Effect of Copper Toxicity on Root Morphology, Ultrastructure, and Copper Accumulation in Moso Bamboo (*Phyllostachys pubescens*)

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A hydroponic culture experiment was conducted to study the effect of copper toxicity on root morphology, ultrastructure, and copper accumulation in Moso bamboo (*Phyllostachys pubescens*). Root ultrastructure of Moso bamboo was studied by transmission electron microscopy and scanning electron microscopy. Application of 200 μM Cu resulted in an accumulation of 810 mg kg^{-1} dry weight and 91 mg kg^{-1} dry weight Cu in roots and shoots, respectively. The majority of the plants did not survive the application of 400 μM Cu. Biomass production declined consistently with application of each additional increment of Cu. Root growth was more severely inhibited than shoot growth. Cu adversely affected the root morphology of the plants, however, root surface area and number of root tips increased slightly at low levels of Cu. Root cell ultrastructure and organelles changed significantly under Cu stress, in particular, cell walls, mitochondria, and xylem parenchyma were affected.

Key words: Moso Bamboo, Phytoremediation, Ultrastructure, Root Morphology

Introduction

Copper (Cu) is an essential element for plants' growth, however, due to human activities large amounts of Cu have been released into the environment which adversely affect plant growth. During the past five decades, about 939,000 t of Cu were released into the environment around the world (Singh *et al.*, 2003). The Cu content in arable lands generally ranges between 5 and 30 mg kg^{-1}, however, in vineyard soils the Cu content ranges from 200 to 500 mg kg^{-1} (Malagoli *et al.*, 2013). Additional release of Cu due to human activities has further enhanced the Cu contents in soils. Cu contents in different crop species are variable, however, the critical level of toxicity in leaves is above 20 – 30 mg kg^{-1} dry weight (Peng *et al.*, 2005). Excessive Cu adversely affects physiological and biochemical processes in plants, and its accumulation in plants negatively affects root morphological parameters and biomass of plants (Weng *et al.*, 2005). Chlorophyll synthesis and photosynthetic activities are inhibited (Jiang *et al.*, 2013), metabolic activities are disordered (Remans *et al.*, 2012), and cell division is inhibited (Jiang *et al.*, 2000). Excess accumulation of Cu in plants will not only damage plant growth, but will also transmit health hazards through the food chain.

Due to potential hazards of Cu pollution and its widespread contamination, soils must be cleaned of Cu at minimal cost and with minimum environmental side effects. Several conventional remediation techniques such as soil washing, ion exchange, and chemical precipitation have been successfully used to clean up Cu-contaminated soils. All these techniques are relatively expensive and generally unable to remove Cu contamination completely (Ansari *et al.*, 2013). In phytoremediation, usually hyperaccumulator plants are employed to remove pollutants from the environment or to render them harmless. This technique has emerged as an alternative for removing toxic metals from soil and offers the benefits of being accomplished *in situ*, cost-effective and environmentally sustainable (Islam *et al.*, 2007). But the biomass of hyperaccumulator plants is generally low due to their slow growth. More than 700 species of metal hyperaccumulators have been identified with only about 37 of them being Cu hyperaccumulator species (Reeves and Baker, 2000). The best hyperaccumulator plants are able to accumulate high levels of contaminants, but at the same time attain maximum growth rate and high biomass production.

We observed normal growth of Moso bamboo (*Phyllostachys pubescens* Mazel ex Houzeau, Bambusoideae, Poaceae) in an old Pb/Zn mine area. This indicated the potential ability of Moso bamboo to tolerate and accumulate heavy metals. Moso bamboo grows fast and can reach its maximum size (average height about 15 m) within two months (Xu *et al.*, 2011) with above-ground-biomass production of almost 59 t dry weight ha^{-1} (Chen *et al.*, 1998). Moso bamboo is widely distributed throughout the tropics and subtropics within latitudes of 46° N and 47° S (Song *et al.*, 2013). The plant can exist in a mean annual temperature range of 15 – 20 °C and precipitation range of 1000 – 2000 mm (Song *et al.*, 2013). The area covered by Moso bamboo plants is more than 3.37 Mha which is about 70% of the area of all species of bamboo in China (Chen X. G. *et al.*, 2009).

Plant roots are the sole organs which are in direct contact with heavy metal-contaminated soils and thus are the first organs to experience the toxic effects (Panou-Filotheou and Bosabalidis, 2004). In this study, we investigated the adverse effects of Cu on the root morphology and ultrastructure of Moso bamboo.

