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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*.

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: $Ca(NO_3)_2{\cdot}4H_2O$ 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, K_2HPO_4 0.5, KCl 0.1, MES 1.0, and KOH 0.5 mmol L^{-1}; and H_3BO_3 10, $Na_2MoO_4{\cdot}2H_2O$ 0.2, $MnSO_4{\cdot}4H_2O$ 1.8, $CuSO_4{\cdot}5H_2O$ 0.31, $NiSO_4{\cdot}6H_2O$ 0.5, Fe-EDDHA 100, and $ZnSO_4{\cdot}7H_2O$ 5 μmol 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 $CuSO_4{\cdot}5H_2O$, i.e. 0.31 (control, the Cu concentration in the nutrient solution), 5, 10, 50, 100, 200 μM for each pot, and 50 μM Cd and 500 μM 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 μM 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 μM). In experiment 2, 0.31 μM and 200 μM Cu were selected together with 50 μM Cd and 500 μM 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 descibed 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 $M(H_2PO_4)_2$; (3) 1 M NaCl, extracting metals associated with pectates and proteins; (4) 2% acetic acid (HAC), extracting the insoluble metal fraction including $MHPO_4$ and $M_3(PO_4)_2$; (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 × *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 × *g* for 10 min, and the three supernatants were pooled. The pooled supernatant solutions from each fraction and