Copper changes the yield and cadmium/zinc accumulation and cellular distribution in the cadmium/zinc hyperaccumulator *Sedum plumbizincicola*

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HIGHLIGHTS

- Low Cu has no significant effect on *Sedum plumbizincicola* plant growth and Cd and Zn uptake.
- Plant held Cu in unactive areas and insoluble forms as de-toxification mechanisms.
- Influence of Cu on Zn and Cd uptake and translocation were different.
- Cu accumulation in leaf veins may restrain Cd/Zn unloading to the leaves

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ABSTRACT

Non-accumulated metals in mixed metal contaminated soils may affect hyperaccumulator growth and metal accumulation and thus remediation efficiency. Two hydroponics experiments were conducted to investigate the effects of copper (Cu) on cadmium (Cd) and zinc (Zn) accumulation by the Cd/Zn hyperaccumulator *Sedum plumbizincicola*. Cu toxicity and plant detoxification using chemical sequential extraction of metals, sub-cellular separation, micro synchrotron radiation based X-ray fluorescence, and transmission electron microscopy. Compared with the control (0.31 μM Cu), 5–50 μM Cu had no significant effect on Cd/Zn accumulation, but Cu at 200 μM induced root cell plasmolysis and disordered chloroplast structure. The plants held Cu in the roots and cell walls and complexed Cu in insoluble forms as their main detoxification mechanisms. Exposure to 200 μM Cu for 4 days inhibited plant Cd uptake and translocation but did not affect Zn concentrations in roots and stems. Moreover, unloading of Cd and Zn from stem to leaf was restrained compared to control plants, perhaps due to Cu accumulation in leaf veins. Copper may thus interfere with root Cd uptake and restrain Cd/Zn unloading to the leaves. Further investigation of how Cu affects plant metal uptake may help elucidate the Cd/Zn hyper-accumulating mechanisms of *S. plumbizincicola*.

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1. Introduction

Mixed and multiple contamination of soils by metals can result from industrial and mining activities and agricultural practices such as land application of contaminated sewage sludges and usually involves several contaminants [1,2]. Phytoremediation using hyperaccumulator plants to remove contaminants from soils is an environmentally friendly and relatively inexpensive technique [3–5]. However, most hyperaccumulators can typically remediate

only a very limited number of pollutants so that other non-accumulated metals (i.e. metals the hyperaccumulator does not have the ability to hyperaccumulate) remaining in the soil may affect the growth, metal accumulation, and hence phytoremediation efficiency of the hyperaccumulator.

There are several published studies on the influence of metal interactions on hyperaccumulators. For example, Ni was found to affect Cu and Fe homeostasis in the Ni-hyperaccumulator *Alyssum inflatum* [6] and Cu, Mn and Zn affected the Mn-hyperaccumulator *Phytolacca americana* [7], with Mn interacting with Cd in the same plant species [8]. Cadmium and Zn interactions occurred during uptake, translocation and subcellular distribution in the hyperaccumulators *Potentilla griffithii*, *Thlaspi caerulescens*, and *Arabidopsis*

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halleri [9–11]. There have been few reports on the effects of Cu on hyperaccumulators and their metal accumulation. Copper is an essential element for plant growth through its role in the composition of many enzymes and proteins, but the normal range of Cu concentrations in plants is very narrow (5–20 mg kg⁻¹) [12] and high Cu concentrations in the growth medium might induce phytotoxicity by interacting with other essential elements, impairing cells through oxidative damage, disrupting the structure of proteins, and inactivating some key enzymes [13]. Metal toxicity to plant can be reflected by plant apparent characters (e.g. plant biomass) and the change of cell and organelles which can be characterized by ultra-structure of cell (transmission electron micrographs) [14]. As metal distribution in different cell parts and different metal chemical forms have different toxicity to plant, metal in cellular distribution and chemical forms can be used to investigate plant detoxification mechanisms [15]. *Sedum plumbizincicola*, a Cd/Zn hyperaccumulator with large biomass and high accumulation of Cd/Zn in the shoots [16,17], is a promising species for the remediation Cd/Zn polluted soils. However, this species was found to have a very high Cu concentration (254 mg kg⁻¹) in shoots and its growth was significantly inhibited when plant grow in acid soil (pH, 4.56; total Cu in soil, 369 mg kg⁻¹) [18]. Also 688 mg kg⁻¹ Cu was detected in *S. plumbizincicola* when EDDS was applied to enhance Cd and Zn phytoextraction in an alkaline soil [19]. It is therefore necessary to investigate the potential effects of Cu on *S. plumbizincicola* and its accumulation of Cd and Zn for the potential phytoremediation in Cd/Zn and Cu co-polluted soils.

