Copper Accumulation and Tolerance in *Chrysanthemum coronarium* L. and *Sorghum sudanense* L.

Lan Wei [1, 2], Chunling Luo [3], Xiangdong Li [3], Zhenguo Shen [1]

[1] *College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China*

[2] *Institute of Soil and Fertilizer, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China*

[3] *Department of Civil and Structural Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong*

Correspondence to: Zhenguo Shen; *email*: zgshen@njau.edu.cn

Abstract. In the present study, the growth of *Chrysanthemum coronarium* L. and *Sorghum sudanense* L., and their copper accumulation were studied using hydroponic experiments. Results showed that the root elongation, dry biomass yield and chlorophyll content in both plant species decreased significantly with the increasing level of Cu in solution. The concentrations of Cu in the two plants increased greatly with the increasing Cu level in the treatments. However, most of Cu was accumulated in roots, and only a small portion was translocated into shoots. Compared with *S. sudanense*, the shoots of *C. coronarium* had significantly higher concentration of Cu. The total amount and percentage of water-soluble Cu, and the non-protein thiol (NPT) were also higher in the shoots of *C. coronarium*. In the roots, however, *S. sudanense* accumulated more Cu than *C. coronarium*. The treatments with 5 to 50 μM L^{-1} Cu significantly increased the uronic acid content in the roots of *S. sudanens*, but did not have any significant effect for *C. coronarium*. Higher concentrations of Cu bound to cell wall and uronic acid in the roots of *S. sudanense* were speculated to be the main reason to restrain Cu translocation from roots to shoots.

Key words: Copper; Cell wall; Non-protein thiol; Uronic acid; Cu-binding protein

Introduction

With the rapid development of agriculture and industry, soil contamination with heavy metals has become a very serious problem throughout the world. Copper is one of common metal contaminants in many parts of the world. Cu contamination is usually

resulted from human activities, such as mining, smelting, industrial waste disposal, sewage sludge application to agricultural soils, and the use of fertilizer and pesticide. In EU countries, the warning and critical limits of Cu in soil are set at 50 and 140 mg kg^{-1}, respectively (Council Directive 86/278/EEC 1986). In China, the contamination with Cu is also very serious, especially for the soils near Cu mines and industrial areas (Li 2006). Although Cu is an essential element to normal plant growth, elevated Cu can produce negative effects on plants through a range of interactions at cellular/molecular levels (Luna *et al*. 1994; Shen *et al*. 1998; Schützendübel and Polle 2002).

In Cu contaminated soils, plants cope with the potential metal stress in different ways. Some plant species adopt exclusion strategy to avoid the excessive uptake and transport of metal ions. These plants usually accumulate relatively low concentrations of metals in their tissues even grown in highly contaminated soils. The roots of some species secrete organic compounds that can bind heavy metals, thereby reducing their uptake into root cells (Hall 2002). Some plant species can hold heavy metals in cell walls (Neumann *et al.* 1995; Bringezu *et al.* 1999; Lou *et al*. 2004; Konno *et al.* 2005), thereby reducing their translocation to shoots. These metal exclusion characteristics make these plants much safer for animals and human beings relying on plant materials. In contrast, some plant roots can take up large amounts of metals, and transport them to the shoots. The accumulated metal ions are detoxified by vacuolar compartmentation or complexing with organic ligands, such as organic acids, amino acid and metal-binding peptides which could greatly ameliorate the toxicity of metals to plant cells (Clemens 2001; Hall 2002). Two types of metal-chelating peptides in plants have been reported, gene-encoded

metallothioneins (MTs) and enzymatically synthesized phytochelatins (PCs). MTs are low molecular weight cysteins-rich proteins. Results obtained by gene expression and functional analysis suggested that plant MTs may be involved in metal homeostasis and tolerance of Cu (van Hoof *et al.* 2001; Roosens *et al.* 2004; Jack *et al.* 2007). PC synthesis, catalyzed by the enzyme PC synthase from reduced glutathione (GSH), is rapidly induced in response to the toxic levels of heavy metals (Clemens 2001). The role of PCs in heavy metal tolerance has been well characterized. However, De Vos *et al.* (1992) found that the synthesis of PCs resulted in a depletion of GSH, causing Cu-mediated oxidative stress. It has been suggested that PCs might not be involved in the detoxification of Cu in plants although they are required for the detoxification of certain non-essential metals, such as Cd and As (Schat *et al.* 2002).