Materials and Methods

Hydroponics experiments

Plants were grown in a glasshouse environment with natural light, day/night temperatures of 25/30 °C, and day/night humidity of 70/90%. Seeds of *Phyllostachys pubescens,* collected from Guilin in Guangxi Province, China, were sown in substrate containing perlite and vermiculite (3:1 v/v) moistened with distilled water. The nutrient solution was applied until seedlings had two leaf pairs. The composition of the nutrient solution was as follows (in $\mu mol\ L^{-1}$): NH_4NO_3 (714), $NaH_2PO_4 \cdot 2H_2O$ (161), K_2SO_4 (256), $CaCl_2$ (499), $MgSO_4 \cdot 7H_2O$ (823), Na_2EDTA (13), $FeSO_4 \cdot 7H_2O$ (13), $MnSO_4 \cdot H_2O$ (5), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.04), H_3BO_3 (9), $ZnSO_4 \cdot 7H_2O$ (0.08), $CuSO_4 \cdot 5H_2O$ (0.08), citric acid (monohydrate) (35), and concentrated H_2SO_4 (0.025 L). After two weeks, seedlings of uniform size were selected and transferred to plastic pots containing nutrient solution. All seedlings were exposed to a uniform growth environment. Three plants were chosen randomly in each pot as one replicate and arranged randomly, with each treatment in triplicate. Excess copper was applied as $CuSO_4 \cdot 5H_2O$ at (1) 0 μM (control), (2) 10 μM, (3) 25 μM, (4) 50 μM, (5) 100 μM, (6) 200 μM, (7) 400 μM. The pH value of the nutrient solution was adjusted to 5.8 with 0.1 M NaOH or 0.1 M HCl. The nutrient solution was continuously aerated and renewed after every 5 d.

Plant harvest and elemental analysis

Plants were harvested 30 d after the start of the treatment, washed with distilled water, and immersed in 20 mM Na_2EDTA for 15 – 20 min to remove Cu adhering to the root surface. Plants were washed three times with distilled water and finally with de-ionized water. The shoots and roots were separated and their fresh weights recorded. Plant parts were oven-dried at 70 °C for approximately 72 h, and the dry weight was recorded. The dried plant parts were powdered using a stainless steel mill and passed through a 0.1-mm nylon sieve for Cu analysis. Approximately 0.1 g of a plant sample were digested in a 10:1 (v/v) mixture of concentrated HNO_3 and concentrated $HClO_4$ at 160 °C. The digest was transferred into a 50-mL flask, and the volume was adjusted to 50 mL by addition of de-ionized water. The concentration of Cu was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500a; Santa Clara, CA, USA).

Root morphology

Root morphological parameters were determined after plants had been grown at different Cu levels for 30 d. In each replicate, three plants were selected randomly, and the length, surface area, and volume of the roots, as well as the number of root tips were determined using a root scan apparatus (Epson Expression 10000XL; Beijing, China) equipped with WinRHIZO (Regent Instruments, Sainte-Foy-Sillery-Cap-Rouge, QC, Canada) software. The average values of three plants were considered as one replicate.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

Control plants and plants exposed to 100 μM Cu for 30 d were selected for the TEM studies. Root sections of 1 – 3 mm in length were fixed in 4 % glutaraldehyde (v/v) in 0.2 M sodium phosphate buffer (pH 7.2) for 6 – 8 h and post-fixed in 1 % OsO_4 for 1 h, and in 0.2 M phosphate-buffered saline (PBS) (pH 7.2) for 1 – 2 h. The fixed sections were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100 %), followed by acetone, and embedded in Spurr's resin. Ultra-thin sections (80 nm) were prepared and mounted on Cu grids for viewing in a transmission electron microscope (JEOL TEM-1200EX; Tokyo, Japan) at an accelerating voltage of 60.0 kV. For SEM, ethanol-dehydrated sections were dehydrated in a Hitachi Model HCP-2 critical point dryer (Tokyo, Japan) with liquid CO_2, coated with gold-palladium in an Eiko Model IB5 ion coater (Shawnee, KS, USA) for 4 – 5 min, and then observed in a Hitachi Model TM-1000 scanning electron microscope.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 21.0). All values reported are the mean ± SD (means of three independent replicates). Data were tested at significance levels of $P < 0.05$ by one-way ANOVA (LSD). Graphical work was carried out using Sigma Plot software v.12.5.

