth inhibition rate, visual damage, and metal sequestration are the basis for selecting plant species for metal-polluted environments (Park *et al*., 2011). Current investigation on *J. effusus* divulged that higher Mn (500 µM) concentration can inhibit plant growth by reducing dry weight biomass accumulation process. The plant growth attributes such as biomass, height and root morphology serve as toxicity indicators of metal (Morel, 1983), and reduced *J. effusus* growth under elevated Mn concentrations evidenced the potential hazards of Mn toxicity to higher plants. Mn-induced toxicity has been reported to disturb plant metabolic processes including protein synthesis, cellular damage and reduced cell size (Najeeb *et al*., 2009; Gangwar *et al*., 2011) that could be responsible for overall plant growth reduction. As the aboveground plant parts are more sensitive to Mn toxicity (Mou *et al*., 2011), we used shoot to root dry biomass ratio (S/R) to assess plant tolerance to Mn stress. Increasing S/R value under elevated Mn levels suggested that *J. effusus* can tolerate Mn toxicity. It also indicated a relatively higher damage to *J. effusus* roots by Mn toxicity stress that was evident from ultra-structural modifications in root tip cells.

As the roots tissue can directly influence the uptake of water, nutrients and heavy metals, determination of root morphological attributes becomes crucial for understanding plant tolerance mechanism to various abiotic stresses. In the present study, exceeded Mn levels in the nutrient media inhibited various root growth parameters such as root surface area, volume and diameter. Our results confirmed the findings of Kováčik *et al*. (2014) who observed significantly greater damage to *Matricaria chamomilla* roots compared with shoots under Mn toxicity (100, 500 and 1000 µM). On the other hand, Rezai & Farboodnia (2008) reported relatively more damage to shoot tissues under higher Mn concentrations. This inconsistency in results is due to the tendency of *J. effusus* to store greater Mn concentration in roots, and lower translocation to aerial parts (Najeeb *et al*., 2011).

Juncus effusus experienced a consistent rise in Mn contents in plant tissues under increasing Mn concentrations seems to follow free ion activity model (FIAM) describing a direct relationship between metals uptake by plant and their bioavailability (McLaughlin, 2002). Translocation factor (TF) that measures the efficiency of plants to transport metals from roots to shoots was reduced by increasing Mn levels in the growth media indicating high potential of plants to sequester metal in root tissues (Zheljazkov *et al*., 2008). Low translocation and metal retention in the roots caused comparatively higher damage to root tissues as was evident from the study on root morphology and ultra-structure suggesting slow Mn mobility from roots to shoots attributed tolerance (Horiguchi, 1988) and protection to Mn-induced injury (Hirschi *et al*., 2000). Wetland plant species including *J. effusus* have a strong propensity to immobilize metals in their roots and rhizosphere, and are appropriate phyto-stabilizers for waterlogged mine tailings and metal-contaminated lands (Deng *et al*., 2004).

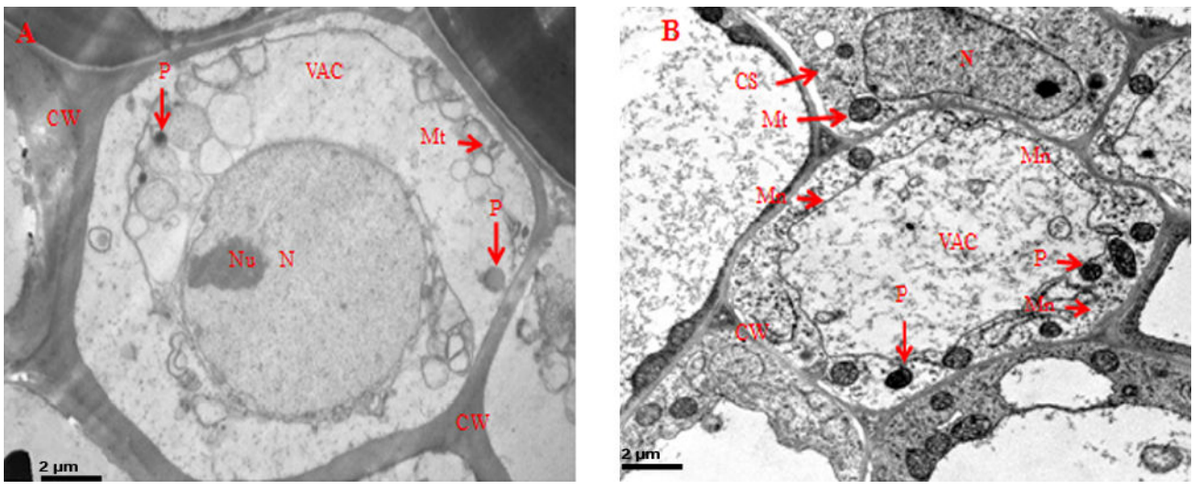

Fig. 1. Root tip cells of *Juncus effusus* in control (A), and in 500 µM Mn treatment (B).
CS, Cytoplasmic shrinkage; CW, Cell wall; Mt, Mitochondria; Mn, Manganese deposition; N, Nucleus; P, Plastids; and Vac, Vacuole. Bar = 2 µm