In the present study, two plant species *C. coronarium* and *S. sudanense* were investigated using hydroponic experiments. *C. coronarium* is a normal vegetable plant in south China, and *S. sudanense* is a typical pasture grass with an extensive distribution in China. Considering the distribution and usage of these two plant species, their capacity to accumulate metals will be directly related to the health of animals and human beings. In addition, *C. coronarium* is a dicotyledon plant and *S. sudanense* belongs to monocotyledon plant. The difference of their response to metal stress may provide some insight on the two plants' distinctive morphology. Therefore, the objectives of the current study were (1) to study the tolerance and accumulation of Cu in the two plant species; and (2) to investigate some parameters responsible for Cu tolerance in the plants.

Materials and Methods

Plant Materials and Cu Treatments

The seeds of *C. coronarium* and *S. sudanense* were purchased form Jiangsu Academy of Agricultural Sciences, China. After sterilized in 0.5% (w / v) $NaClO_3$ for 5 min, and rinsed four times in deionized water, the seeds were placed in vermiculite for germination at room temperature (25 °C). After germination, plants of the same size were selected and transferred to 2 L polyethylene vessels containing 1/2-strength Hoagland nutrient solution. One week later, the plants were cultured with full Hoagland nutrient solution at pH 5.5. The nutrient solutions were aerated continuously and renewed every 3 days. The plants were grown in a green house with natural light. On Day 30 after the transplant, the seedlings were treated with different concentrations of Cu supplied in $CuSO_4 \cdot 5H_2O$ solution: 0.32 (control), 5, 10, 25 and 50 μmol L^{-1}. Three replicates were used in each treatment. The root length was recorded on 0, 3, 5 and 7 d after the treatment. After 7 days of exposure, the plants were harvested. Shoots and roots were separated, washed with tap water, and then with deionized water, and blotted dry with tissue paper. Plant samples were further dried in an oven to a constant weight for dry biomass measurement.

Determination of Cu Accumulation

The total concentrations of Cu in the plants were determined according to Zhao *et al*. (1994). The dried samples were digested in a mixture of concentrated HNO_3 and $HClO_4$ (87:13, by volume), and the total concentration of Cu was determined using an atomic absorption spectrophotometer (AAS) (TAS-986, Purkinje General Co., Beijing, China). For

the Cu levels in root cell walls, the cell walls were first isolated with methanol and chloroform (MC) mixed solution, and then the isolated cell walls were digested according to the method described by Hart *et al*. (1992).. The total amounts of Cu in the whole roots were measured in the roots not treated with the solvent.

Water-soluble Cu in the shoots was estimated according to Cakmak and Marschner (1987), in which 0.2 g dried shoot samples were extracted with 10 ml 1 mmol L^{-1} MES-Tris buffer solutions at pH = 6.0 for 5 h. The extraction solution was filtered through a 0.45 μm filter paper (Whatman [Maidstone, UK] 42), acidified with concentrated HNO_3, and analyzed for Cu concentrations in the same method as above.

Estimation of Chlorophyll Content

The level of chlorophyll was determined according to the method described by Arnon (1949). About 0.1 g fresh leaf was extracted with 10 ml ethanol solution, and absorbance was measured at 645 and 663 nm. The chlorophyll content was calculated according to the formula:

$$\text{Chlorophyll concentration (mg g}^{-1}) = (8.04A633 + 20.29A645) \times \frac{V}{1000W}$$