Results and Discussion

Effects of copper on plant growth

Exposure of Moso bamboo plants to increasing levels of Cu for 30 days produced severe toxic symptoms especially at 400 μM Cu treatment, in which the majority of plants had died before harvest time (Figs. 1 – 3). Cu stress inhibits cellular metabolic processes of plants (Liu *et al.*, 2009). At 10 μM Cu stress, the bamboo plants had a reduced number of leaves. The leaves turned yellow, the plants became smaller with each increment of Cu concentration (Fig. 1), and likewise, root and shoot dry weights were reduced compared with the control (Fig. 3). Similar results were reported by Collin *et al.* (2014) for *Phyllostachys fastuosa* under Cu stress. In *Elymus elongatus* subsp. *ponticus* cv. Szarvasi-1 (Poaceae), which was taken into consideration for phytoremediation, 10 μM Cu stress for 37 days caused dry mass reduction of 90 % and 75 % in roots and shoots, respectively (Sipos *et al.*, 2013). In our experiment, 200 μM Cu stress led to 17 % and 50 % reduction in shoot and root length, respectively (Fig. 2). Root growth was increasingly affected by Cu at concentrations up to 25 μM. In the treatment range of 25 – 200 μM Cu, root growth was inhibited, and no significant difference could be observed in these treatments (Fig. 2).

Effects of copper on root morphology

The root system reacts very sensitively to different environmental factors (Bona *et al.*, 2007). The root morphological characteristics is directly affected by the uptake of heavy metals (Li *et al.*, 2005). Several

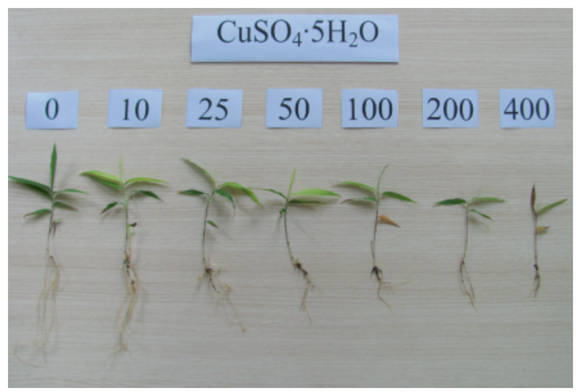

Fig. 1. Morphology of Moso bamboo seedlings grown for 30 days in the presence of increasing concentrations (in μM) of $CuSO_4$.

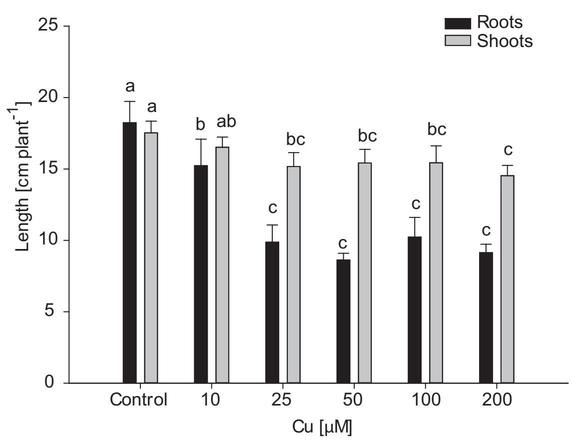

Fig. 2. Shoot and root length of Moso bamboo grown in the presence of increasing concentrations of $CuSO_4$. All values are means of three independent replications ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between treatment and control.

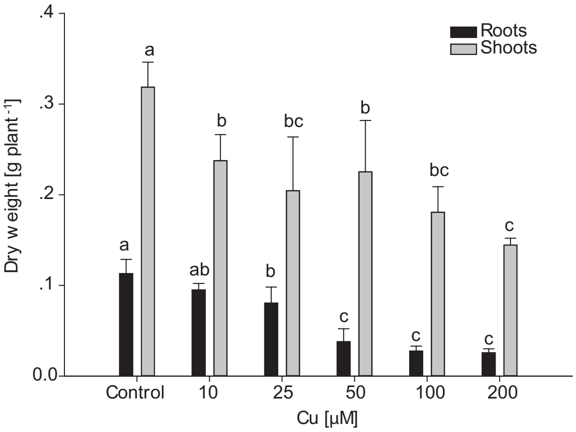

Fig. 3. Dry weight of shoots and roots of Moso bamboo grown for 30 days in the presence of increasing concentrations of $CuSO_4$. All values are means of three independent replications ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between treatment and control.