In the present study, two hydroponics experiments were conducted and the methods employed included chemical sequential extraction of plant metals and subcellular separation based on differential centrifugation, micro synchrotron radiation based X-ray fluorescence (μ -SXRF) and transmission electron microscopy of plant samples. The aims were to determine how Cu influences Cd and Zn uptake and accumulation by *S. plumbizincicola* and to investigate the possibility of Cu toxicity and plant detoxification.

2. Materials and methods

2.1. Hydroponics experiments

2.1.1. Plant preparation

S. plumbizincicola shoots were collected from a seedbed of *S. plumbizincicola* in a field experimental facility (located in the suburbs of Hangzhou city, Zhejiang province, east China), cut into uniform pieces and washed with tap water, and then a plastic pot (10 L) was used to culture the plant shoots using fresh nutrient solution. The solution was modified Hoagland nutrient solution: Ca(NO₃)₂·4H₂O 1.0, MgSO₄·7H₂O 0.5, K₂HPO₄ 0.5, KCl 0.1, MES 1.0, and KOH 0.5 mmol L⁻¹; and H₃BO₃ 10, Na₂MoO₄·2H₂O 0.2, MnSO₄·4H₂O 1.8, CuSO₄·5H₂O 0.31, NiSO₄·6H₂O 0.5, Fe-EDDHA 100, and ZnSO₄·7H₂O 5 μ mol L⁻¹. The replacement of EDTA with EDDHA is because EDDHA has higher complex ability with iron than EDTA, which resulting high available iron for plant [20]. The pH value was adjusted to 5.8 with 0.1 M sodium hydroxide (NaOH) and/or 0.1 M hydrochloric acid (HCl) and the solution was continuously aerated with a pump during plant growth. The solution was replaced with fresh medium every three days. The plants grew in a growth chamber with a day/night temperature regime of 25/20 °C and a photoperiod of 14 h at a photosynthetically active radiation flux of 60 W m⁻². The plant shoots produced roots after two weeks and healthy looking plants of uniform size were chosen for the hydroponics experiments. One sample was separated into leaf, stem and root and the plant parts were washed with double distilled water and dried at 80 °C prior to determination of the metal concentrations before the experiments began.

2.1.2. Experiment 1: plant treatment with Cu, Zn and Cd

The prepared plants were transferred to 2-L plastic containers with polystyrene covers, each with 6 evenly spaced holes and 1 smaller hole in the center. Before plants were transferred to 2-L plastic containers, six levels of Cu as CuSO₄·5H₂O, i.e. 0.31 (control, the Cu concentration in the nutrient solution), 5, 10, 50, 100, 200 μ M for each pot, and 50 μ M Cd and 500 μ M Zn (as the nitrates) for all pots were also added to the nutrient solution and the pH was adjusted to about 5.8 with 0.1 M NaOH and/or 0.1 M HCl. There were six plants in each pot and four replicates of each treatment were randomly arranged on a bench inside the growth chamber and the conditions were the same as described above for plant preparation. Fourteen days after the plants were exposed to the Cu the plants exposed to 100 or 200 μ M Cu displayed visual toxicity symptoms and all plants were harvested. The roots were soaked in 20 mmol L⁻¹ EDTA solution for 30 min and rinsed with distilled water. Each plant sample was divided into leaves, stems and roots and all plant parts were washed thoroughly with distilled water and then oven dried at 80 °C.

2.1.3. Experiment 2: plant treatment with Cu, Zn and Cd for short time

In hydroponics experiment 1 *S. plumbizincicola* showed toxicity symptoms under high Cu (200 μ M). In experiment 2, 0.31 μ M and 200 μ M Cu were selected together with 50 μ M Cd and 500 μ M Zn to test the further metal effects. Each treatment had four replicates, and there were six plants in each pot and the growth conditions were the same as described above for plant preparation. After the metals were added to the nutrient solution the pH was adjusted as described above for experiment 1. Four days after the metals were added to the nutrient solution the experiment was ended because plants in the high Cu treatment showed clear toxicity symptoms and the roots secreted copious white material. After the roots were soaked in a solution of 20 mmol L⁻¹ EDTA for 30 min the plant samples were separated into leaves, stems and roots. All plant samples were rinsed thoroughly with distilled water and dried with paper tissue. The fresh samples were prepared for transmission electron microscopy and μ -SXRF (details below) and then 2.00-g sub-samples (cut into 1–2 mm pieces) were weighed accurately and stored at –20 °C for metal sequential extraction and plant tissue separation. The remainder of each sample was oven dried at 80 °C.