Assay for Total and Non-protein Thiol Contents

Thiol contents were measured using the method of Nagalakshmi and Prasad (2001) with some modifications. 0.5 g root or shoot was homogenized in 4 ml 20 mM L^{-1} ethylenediaminetetraacetic acid (EDTA), and centrifuged at 10,000 × g for 30 min at 4 °C. Aliquots of 0.5 ml of the supernatants were mixed with 2.5 ml of 0.2 M phosphate

buffer solution (PBS) (pH = 8.9) and 0.1 ml of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The color was allowed to develop for 30 min at room temperature (25 °C), and the absorbance of the clear supernatant was measured at 412 nm. Aliquots of 2.0 ml of the supernatants were mixed with 1.6 ml distilled water and 0.4 ml 50% trichloroacetic acid (TCA). After centrifuging at 3,000 × g for 15 min, the contents were mixed well and incubated for 15 min. Aliquots of 1.0 ml of the supernatant was mixed with 2.0 ml of 400 mM PBS and 0.1 ml of 0.01 mM DTNB, and the absorbance was recorded at 412 nm within 5 min against an appropriate blank. The protein-bound thiols were calculated by subtracting the NPT form the total thiols (TT).

Measurement of Uronic Acid Content

The plant samples for uronic acid analysis were heated at 105 °C for 2 h, and then dried at 80 °C for 72 h. 0.2 g dry plant roots, grounded and past through a 0.2 mm diameter sieve, were undergone successive extractions with ethanol/toluene (1:2, v/v) and water (8 h each), and then were dissolved into 4 mL of 72% H_2SO_4 at 50 °C for 10 min. After heating, the solution was diluted with water to 25 ml followed by heating at 120 °C for 1 h in an autoclave. The uronic acids content of the hydrolysate was determined spectrophotometrically using the m-phenylphenol method outlined by Blumenkrantz and Asboe-Hansen (1973) with some modifications. In the process, glucuronic acid was used to develop a standard curve. Photometric absorbance (515 nm) was determined for all samples on a spectrophotometer (*UV-2450/2550*, *Shimadzu*, Kyoto, Japan).

Evaluation of the Cu-binding Proteins

Copper-binding proteins were assayed according to Wong and Qiu (1998) with some modifications. Plant materials (5 g) were homogenized in 50 mmol L^{-1} ice-cold PBS (pH 7.4) containing 10 mmol L^{-1} mercaptoethanol. After filtrating through four layers of gauze cloth, the filtrate was centrifuged at 80,000×*g* for 60 min at 4°C. The supernatant fraction of 2.0 ml was loaded on Sephadex G-50 column (1.6 × 65 cm) pre-equilibrated with 10 mM PBS (pH 7.4), and eluted with the same buffer at a flow rate of 0.5 ml min^{-1}, The elutant solution was collected in a 5 ml fraction, and the absorbance was recorded at 254, 280 and 412 nm followed by the determination of Cu concentrations by AAS (Lou *et al.* 2004).

Statistical Analysis

Statistical analyses of the experimental data, such as of the correlation and significant differences, were performed using SPSS® 11.0 statistical software (SPSS, Chicago, IL, USA). All the values reported in this paper were the means of three replicates. Probability level of 0.05 was considered to be statistically significant for the data set.

Results

Plant Growth

The treatment with 5 μM Cu L^{-1} for 7 d significantly inhibited the net elongation of the roots of *S. sudanense* and *C. coronarium* (see Table 1). The decrease of the root elongation was more pronounced in *S. sudanense* than in *C. coronarium* when the plants

were treated with 10 μM L^{-1} Cu and above. The dry matter (DM) yields of both roots and shoots decreased with the increasing concentration of Cu in solutions. Compared with the shoots, the growth of the roots was more sensitive to the Cu concentration (Table 1).

The levels of chlorophyll in the two plant species also decreased when the Cu concentration in solution increased. At the same Cu treatment, the amounts of chlorophyll in the leaves of *C. coronarium* were lower than that of *S. sudanense* ($P < 0.05$).

Cu Accumulation in Plants

The concentrations of Cu in the shoots and roots of the two plants increased with the increasing Cu supply in the nutrient solutions (see Figs. 1 and 2). In all treatments, most of Cu taken up by plants was concentrated in roots. In comparison with *S. sudanense*, more Cu was found in the shoots of *C. coronarium*. But, in roots, the tendency was *S. sudanens* accumulated more Cu than *C. coronarium* ($P < 0.05$). The highest concentration of Cu in the shoots and roots of *C. coronarium* were 68 and 4290 mg kg^{-1}, and 24 and 5330 mg kg^{-1} for *S. sudanense*, respectively, when they were exposed in the 50 μM L^{-1} Cu solutions.