studies have reported an adverse influence of heavy metals on root morphology, including Zn (Chen W. R. *et al.*, 2009), Pb (Islam *et al.*, 2007), Cd (He *et al.*, 2007; Wei *et al.*, 2012), and Cu (Huang *et al.*, 2013). In case of Moso bamboo, the root volume (Fig. 4C) and number of roots tips (Fig. 4D) were affected sensitively compared with other root morphological parameters. Application of 50 μM Cu resulted in 86% and 88% reduction in root volume and number of root tips, respectively, compared with the control. The number of root tips (Fig. 4D) increased at the lowest Cu level (10 μM), but was greatly decreased at 25 μM Cu and higher concentrations. The total root length (Fig. 4A) and root volume (Fig. 4C) were inhibited at all Cu concentrations. No significant differences were observed in all four root morphological parameters in the concentration range of 50 to 200 μM. The concentration of 25 μM Cu is a crucial threshold in detrimental effects of Cu on root morphological

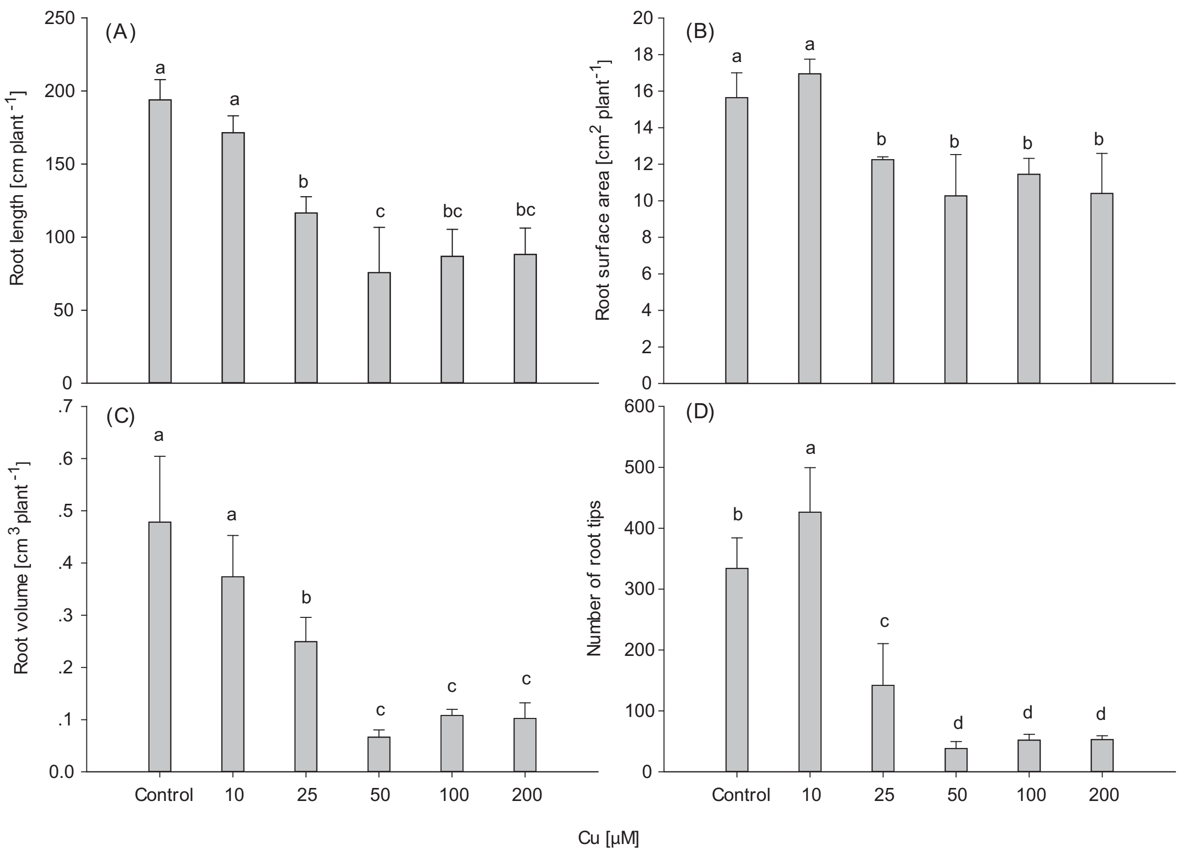

Fig. 4. Root morphology of Moso bamboo grown for 30 days in the presence of increasing concentrations of $CuSO_4$: (A) total root length, (B) root surface area, (C) root volume, and (D) number of root tips. Error bars are standard deviations ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between treatment and control.