2.2. Sequential extraction of metals

Sequential extraction of Cu, Cd and Zn in plants was conducted by the methods of Wang et al. [15] and Wu et al. [21]. Metals in plants were divided into six chemical forms according to the different extractant solutions in the following order: (1) 80% ethanol, extracting the inorganic metal fraction including metal-nitrate, chloride and aminophenol; (2) distilled water, extracting the water soluble fraction including metal complexed with organic acids and M(H₂PO₄)₂; (3) 1 M NaCl, extracting metals associated with pectates and proteins; (4) 2% acetic acid (HAC), extracting the insoluble metal fraction including MHPO₄ and M₃(PO₄)₂; (5) 0.6 M HCl, extracting M-oxalate; and (6) metal in residues. The frozen plant leaf, stem and root tissues with a fresh weight of 2.00 g were homogenized in extractant solution with a glass mortar and pestle, diluted at a ratio of 1:10 (w/v), and shaken for 22 h at 25 °C. The homogenate was centrifuged (Nr. 12150-H, Sigma, Osterode am Harz, Germany) at 5000 $\times$ g for 10 min and the supernatant was carefully transferred to a 100 ml flask. The pellet was re-suspended twice in the same extractant solution and shaken for 2 h at 25 °C, centrifuged at 5000 $\times$ g for 10 min, and the three supernatants were pooled. The pooled supernatant solutions from each fraction and

the residual fractions were then evaporated at 70 °C to nearly constant weight prior to digestion.

2.3. Plant tissue separation

Differential centrifugation was used to separate plant tissues following the methods of Wu et al. [21] and Weigel and Jäeger [22]. The frozen plant leaf, stem and root tissues with a fresh weight of 2.00 g were homogenized in pre-cooled extraction buffer (200 mM sucrose, 50 mM Tris-HCl, 1.0 mM DET (C₄H₁₀O₂S₂), 5.0 mM ascorbic acid and 1.0% (w:v) Polyclar AT PVPP, pH 7.5) with a chilled glass mortar and pestle. The homogenate was transferred to a 50 ml centrifuge tube for separation. The first centrifugation (Nr. 12150-H, Sigma, Osterode am Harz, Germany) was at 300 × g for 30 s and the pellet was defined as F1 containing mainly cell walls and cell wall debris; the second at 1500 × g for 15 min (root samples at 2500 × g for 20 min) and the pellet was regarded as F2 containing chloroplasts from leaves and shoots or trophoplasts from roots; and finally at 15,000 × g for 35 min and the pellet was membranes and organelles defined as F3, and the supernatant was considered to be the soluble fraction, F4. All the above steps were performed at 4 °C. The pellets obtained at each centrifugation step were re-suspended in a glass flask and dried to nearly constant weight at 70 °C and digested by the method described below for whole plant samples.

2.4. μ -SXRF spectroscopy data collection

The μ -SXRF data were collected on beam line 15 U (3.5 GeV and 300 mA) at Shanghai Synchrotron Radiation Facility. The sample was oriented 45° to the incident X-ray beam. Fluorescence signals were collected using a Si (III) solid-state multi-element detector. The beam energy was set to 11 keV to detect the elements in samples and the fluorescence maps were collected with a beam size 2 × 2 μ m² using a step size of 10 μ m × 12 μ m at a integration time of 100 ms. The samples were prepared as follows. The fifth (from top) well developed leaf in hydroponics experiment 2 was selected and the surface was rinsed with d-water. The leaves were cut into pieces with a razor blade and cryofixed by a freezing method with liquid nitrogen (LN₂). The cryofixed leaf samples were transferred into 5 ml plastic vials with a hole in the cover and then dried in an Emitech K750 Freeze Drier (Ashford, Kent, UK) at –60 °C at a pressure of 0.02 mbar for 3 days. The freeze-dried leaf samples were hand sectioned using unused and cleaned razor blades and placed on an adhesive tape for detection.

2.5. Transmission electron microscopy

The fifth well-developed leaf (from the plant top) and root tips were selected as detection samples. Small sections of samples 1–2 mm² in area (leaf) or 1–3 mm long (root) were cut and fixed in 6% glutaraldehyde (v/v) in 0.2 M sodium phosphate buffer (pH 7.2) for 10 h and post-fixed in 2% OsO₄ for 1 h. The dehydration samples used graded ethanol with concentration series: 50, 60, 70, 80, 90, 95 and 100% and acetone; then samples were infiltrated and embedded in ethoxy resin (E-pon812). Ultrathin sections (70 nm) were prepared and placed on copper grids for viewing in the transmission electron microscope (Hitachi H-7650, Japan).