The level of Cu in the cell wall fractions in roots and shoots also showed an increasing trend with the change of Cu concentration in the nutrient solution (see Figs. 1 and 2). The proportion of Cu on the cell walls in the shoots remained consistent among different treatments. But, it increased significantly in the roots with the increasing Cu supply in the nutrient solution. Between the two plant species, Cu amounts in the shoot cell wall fraction were higher in *C. coronarium* than *S. sudanense*. However, the

proportion of Cu on the shoot cell walls was lower in *C. coronarium* than in *S. sudanense*. In the roots, the amount and the proportion of Cu on root cell walls were both lower in *C. coronarium* than in *S. sudanense*.

The water-soluble Cu in the shoots of both plants increased with the increasing of Cu concentration in solution. However, the percentage of water-soluble Cu in the shoots of *S. sudanense* decreased with the increasing Cu concentration in the treatments. At the same Cu treatment, the percentage of water-soluble Cu was higher in the shoots of *C. coronarium* than in *S. sudanense*.

Uronic Acid Contents in the Roots

There was a significant increase in uronic acid contents in the roots of *S. sudanense* when the Cu concentration in the solution increased from 0.32 to 5 μM L^{-1} (Fig. 3). No further increase was observed at higher Cu concentrations. However, different Cu treatments had no significant effect on the uronic acid content in the roots of *C. coronarium*. When the two plants were treated with the same Cu concentration, the concentrations of uronic acid in the roots of *S. sudanense* were always higher than that in *C. coronarium*.

Non-protein Thiol Contents

Compared with the control groups, the increase of Cu supply in the nutrient solutions from 10 to 50 μM L^{-1} significantly increased non-protein thiol contents in the shoots of *C. coronarium*. (Fig. 4). However, in the shoots of *S. sudanense*, no significant effect was observed on non-protein thiol contents among different Cu treatments. In general, the

contents of non-protein thiol were higher in the shoots of *C. coronarium* than that in *S. sudanense*. However, in the roots, *S. sudanense* had higher non-protein thiols than *C. coronarium* at all Cu treatments. The contents of non-protein thiols in the roots of both plant species showed a decreased trend with the increasing Cu supply in the nutrient solutions.

Profiles of Cu-binding Proteins

The elution fraction of the root and shoot extracts, and Cu contents in the extracts from the two plant species are shown in Figs. 5 and 6. In general, two UV absorbing peaks were observed in Volume 40-55 ml and 110-140 ml, respectively, in both roots and shoots. The second peak in the shoots of *S. sudanense* was far higher than that in *C. coronarium*. Compared with the control group, the treatment with 50 μM L^{-1} of Cu significantly increased the second peak. In the shoots, a substantial amount of Cu was detected in the first peak. However, there was no Cu observed in the second peak. By contrast, Cu showed two peaks in the roots, which were consistent with the peaks of proteins.

Discussion

Excess Cu can produce toxic effects on plants, such as inhibiting plant growth, causing chlorosis of leaves, increasing root cell membranes leakage (Devos et al. 1989; Lidon and Henriques 1992; Murphy and Taiz 1997; Shen *et al*. 1998; Murphy *et al.* 1999). The present results showed that when the plants of *C. coronarium* and *S. sudanense* were

treated with Cu, the dry weights of roots and shoots decreased, and the roots were more sensitive to the Cu toxicity than the shoots (see Table 1). In comparison with *C. coronarium*, Cu treatments had a more negative effect on the root elongation of *S. sudanense*. However, more lateral roots were induced by the Cu treatment in *S. sudanense*, which to some extent compensated for the loss of root dry weight caused by the decreased root elongation, and resulted in a less significant decrease in dry weight than *C. coronarium*. In addition, the shoots of *S. sudanense* responded less to the Cu treatment than that of *C. coronarium*. The results demonstrated *S. sudanense* was more tolerant to Cu stress than *C. coronarium*.