parameters (Huang *et al.*, 2013). The numbers of root tips of two *Rumex japonicus* populations, adapted and non-adapted to Cu, were significantly inhibited by Cu treatments. The root surface area of the non-adapted population of *Rumex japonicus* was significantly reduced by Cu treatment, while that of the adapted population did not differ from the control. Our results agree with those of Cai *et al.* (2014), who reported that total root length and root surface area of *Elsholtzia haichowensis* (Lamiaceae) were affected by Cu stress.

Copper content in the plants

Copper, as an essential microelement, plays an important role in growth and development of plants, but at the same time plants are very sensitive to excess Cu. Exposure of plants to slightly elevated Cu levels provokes symptoms of stress-induced morphogenic responses (SIMR) such as inhibition of cell elongation, local stimulation of cell division, and alterations in the cell differentiation status (Feigl *et al.*, 2013). Figure 5 shows that Cu concentrations were significantly ($P < 0.05$) elevated in roots and shoots of Moso bamboo at all applied Cu concentrations compared with the control. At external Cu concentrations of 10 to 100 μM, there were no significant differences in the Cu contents of shoots (Fig. 5A). Application of 200 μM Cu resulted in a sharp rise of the Cu content of the shoots which was similar to that reported for Cu-exposed *Elsholtzia splendens* (Tian *et al.*, 2008). On the other hand, the Cu content of roots exposed to 50 μM Cu was about 57 times higher than that of the control. No consistent trend was observed in the Cu contents of plants exposed to different Cu concentrations. The range of the Cu contents in roots varied from 524 to 809 mg kg^{-1} dry weight with no obvious dependence on the concentration of exogenous Cu (Fig. 5B). Collin *et al.* (2014) observed that the Cu concentration in the roots of another bamboo species, *Phyllostachys fastuosa*, can reach up to 3171 mg kg^{-1} after 70 days with 100 μM Cu in the nutrient solution. In case of *Phragmites australis* (Poaceae), it can reach 8309 mg kg^{-1} in roots of plants grown in 157 μM Cu for 15 days (Ait Ali *et al.*, 2002). The treatment with 400 μM Cu resulted in almost dead plants (Fig. 1). Shu *et al.* (2002) studied the Cu accumulation and tolerance in *Paspalum distichum* (Poaceae) and reported an EC_{50} value of 3 μM. We conclude that Moso bamboo has strong tolerance to Cu stress. While this plant survives 200 μM Cu, a suitable concentration for Moso bamboo as phytoremediation material is 10 μM Cu under hydroponic conditions. The resulting Cu content in Moso bamboo plants is low compared to other Cu accumulators. Yang *et al.* (2002) considered *Elsholtzia splendens* as Cu hyperaccumulator for the reason that shoot Cu contents of 1133 and 3417 mg kg^{-1} dry weight were achieved with 500 and 1000 μM Cu supply, respectively. Application of 100 μM Cu resulted in 2294 and 144 mg Cu kg^{-1} dry weight, respectively, in roots and shoots of *Elsholtzia splendens* (Weng *et al.*, 2005). Compared to our results, the capacity of Moso bamboo to accumulate Cu is low. However, considering the much larger biomass and other advantages of Moso bamboo, compared to *Elsholtzia splendens*, Moso bamboo has great potential for use in phytoremediation.