2.6. Determination of metals in plant and soil samples

Dry plant samples (~0.25 g DM) and the residues from the sequential extraction were digested with a mixture of 6 ml HNO₃ and 4 ml HClO₄. Digestion solutions were determined for Cd, Zn and Cu using AAS (Varian SpectrAA 220 FS). A certified reference material (GBW07603, provided by the Institute of Geophysical and

Table 1

Biomass of leaves, stems and roots of *S. plumbizincicola* exposed to different Cu concentrations in the nutrient solution for 14 days (g pot^{−1}). Data presented are mean ± standard error (n = 4). Different letters in a column indicate significant difference from each other (P < 0.05).

Cu in solution (μ M)	Leaf	Stem	Root
0.31	0.61 ± 0.06 a	0.34 ± 0.04 a	0.22 ± 0.02 a
5	0.72 ± 0.07 a	0.44 ± 0.04 a	0.28 ± 0.04 a
10	0.69 ± 0.06 a	0.39 ± 0.04 a	0.20 ± 0.06 a
50	0.70 ± 0.13 a	0.41 ± 0.06 a	0.30 ± 0.06 a
100	0.43 ± 0.02 b	0.27 ± 0.03 a	0.23 ± 0.02 a
200	0.36 ± 0.02 b	0.31 ± 0.04 a	0.22 ± 0.02 a

Geochemical Exploration, Langfang, Hebei Province, China) was used for quality control. The data obtained by the methods above were within the certified ranges for Cu, Zn and Cd.

2.7. Statistical analysis

Data were presented as mean ± standard error of the mean and were analyzed by one-way analysis of variance using SPSS version 16.0 for Windows. Differences in mean shoot biomass or metal concentrations between treatments were tested by Duncan's multiple range test at the 5% level. The maps of μ -SXRF were made with Igor Pro 6.10A and other figures with Microsoft Excel 2003 or Origin 8.0.

3. Results

3.1. Plant biomass and metal concentrations under different Cu treatments

The Cu in the nutrient solution exerted a significant influence on the growth of *S. plumbizincicola* exposed to different levels of Cu for 14 days (Table 1). Compared to the control (0.31 μ M Cu) the average leaf biomass in the 5–50 μ M Cu treatments did not increase significantly. However, when the solution Cu concentration was 100 μ M or 200 μ M the plants exhibited growth inhibition and the leaf biomass was significantly lower than the control (Table 1). However, in experiment 2 the plants were exposed to the same Cu concentrations for only 4 days and there was no significant difference in biomass (data not shown), possibly due to the short exposure time.

Plant metal concentrations after 14 d exposure to different levels of Cu (from 0.31 to 200 μ M) at the same Cd (50 μ M) and Zn (500 μ M) levels in experiment 1 are shown in Fig. 1. Leaf, stem, and root Cu concentrations increased linearly with increasing Cu concentration in the nutrient solution and the rate of increase in roots was much higher than in stems and leaves (Fig. 1A) and most of the Cu was accumulated in the roots. There was no significant difference among treatments 0.31–50 μ M Cu in Cd in the leaf, stem and root, but a clear decline was found when the Cu concentration increased to 100 or 200 μ M (Fig. 1B). Zn showed similar trends to Cd. Compared with control 5–50 μ M Cu levels in the solution produced no significant effect on the Zn concentrations in leaf, stem or root but 100 or 200 μ M Cu produced significantly lower leaf and stem Zn concentrations compared to the other treatments, but no significant change in the root (Fig. 1C).

In experiment 2, 200 μ M Cu also depressed plant Cd accumulation compared to the control when the plants were exposed to the Cu for only 4 days. As with Zn, 200 μ M Cu did not show any significant influence on the Zn concentration in stems or roots compared with the control (Fig. 2), but inhibited leaf Zn uptake. Neither Cd nor Zn in the leaves in 200 μ M Cu treatment showed any significant difference from the control (Fig. 2).

Fig. 1. Cu (A), Cd (B), and Zn (C) concentrations in plants grown in solution with different Cu concentrations for 14 days. For (A), the linear regression analysis shows that Cu concentration in plants increased with Cu concentration in solution in the leaf ($C_{\text{leaf}} = 0.39 \times C_{\text{solution}} + 21.4$, $R^2 = 0.88$), stem ($C_{\text{stem}} = 0.91 \times C_{\text{solution}} + 5.30$, $R^2 = 0.98$), and root ($C_{\text{root}} = 11.0 \times C_{\text{solution}} + 88.4$, $R^2 = 0.98$). Data presented are mean $\pm$ standard error ($n = 4$).

3.2. Effects of Cu on chemical forms of metals in the plant

The chemical forms of Cu, Cd and Zn in *S. plumbizincicola* in hydroponics experiment 2 are shown in Fig. 3.