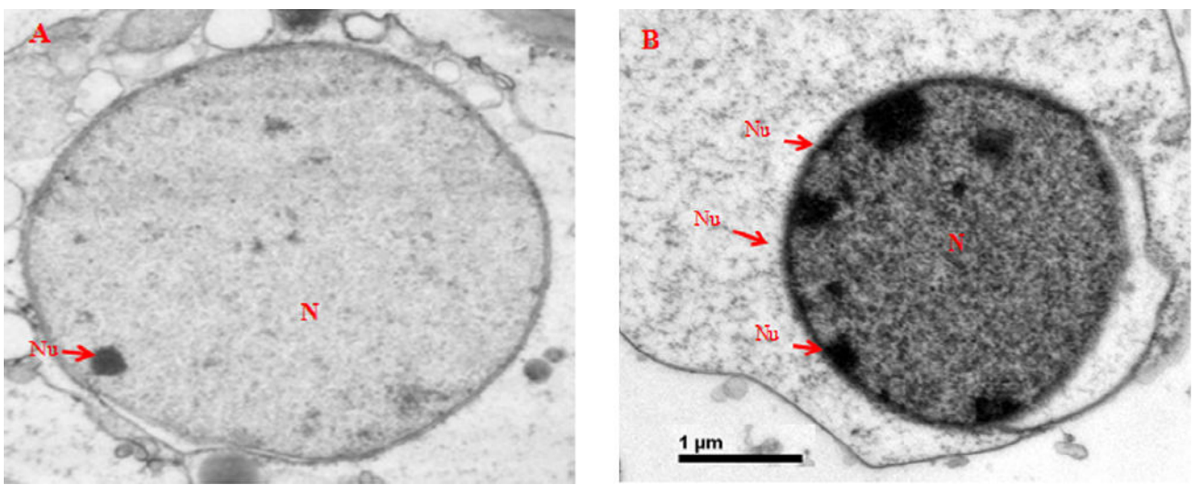

Fig. 2. Nucleus of root tip cells of *Juncus effusus* in control (A), and in 500 µM Mn treatment (B).
N = Nucleus; and Nu = Nucleolus. Bar = 1 µm

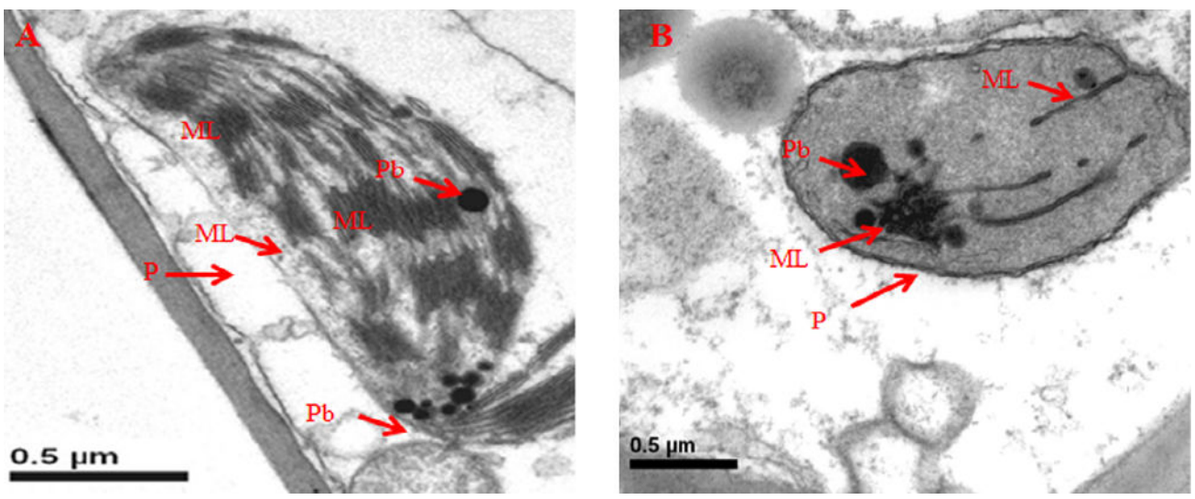

Fig. 3. Plastids of root tip cells of *Juncus effusus* in control (A), and in 500 µM Mn treatment (B).
CW, Cell wall; LM, Lamellar membrane; P, Plastids; and Pb, Plastoglobuli. Bar = 0.5 µm

Higher Mn concentrations have been reported to induce irregularities including changes in cell shape, increase vacuolation and swollen mitochondria in *Acer saccharum* roots (McQuattie & Schier, 2000). Electron micrographs of *J. effusus* root tip cells revealed several modifications such as increased number of mitochondria and nucleoli, Mn deposition inside vacuole, and cytoplasmic shrink age under Mn stress. Increased number of nucleoli under elevated Mn level might amplify ribosomes and mRNA production, and consequently synthesis of proteins to tolerate heavy metal stress (Sresty & Rao, 1999). Localization of excessive Mn concentration has been reported to different parts of the cells such as vacuoles, cell walls or golgi vesicles (Papadakis *et al*., 2007), which can induce tolerance to Mn toxicity by limiting immobilizing Mn ions in cytosol.

Conclusion

In this study, *Juncus effusus* exhibited no apparent symptom of phyto-toxicity; although, higher Mn concentrations negatively affected plant growth and root ultra-structure. These plants accumulated substantial amounts of Mn in root tissues helping them overcome Mn toxicity. Electron microscopy manifested the role of ultra-structural modifications in root cells suggesting that *J. effusus* can trounce Mn toxicity by immobilizing Mn ions in root tissues.

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