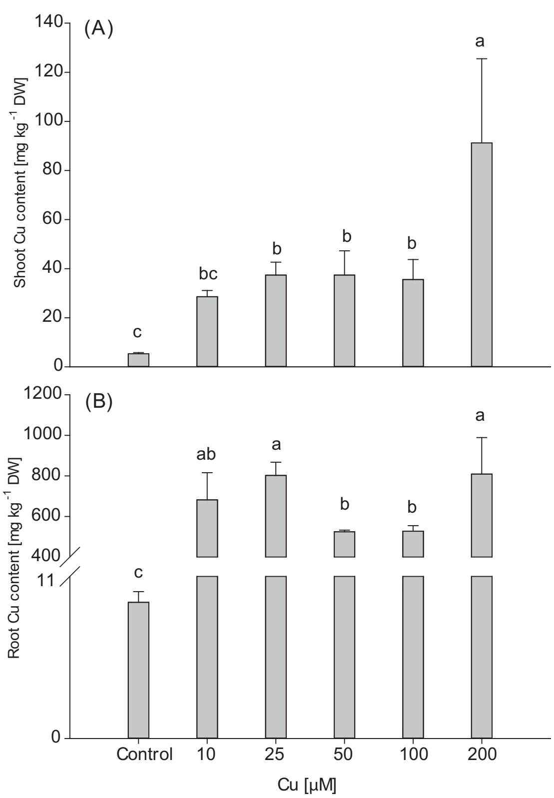

Fig. 5. Cu content in (A) shoots and (B) roots of Moso bamboo grown for 30 days in the presence of increasing concentrations of $CuSO_4$. Error bars are standard deviations ($n = 3$). Different letters indicate significant differences ($P < 0.05$) between treatment and control. DW, dry weight.

TEM and SEM

Transmission and scanning electron micrographs of the roots of control plants and plants exposed to

100 μM Cu are presented in Figs. 6 and 7. It had previously been found that under Cu stress root cell walls of *Elsholtzia splendens* became thicker, and in root tip cells of wheat seedlings (Yannong 19, which is widely planted in Xuzhou, China) mitochondrial structure became indistinct (Peng, 2005; Yu *et al.*, 2010). In our