Cu extracted by 80% ethanol was the dominant fraction in the leaves followed by 0.6M HCl, and there were no significant differences between the other fractions in the control. 200 μM Cu induced significant increase in the fractions extracted by distilled water and 2% HAc. There was no difference between the different chemical forms in the stems except for the lower fraction extracted by 1 M NaCl in the control. 2% HAc extractable fractions were dominant followed by distilled water and 80% ethanol in the 200 μM Cu treatment. Compared to the control, these three fractions increased markedly and other forms increased slightly, but the residual fraction showed no apparent change. In terms of the percentage of the total, the fraction extracted by 2% HAc also increased greatly, from 13.5% in the control to 36.8% in the 200 μM Cu treatment on average. Similar to the stems, 200 μM Cu induced significant increases in the first five Cu chemical forms in roots with respect to the control. The form extracted by 2% HAc showed the largest increase in percentage of the total from 7.07% (control) to 24.2% (200 μM Cu) on average. However, the dominant chemical form of Cu was still the fraction extracted by 0.6 M HCl in both Cu treatments.

The distribution of Cd chemical forms in *S. plumbizincicola* differed from Cu. Plant Cd consisted mainly of the forms extracted by

80% ethanol, d-water and 1 M NaCl, representing 88–98% of the total Cd in all plant organs, and other Cd chemical fractions were very low (Fig. 3 center). In leaves and stems 80% ethanol and 1 M NaCl extractable fractions dominated followed by d-water extractable, but in the roots the Cd extracted by 1 M NaCl was dominant at >60% of the total Cd. Although 200 μM Cu greatly and significantly lowered the concentration of Cd in the main chemical forms (the first three), their percentages of total Cd showed no marked change with respect to the control.

In common with Cd, plant Zn mainly consisted of the forms extracted by 80% ethanol, d-water and 1 M NaCl, representing 92–97% of the total Cd in all plant organs of both treatments. Although 200 μM Cu had no significant influence on the quantities of Zn chemical forms, a decrease on average in Zn extracted by 80% ethanol in stems and roots was observed. 200 μM Cu appeared to decrease the first four chemical fractions in the leaves but the apparent trend was not significant (Fig. 3 bottom).

3.3. Effects of Cu on plant elemental distributions by differential centrifugation and μ-SXRF

The subcellular tissues of *S. plumbizincicola* were obtained by differential centrifugation and the corresponding percentages of metal concentrations in each fraction to the total are shown in Fig. 4. The percentage Cu in each fraction of total Cu in the leaves

Fig. 2. Cu (A), Cd (B), and Zn (C) concentrations in plants before metal treatment (background) and in plants exposed to 0.31 (control) and 200 μM Cu in the nutrient solution for 4 days. Different letters in plant parts indicate significant difference from each other ($P < 0.05$). Data presented are mean $\pm$ standard error ($n = 4$).

did not change ($P > 0.05$) compared to the control. However fraction F1 in the stems increased by 10% and F2 decreased by 9%. In the roots the Cu percentage in F1 increased by 7% increase and in F4 decreased by 6%. The proportion of Cd in the F1 fraction in the of the leaves and roots in 200 μM treatment increased by about 12 and 7% and in fraction F4 decreased by 5 and 8%, respectively. However, the percentage of Cd in all fractions in the stems did not change ($P > 0.05$). There was much more Zn accumulation in fraction F1 and less in fraction F4 in the leaves in the 200 μM Cu treatment than in the control, with a 12% increase and 10% decrease respectively. In stems and roots in the 200 μM Cu treatment the Zn percentage distributions increased slightly in fraction F1 with respective to the control, by 8% and 5% respectively, and changes in the other fractions in stems and roots were less than 5%.

The effects of Cu on elemental distributions in leaves were also investigated by μ -synchrotron XRF, and the Cu, Zn, P, and K distribution maps in the scanned area of leaf cross-sections of both levels of Cu treatment are shown in Fig. 5 together with the optical micrographs. Cd distribution would have been affected by interference from K and the results are not shown here. The X-ray fluorescence intensities are shown with different colors, from purple (maximum) to red (minimum) for the individual elements. Each map showed the relative distribution of elements and the count scales were set same for the same element in both Cu treatments. In the control Cu and Zn accumulated mainly in the leaf margins and epidermis and were evenly distributed in other regions. K distribution was concentrated in the leaf veins together with other peaks in the leaf margin and the epidermis. P was evenly distributed within

the leaf cross-section. In the 200 μM Cu treatment, Cu was concentrated mainly in the leaf veins and was also distributed in the leaf margin and upper epidermis. Zn occurred mainly in the leaf margin and epidermis. K showed a similar distribution to Cu, mostly at the leaf veins. P was evenly located within the leaf cross-section as in the control. Comparing two treatment images indicated that the leaf cross-section in the 200 μM Cu treatment had lower accumulation of P, K, and Zn and higher was in Cu than the control.