In order to study possible different Cu tolerance mechanisms between *S. sudanense* and *C. coronarium*, several parameters on metal uptake and distribution were investigated. Usually, when plants are exposed to high concentrations of Cu, most of Cu is accumulated in roots. Cu translocated into shoots only accounts for a very small proportion of total metal uptake (Baker and Walker 1990; Wenzel and Jockwer 1999; Stoltz and Greger 2002). In our present study, more than 90% of Cu was concentrated in the roots of the two plants (see Fig. 2). In comparison with *S. sudanense*, more Cu was found in the shoots of *C. coronarium*. When the two plants were treated with 50 μM L^{-1} of Cu, the content of Cu reached 68 mg kg^{-1} in the shoots of *C. coronarium* L. However, only 24 mg kg^{-1} of Cu was recorded in the shoots of *S.sudanense* L. (see Fig. 1). On the contrary, in the roots, more Cu was found in *S. sudanense*, and the root cell walls absorbed more Cu than that of *C. coronarium* (Fig. 2). It is known that cell wall is the first obstacle for Cu entering into plant cells. Absorption to cell walls can decrease Cu

availability, which in turn decrease the ability of Cu^{2+} to penetrate the plasma membrane, and increase the resistance of plants to Cu toxicity. Heavy metals precipitation on cell walls may be a defending mechanism adopted by plants to tolerate high concentrations of metals, which can reduce the potential damage to cell membrane, and prevented heavy metal ions from entering plant cell plasma (Macfie and Welbourn 2000; Nedelkoska and Doran 2000). Lou *et al*. (2004) reported the concentration of Cu in the roots of *Elsholtzia haichowensis* increased sharply when the plant was exposed to 100 μM L^{-1} Cu nutrient solutions, and most Cu was distributed on the cell walls. Branquinho *et al*. (1997) also observed about 70-90% of Cu was bound to cell walls of the lichen (*Usnea* spp.) The present study shows that the percentage of Cu on the root cell walls increased with the increasing Cu supply in the nutrient solutions. When being treated with 50 μM L^{-1} of Cu, about 76% of the Cu was bound to the root cell walls in *S. sudanense*. However, a significant lower amount (e.g. 60%) of Cu was found on root cell walls of *C. coronarium*. In *S. sudanense*, most of Cu was adsorbed on cell walls, and was not transported upward, which may be responsible for the higher tolerance to Cu toxicity than *C. coronarium*. The ability to resist Cu toxicity in plant could be attributed to the composition of cell walls. A generalized plant cell wall is composed of cellulose, hemicellulose, pectin, and glycoprotein (Wang and Evangelou 1995). Usually, the content of pectin on cell walls is 7-10 times higher than glycoprotein. Uronic acid residues from pectin could dissociate into negatively charged carboxyl. Therefore, the root cell walls are dominated by negatively charged sites. Positively charged Cu^{2+} could be adsorbed to cell walls by electrostatic force. Further negative charge was produced by the dissociation of uronic

acid residues, and more Cu^{2+} would be adsorbed. Hence, much higher tolerance to Cu would be achieved in the plants which can adsorb metals on cell walls (Nishizono *et al*. 1987; Branquinho *et al*. 1997; Bringezu *et al*. 1999). Our present study showed that the uronic acid content was higher in the roots of *S. sudanense* from the 5-50 μmol L^{-1} Cu treatments than that from the 0.32 μmol L^{-1}Cu treatment, and it was also higher than that in *C. coronarium* at the same Cu treatment. Uronic acid residues in the roots may provide more binding sites to absorb Cu^{2+} on cell walls

Compartmentation of heavy metal ions into the vacuole is another important mechanism employed by the plants to reduce metal toxicity through limiting free heavy metal ions entering into the cytosol. When *Thlaspi caerulescens* was exposed to high Zn solutions, Zhao *et al*. (1998) found that more than 80% of Zn in the shoots was present in water-soluble forms. The high percentage of water-soluble Zn in the shoots suggested the excessive Zn was complexed with soluble organic compounds and stored in the vacuoles (Zhao *et al*. 1998). In addition, Liu *et al*. (2007) reported that the water-soluble Cd in the shoots of *Brassica pekinensis* accounted for more than 60% of the total Cd in the shoots when the plant was exposed to 50 and 100 μM L^{-1} Cd solutions. The high tolerance to Cd observed in *B. pekinensis* was probably attributed to the effective vacuolar compartmentation of the metal in its shoots. In the current study, 67-88% of the Cu in the shoots of *C. coronarium* was in the water-soluble form under all Cu treatments, which was significantly higher than that in the shoots of *S. sudanense*. Moreover, the contents of non-protein thiols in the shoots of *C. coronarium* increased dramatically with the increasing Cu supply from 0.32 to 20 μM L^{-1} in solution. Higher NPT levels in the shoots