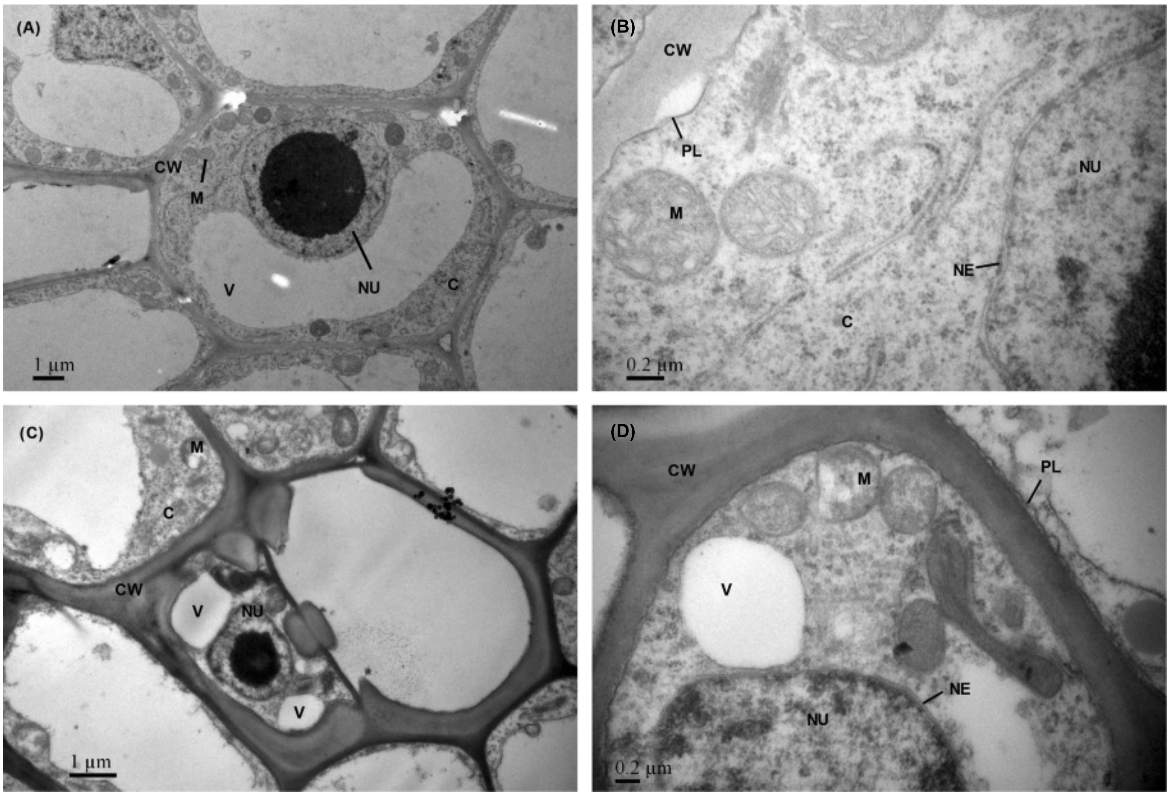

Fig. 6. Transmission electron micrographs of root tip cells of Moso bamboo. (A and B) Control and (C and D) exposed to 100 μM Cu for 30 days. Abbreviations: CW, cell wall; NU, nucleus; C, cytoplasm; V, vacuole; PL, plasmalemma; NE, nuclear envelope; M, mitochondrium.

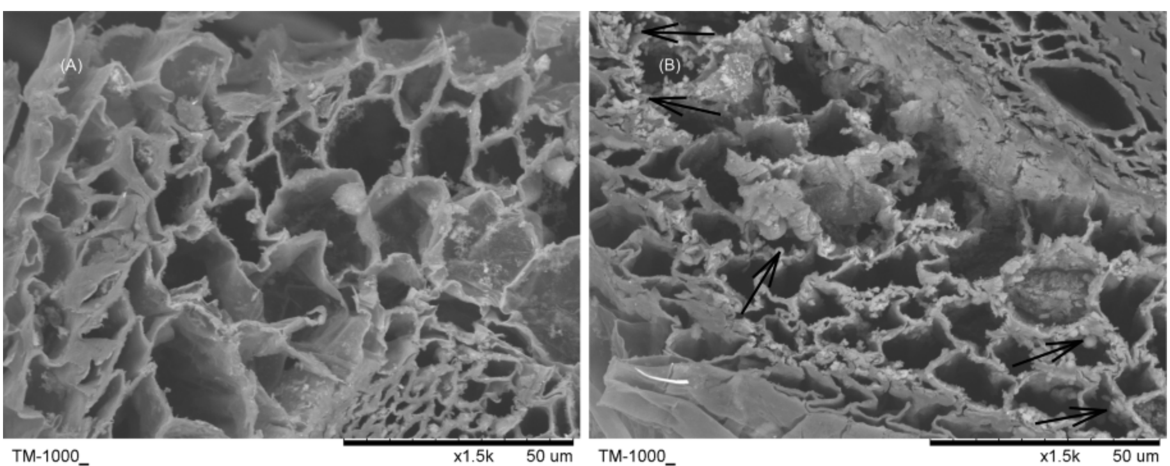

Fig. 7. Scanning electron micrographs of root tips of Moso bamboo exposed to (A) no Cu and (B) 100 μM Cu for 30 days. Small crystals are indicated by arrows.

study, the cell walls of the controls (Figs. 6A and B) were smooth, and the mitochondria were intact. When roots were exposed to 100 μM Cu (Figs. 6C and D), the cell walls were more irregular and thicker, and mitochondria appeared distorted. Without copper stress (Figs. 6A and B), we could observe a dense cytoplasm with normal organelles, large nucleolus, nuclear envelope, tonoplast, and plasmalemma. Under 100 μM Cu stress, nuclei appeared distorted and the cytoplasm was less dense (Figs. 6C and D). Scanning electron micrographs of root sections (Fig. 7) showed toxic effects due to exposure to Cu stress. In the control, the structure of the root xylem can be seen clearly, while Cu treatment produced several small crystals (shown by arrows) in the root xylem which may block the nutrient transport. Cestone *et al.* (2012) found that upon treatment of *Brassica carinata* with 150 μM Cu, Cu was mainly concentrated in the vascular tissue, and they considered an increased Cu uptake into the symplast and further into the xylem. We assume that excess Cu may cause cells to secrete various substances, such as proteins, amino acids, and organic acids, which chelate with Cu and form small crystals in the vascular tissue of the root xylem. However, the EDX technique is required in further research to confirm the presence of Cu in the crystals.

Conclusions

Moso bamboo is a suitable plant to immobilize Cu in contaminated soils, and it can be grown as phytoremediation material due to high biomass of its shoots in contaminated soils. A low Cu stress will stimulate root growth (root surface area and root tips), compared to the adverse effects of high concentrations. Biomass, cell structure, and cell organelles are significantly affected by Cu. Crystals observed in the root xylem may contain Cu, possibly as a strategy to reduce the toxicity of soluble Cu in Moso bamboo.

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