3.4. Effects of Cu on ultra-structure of leaves and roots of *S. plumbizincicola*

The transmission electron micrographs of leaf (Fig. 6A and B) and root (Fig. 6C and D) cells of *S. plumbizincicola* exposed to 0.31 μM (Fig. 6A and C) and 200 μM Cu (Fig. 6B and D) showed that rare organelles occurred in the mesophyll cells of both two treatments, with only chloroplasts and mitochondria observed (Fig. 6A and B). After 4 days, the plants exposed to 200 μM Cu showed clear differences in the chloroplasts compared to the control. In the control one or two large starch grains were present in the chloroplasts and the thylakoids were orderly, loosely and densely arranged. However, in the 200 μM Cu treatment the chloroplasts had small starch grains and the thylakoids were disordered. Transmission electron micrographs of cells of the root tip show that copious organelles were present in the cytoplasm (Fig. 6C and D). Compared to the control the organelles in *S. plumbizincicola* roots in the 200 μM Cu treatment showed no visible damage but clear incipient plasmolysis was observed. Moreover, some particles were found regularly deposited

Fig. 3. Cu (upper), Cd (center), and Zn (bottom) concentrations of different chemical forms (extracted by 80% ethanol, d-water, 1 M NaCl, 2% HAc, and 0.6 M HCl, and residual forms) in leaves, stems and roots under 0.31 μM (control) and 200 μM Cu in the nutrient solution for 4 days. Different letters for chemical forms in one treatment indicate significant difference from each other ($P < 0.05$). Data presented are mean $\pm$ standard error ($n = 4$).

Fig. 4. Cu (A), Cd (B), and Zn (C) subcellular distribution in tissues of *S. plumbizincicola* grown in 0.31 μM (control) and 200 μM Cu in the nutrient solution for 4 days. F1, F2, F3, and F4 denote metal in cell walls and cell wall debris, chloroplasts (leave and shoot) or trophoplasts (root), membranes and organelles, soluble fraction, respectively. Data presented are mean $\pm$ standard error ($n = 4$).

Fig. 5. μ -Synchrotron XRF elemental maps for Cu, Zn, K, and P of leaf cross sections from *S. plumbizincicola* grown in 0.31 and 200 μ M Cu in the nutrient solution for 4 days. The red color was scaled to the background value for each map; the same color depicts the same concentration for the same element comparing two Cu treatments. UE, upper epidermis; LE, lower epidermis; and LM, leaf margin. Scale bar, 100 μ m.

on the plasma membrane and these may have been connected with the plasmolysis.

4. Discussion

4.1. Effects of copper on *S. plumbizincicola* and plant detoxification

Copper is an essential plant micronutrient that can promote growth at low concentrations in the growth medium but can be potentially toxic at high concentrations. In the present study 5–50 μ M Cu in the nutrient solution did not increase plant leaf biomass significantly (Table 1), perhaps indicating that the Cu in the unamended solution was adequate for plant growth. A previous study found that a 3.61 mg kg^{−1} Cu in soil inhibited *S. plumbizincicola* growth and addition of Cu to the soil increased the biomass significantly [23]. However, 100 μ M or 200 μ M Cu in nutrient solution significantly inhibited *S. plumbizincicola* biomass (Table 1). Copper concentrations in normal plants without toxicity symptoms vary with plant species, with an average Cu concentration in the leaves of 10 mg kg^{−1} and ranging from 5 to 20 mg kg^{−1} [12]. In Cu accumulator or tolerant plants the Cu concentration in the shoots can be >1000 mg kg^{−1} [24]. In the Cd/Zn hyperaccumulator *S. plumbizincicola* the Cu concentrations can be 37.1, 30.9, and 771 mg kg^{−1} in leaves, stems, and roots, respectively, without toxicity symptoms after growth for 14 days in nutrient solution with 50 μ M Cu (Fig. 1). This foliar Cu concentration is higher than the critical toxicity level of Cu for vascular plants in general, 15–20 mg kg^{−1} [25], and is similar to that of the much-studied Cd/Zn hyperaccumulator *T. caerulescens* exposed to 10 μ M Cu for 8 weeks, but with *T. caerulescens* showing significant toxicity [26]. Although *S. plumbizincicola* had a high tolerance to Cu, when the Cu concentration in the nutrient solution was as high as 100

or 200 μ M, it showed substantial toxicity and decrease in biomass (Table 1).