may increase Cu tolerance by forming a NPT-Cu complex and by protecting plant cells from metal-related oxidation stress damage. It was reported under Cd stress condition, Cd could be transported over the tonoplast as Cd-PCs, and was stored in the vacuoles (Ernst *et al*. 1992). Probably, Cu was transported over the tonolplast as an NPT-Cu complex, and was finally stored in the vacuole in the detoxification process.

When plants are exposed to toxic metal stress, intracellular metal-binding peptides, MTs and PCs, could be synthesized to defend themselves from possible damage (Clemens 2001; Hall 2002). MTs and PCs can effectively bind metals through thiolate cluster formation. Enhanced MT expression was observed in metal tolerant plants such as *Silene paradoxa*, *Silene vulgaris* and *Thlaspi caerulescens* (van Hoof *et al.* 2001; Roosens *et al.* 2004; Jack *et al.* 2007). Lou *et al*. (2004) found that *E. haichowensis* exposed in 20 and 100 μM L^{-1} of Cu solution had a high level of Cu-binding proteins in the roots. Our present results also showed the Cu treatments could induce Cu-binding protein synthesis in *C. coronarium* and *S. sudanense* (Figs. 5 and 6). The first peak between 40 and 55 ml represented high molecular weight proteins. The second peak (110-140 ml) may contain some low molecular substances, such as amino acids, GSH (Wong and Qiu 1998; Lou *et al*. 2004). When the plants were exposed to high Cu supply (50 μM L^{-1}), the protein peak and Cu concentrations in the 40-55 ml increased in the roots of *S. sudanense*. Meanwhile, in the 40-55 ml, the Cu concentration in the roots of *S. sudanense* was significantly higher than that in *C. coronarium*, indicating that more Cu was probably restrained in the roots associated with high molecular proteins in *S. sudanense*. While in the roots of *C. coronarium*, Cu appeared mainly in the second peak,

which may imply that more Cu had been complexed with low molecular weight compounds like GSH, which were transported into the shoots in the forms of these complexes. GSH is one of low-molecular weight non-protein thiol in plants, and it functions as an antioxidant by scavenging free radicals. GSH is also a direct precursor PCs. PC production may result from oxidative stress due to the depletion of GSH (De Vos et al. 1992). Additional investigations are necessary to characterize in details the metal-binding complexes induced in *C. coronarium* and *S. sudanense* in response to Cu stress.

Conclusions

S. sudanense showed higher tolerance to Cu stress than *C. coronarium*. This could be attributed to that more Cu was bound to the root cell walls, and a higher uronic acid in the roots of *S. sudanense*, which could restrain the translocation of Cu into the shoots. On the other hand, the total Cu and the percentage of water-soluble Cu, and the NPT were all higher in the shoots of *C. coronarium* than those in *S. sudanense*. The high percentage of water-soluble Cu in the shoots might indicate excessive Cu was complexed with soluble organic compounds and stored in the vacuoles. The involvement of these compounds in Cu detoxification mechanisms in *C. coronarium* and *S. sudanense* needs further detailed studies.

Acknowledgements

This research project was supported by the National Natural Science Foundation of China (No. 30471036), the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institutions of MOE, and a Postdoctoral Research Fellowship from The Hong Kong Polytechnic University (G-YX88).

