Journal of Hazardous Materials 170 (2009) 1156–1163

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

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

U. Najeeb[a], L. Xu[a], Shafaqat Ali[a], Ghulam Jilani[b,*], H.J. Gong[a], W.Q. Shen[c], W.J. Zhou[a,**]

[a] *Institute of Crop Science, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, PR China*
[b] *Department of Soil Science, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Punjab 46300, Pakistan*
[c] *The University of Nottingham at Ningbo, Ningbo 315100, PR China*

ARTICLE INFO

Article history:
Received 27 February 2009
Received in revised form 15 May 2009
Accepted 18 May 2009
Available online 22 May 2009

Keywords:
Plant biometry
Metal accumulation
Antioxidative enzymes
Chloroplast ultrastructure
Lipid peroxidation
Thylakoid disruption

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-

* Corresponding author. Tel.: +92 51 9062241; fax: +92 51 9290160.
** Corresponding author. Tel.: +86 571 86971 770; fax: +86 571 86971 117.
E-mail addresses: jilani@uaar.edu.pk (G. Jilani), wjzhou@zju.edu.cn (W.J. Zhou).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.05.084

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