Root is the one plant organ that has direct contact with Cu in the growth medium. Exposed to excessive Cu in the growth medium, the root might be the first part of the plant to experience toxicity and to exhibit symptoms. After four days' exposure to 200 μ M Cu *S. plumbizincicola* roots displayed toxicity symptoms and exuded white organic materials and this may have been associated with attempts to control Cu availability in the rhizosphere. This function of root exudation of organic matters to decrease Mn availability in the rhizosphere of ryegrass (*Lolium perenne* L.) was also found with increasing Mn concentration in nutrient solution [27]. Transmission electron micrographs indicate that the first toxicity symptom in root cells may be plasmolysis (Fig. 6D). The Cu concentration in leaves was very high (56 mg kg^{−1}) after four days' exposure to 200 μ M Cu and this had a marked influence on the chloroplasts. Compared to the control, starch accumulation in the chloroplasts of leaves exposed to Cu were fewer and smaller and with disorganized thylakoids (Fig. 6A and B). This decrease in starch has also been observed in soybean leaf cells after exposure to excessive Cu [28]. Some studies have shown significant increases in starch in *A. thaliana* and cucumber plants exposed to excessive Cu [29,30]. Accumulation or decrease in starch may result from a balance between synthesis and utilization or translocation. In our study a decrease in synthesis and fast assimilation may both have occurred but the former was clearly indicated by the disordered thylakoids which would have undermined photosynthesis.

Metal distribution in the plant and its sub-cellular distribution and chemical speciation can reflect plant detoxification mechanisms. For the metal subcellular distribution plant usually bind most of metal to the cell wall or accumulate metal in the vacuoles instead of in high active part of cell (e.g. organelles), on the other hand, for metal chemical forms, the metal fractions extracted by 80% ethanol and d-water usually have higher migration and

Fig. 6. Transmission electron micrographs of leaf (up) and root (down) cells of *S. plumbizincicola* exposed to 0.31 μM (A and C) and 200 μM Cu (B and D). Labels: Ch, chloroplast; ST, starch grain; IC, internal cystidium; C, cytoplasm; CW, cell wall; MC, mitochondria; K, karyon; GB, Golgi body; N, nucleoli; and P, particle deposits. Scale bar in each graph, 1 μm .

toxicity than the metal extracted by 2% HAc and 0.6 M HCl [15]. In terms of the whole plant Cu accumulated mainly in the roots when exposed to excessive Cu (Figs. 1 and 2), indicating that *S. plumbizincicola* is a Cu-excluder [31]. Immobilization of Cu in the roots and restrained Cu translocation to the shoot is a type of Cu detoxification mechanism. At the subcellular level, root Cu was mainly present in the cell walls, representing >60% in both treatments (Fig. 4). Furthermore, chemical extraction results show that 65% and 75% Cu occurred as Cu-oxalate (extracted by 0.6 M HCl) and insoluble metal fractions including MHPO_4 and $\text{M}_3(\text{PO}_4)_2$ (extracted by 2% HAc) in the roots of the control and Cu treatments respectively (Fig. 3). Mijovilovich et al. [26] also found a strong metal-metal contribution to Cu-EXAFS spectra in *T. caerulescens* and regarded this as likely to be Cu-oxalate and Cu oxides. In stems and leaves of *S. plumbizincicola* insoluble Cu-phosphate increased much more than did Cu-oxalate compared to the control, perhaps indicating that insoluble Cu-phosphate had an important role in detoxification of stems and leaves (Fig. 3). Moreover, dark deposits on root cell membranes were found in the 200 μM Cu treatments (Fig. 6). Similar deposits at cell walls or in vacuoles have also been found in other studies [28,32]. These deposits might be a form of chelate between the toxic metal and organic acids acting as a response to metal toxicity [33]. These results indicate that Cu immobilization in cell walls and fixation as insoluble forms in cells may be cellular level detoxification mechanisms in *S. plumbizincicola*.

4.2. Effect of Cu on Cd and Zn uptake and translocation in *S. plumbizincicola*

In general, metals are uptake by the plant roots, delivered to the xylem, and transported and unloaded into storage parts such as the leaves. Interactions among metals can occur during these processes. Interactions might include competition for metal transporters during membrane transport where one transporter can carry different metals or disturbance of normal plant physiological functions affecting the uptake or translocation of other metals in the plant [7,13]. 5–50 μM Cu had no significant effect on plant Cd or Zn uptake or translocation compared to the control. This suggests that the transporters for Cd and Zn had high selective affinities or that the Cd and Zn transporter(s) were different from the Cu transporter(s) and this may explain why *S. plumbizincicola* is a hyperaccumulator of only Cd and Zn. However, 100 or 200 μM Cu led to clear inhibition of Cd and Zn uptake (Figs. 1 and 2). Plants exposed to 200 μM Cu for 14 d had significantly lower leaf and stem Zn and Cd concentrations than the controls, but after exposure for 4 days this inhibition occurred only in the leaves for Zn. The different effects of different times of exposure to Cu on the Zn concentrations in the stems are easy to understand and illustrate. Long exposure times disrupt physiological functions such as photosynthesis and transpiration which in turn influence energy metabolism involved in metal transfer processes across membranes and in the xylem.

Interestingly, 200 μM Cu concentrations significantly lowered Cd concentrations in roots but had no effect on Zn after both short and long exposure times to Cu (Figs. 1 and 2). Cadmium is not essential to plants and studies on different Cd hyperaccumulator species have indicated that Cd uptake by roots and its transport to the xylem is by the symplastic pathway [34–36]. If this pathway also applies to Cd in *S. plumbizincicola*, high Cu concentrations would affect Cd transport across membranes. In addition, as discussed above, the formation of insoluble Cu phosphate is one the detoxification mechanisms of *S. plumbizincicola* and this deposition could affect ATP metabolism. Furthermore, plasmolysis in root cells could also physically disturb the transport of Cd across membranes (Fig. 6D). Although these mechanisms might also apply to Zn, root Zn uptake showed no significant change from the control under the influence of 200 μM Cu. This indicates that Zn and Cd have different transporters based on changes in energy metabolism and plasmolysis having no effect on Zn membrane transport or Zn and Cd having different transport pathway in the roots of *S. plumbizincicola*. There is some controversy as to whether Zn transport in the roots of the Zn hyperaccumulator *T. caerulescens* is mainly apoplastic or symplastic [37,38]. Moreover, metal subcellular distribution results show that the percentages of Cd and Zn in the cell walls of roots and leaves increased significantly (Fig. 4), possibly indicating that the symplastic pathway was inhibited for both Zn and Cd by high Cu concentrations. Although in this study the exact pathways of Cd and Zn in *S. plumbizincicola* roots cannot be fully elucidated, it is clear that Cd and Zn had different uptake mechanisms in the roots.

Unloading of metals to the leaves from the xylem and their subsequent storage is one of the mechanisms by which plants hyper-accumulate metals [39], and thus the concentration of metal in the leaves has a direct influence on the function of hyperaccumulators and on the practice of phytoremediation. Four days of 200 μM Cu treatment induced significantly lower Cd and Zn concentrations in the leaves compared to the control with no significant difference from plant background concentrations (Fig. 2). This suggests that Cu restrained Cd and Zn translocation and accumulation in *S. plumbizincicola* leaves. The vascular bundles include the veins where metals are unloaded from the xylem to other leaf cells and are the source of metals in the leaves. The XRF maps show considerable Cu accumulation in the leaf veins (Fig. 5B Cu) and chemical extraction shows that insoluble Cu phosphate increased significantly in both stems and leaves (Fig. 3). This indicates that an insoluble Cu complex deposited in the leaf veins can restrain the metabolism of key transporters or enzymes responsible for Cd and Zn unloading from the xylem to the leaves. However, the further investigations are required to determine how Cu influences this process. This information would help us to understand the mechanism of Cd/Zn accumulation by *S. plumbizincicola* in terms of metal unloading from stems to leaves.

5. Conclusions

Copper at 0.31–50 μM had no significant effect on the growth or Cd or Zn uptake and accumulation of *S. plumbizincicola* but 100 or 200 μM Cu showed inhibition of metal uptake by induction of plasmolysis in the root cells and damage to chloroplast structure. Moreover, *S. plumbizincicola* absorbed Cu in its roots but restricted translocation to the shoots, fixed Cu in the cell walls, and complexed Cu in insoluble forms as its detoxification mechanisms. 100 or 200 μM Cu concentrations exerted different effects on Cd and Zn uptake by the roots and this indicates that the Cd/Zn hyperaccumulator had different uptake mechanisms for Cd and Zn. However, 200 μM Cu restrained unloading of both Cd and Zn from the xylem to the leaves and this may be related to substantial Cu

accumulation in the leaf veins. All these results indicate that effects of Cu need to be considered when using *S. plumbizincicola* to remediate soils polluted with Cd, Zn and Cu and further investigation of the effects of Cu Cd and Zn uptake and translocation mechanisms would help in understanding the mechanisms by which *S. plumbizincicola* hyperaccumulates both Cd and Zn.

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