Reference

Arnon DI (1949) Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris* L. Plant Physiol 24:1-15

Baker AJM, Walker PL (1990) Ecophysiology of metal uptake by tolerant plants. In: AJ Shaw (ed) Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Boca Raton, Florida, USA, pp 155-178

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uranic acids. Anal Biochem 54:484-489

Branquinho C, Brown DH, Catarino F (1997) The cellular location of Cu in lichens and its effects on membrane integrity and chlorophyll fluorescence. Environ Exp Bot 38:165-179

Bringezu K, Lichtenberger O, Leopold I, Neumann D (1999) Heavy metal tolerance of *Silene vulgaris*. J Plant Physiol 154:536-546

Cakmak I, Marschner H (1987) Mechanism of phosphorus-induced zinc deficiency in cotton. III. Changes in physiological availability of zinc in plants. Physiol Plant 70:13-20

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta

212:475-486

Devos, CHR, Schat H, Vooijs R, Ernst WHO (1989) Copper-induced damage to the permeability barrier in roots of Silene-cucubalus. . J Plant Physiol 135:164169.

De Vos CHR, Vonk MJ, Vooijs R, Schat H (1992) Glutathione deleption due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol. 98:853-858

Ernst WHO, Verkleij JAC, Schat H (1992) Metal tolerance in plants. Acta Bot Neerl 41:229-248

European Commission (1986) Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. Brussels

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1-11

Hart JJ, DiTomaso JM, Linscott DL, Kochian LV (1992) Characterization of the transport and cellular compartmentation of paraquat in intact maize seedlings. Pestic Biochem Physiol 43:212-222

Jack E, Hakvoort HWJ, Reumer A, Verkleij JAC, Schat H, Ernst WHO (2007) Real-time PCR analysis of metallothionein-2b expression in metalliocolous and non-metallicolous populations of Silene vulgaris (Moench) Garcke. Environ Exp Bot 59:84-91

Konno H, Nakato T, Nakashima S, Katoh K (2005) *Lygodium japonicum* fern accumulates copper in the cell wall pectin. J Exp Bot 56:1923-1931

Li MS (2006) Ecological restoration of mine land with particular reference to the metalliferous mine wasteland in China: a review of research and practice. Sci Total Environ 357:38-53

Lidon FC, Henriques FS (1992) Copper toxicity in rice: Diagnostic criteria and effect on tissue Mn and Fe. Soil Sci 154:130-135

Liu CP, Shen ZG, Li XD (2007) Accumulation and detoxification of cadmium in *Brassica pekinensis* and *B. chinensis*. Biol Plant 51:116-120

Lou LQ, Shen ZG, Li XD (2004) The copper tolerance mechanisms of *Elsholtzia haichowensis*, a plant from copper-enriched soils. Environ Exp Bot 51:111-120

Luna CM, Gonzalez CA, Trippi VS (1994) Oxidative damage caused by an excess of copper in oat leaves. Plant Cell Physiol 35:11-15

Macfie SM, Welbourn PM (2000) The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Arch Environ Contam Toxicol 39:413-419

Murphy AS, Eisinger WR, Shaff JE, Kochian LV, Taiz L (1999) Early copper-induced leakage of K^+ from Arabidopsis seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol 121:1375-1382

Murphy A, Taiz L (1997) Correlation between potassium efflux and copper sensitivity in 10 Arabidopsis ecotypes. New Phytol 136:211-222

Nagalakshmi N, Prasad MNV (2001) Response of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci 160:291-299

Nedelkoska TV, Doran PM (2000) Characteristics of heavy metal uptake by plant species with potential for phytoremediation and phytomining. Miner Eng 13 (5):549-561

Neumann D, Nieden UZ, Lichtenberger O, Leopold I (1995) How does *Armeria maritima* tolerate high heavy metal concentrations? J Plant Physiol 146:707-717

Nishizono H, Ichikawa H, Suziki S (1987) The role of the root cell wall in the heavy metal tolerance of *Athyrium yokoscense*. Plant Soil 101:15-20

Roosens NH, Bernard C, Lephae R, Verbruggen N (2004) Evidence for copper homeostasis function of metallothionnein (MT3) in the hyperaccumulator Thlaspi caerulescens. FEBS Lett 577:9-16

Schat H, Liugany M, Vooijs R, Hartley-Whitaker J, Bleeker PM (2002) The role of phytochelatins in constitutive and adaptive heavy metals tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. J Exp Bot 53:2381-2392

Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351-1365

Shen ZG, Zhang FQ, Zhang FS (1998) Toxicity of copper and zinc in seedings of Mung Bean and inducing accumulation of polyamine. J Plant Nutr 21(6):1153-1162

Stoltz E, Greger M (2002) Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. Environ Exp Bot 47:271-280

van Hoof NALM, Hassinen VH, Hakvoort HWJ, Ballintijn KF, Schat H, Verkleij JAC, Ernst WHO, Karenlampi SO, Tervahauta AI (2001) Enhanced copper tolerance in

Silene vulgaris (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Plant Physiol. 126:1519-1526

Wang J, Evangelou VP (1995) Metal tolerance aspects of plant cell wall and vacuole [A]. In: Pessarakli M (ed) Handbook of Plant and Crop Physiology. Marcel Dekker, Inc, New York, pp 695-717

Wenzel WW, Jockwer F (1999) Accumulation of heavy metals in plants grown on mineralized soils of the Austrian Alps. Environ Pollut 104:145-155

Wong YS, Qiu GH (1998) Isolation and purification of metal binding peptide from copper-treated *Festuca rubra* cv. Merlin roots (in Chinese). Chin J Appl Environ Biol 4: 335-339

Zhao FJ, McGrath SP, Crosland AR (1994) Comparison of three wet digestion methods for the determination of plant sulphur by inductively coupled plasma atomic emission spectrometry (ICP-AES). Commun Soil Sci Plan Anal 25:407-418

Zhao FJ, Shen ZG, McGrath SP (1998) Solubility of zinc and interactions between zinc and phosphorus in the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 21 (1):108-114

Table 1. The effects of various Cu treatments on the dry mass, root elongation, and chlorophyll content of *C. coronarium* and *S. sudanense*. 7 d after the treatments. Different letters in the same column indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

Cu concentration (μmol L^{-1}) in the treatment	*C. coronarium*				*S sudanense*			
	shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Net root elongation (cm)	Chl (a+b) (mg g^{-1})	shoot dry weight (g $plant^{-1}$)	Root dry weight (g $plant^{-1}$)	Net root elongation (cm)	Chl (a+b) (mg g^{-1})
0.32	0.402^{a}	0.090^{a}	8.8^{a}	0.75^{a}	0.753^{a}	0.227^{a}	6.6^{a}	0.88^{a}
5	0.399^{a}	0.080^{b}	6.4^{b}	0.52^{b}	0.711^{a}	0.209^{ab}	4.9^{b}	0.86^{ab}
10	0.370^{b}	0.062^{c}	5.9^{bc}	0.42^{c}	0.654^{ab}	0.203^{ab}	2.8^{c}	0.81^{b}
25	0.365^{b}	0.053^{d}	3.7^{c}	0.43^{c}	0.595^{b}	0.198^{b}	1.6^{d}	0.69^{c}
50	0.364^{b}	0.048^{d}	1.4^{d}	0.38^{c}	0.590^{b}	0.173^{c}	0.5^{e}	0.54^{d}

Figure Captions

Fig. 1. The effects of various Cu treatments on the total Cu concentrations (A), cell wall Cu concentrations (B), the percentage of Cu on cell walls (C) and the percentage of water-soluble Cu (D) in the shoot of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

Fig. 2. The effects of various Cu treatments on the total Cu concentrations (A), cell wall Cu concentrations (B) and the percentage of Cu on cell walls (C) in the root of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

Fig. 3. The effects of various Cu treatments on the uronic acid content in the roots of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

Fig. 4. The effects of various Cu treatments on the non-protein thials contents in the shoots (A) and roots (B) of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

Fig. 5. Gel filtration of Cu-binding protein and Cu concentrations in the shoots (A) and roots (B) of *C. coronarium* treated with 0.32 μmol L^{-1} (control) or 50 μmol L^{-1} Cu on Sephadex G-50 in different collection volume. (OD280 represents the absorbance of 280 nm)

Fig. 6. Gel filtration of Cu-binding protein and Cu concentrations in the shoots (A) and roots (B) of *S. sudanense* treated with 0.32 μmol L^{-1} (control) or 50 μmol L^{-1} Cu on Sephadex G-50 in different collection volume. (OD280 represents the absorbance of 280 nm)

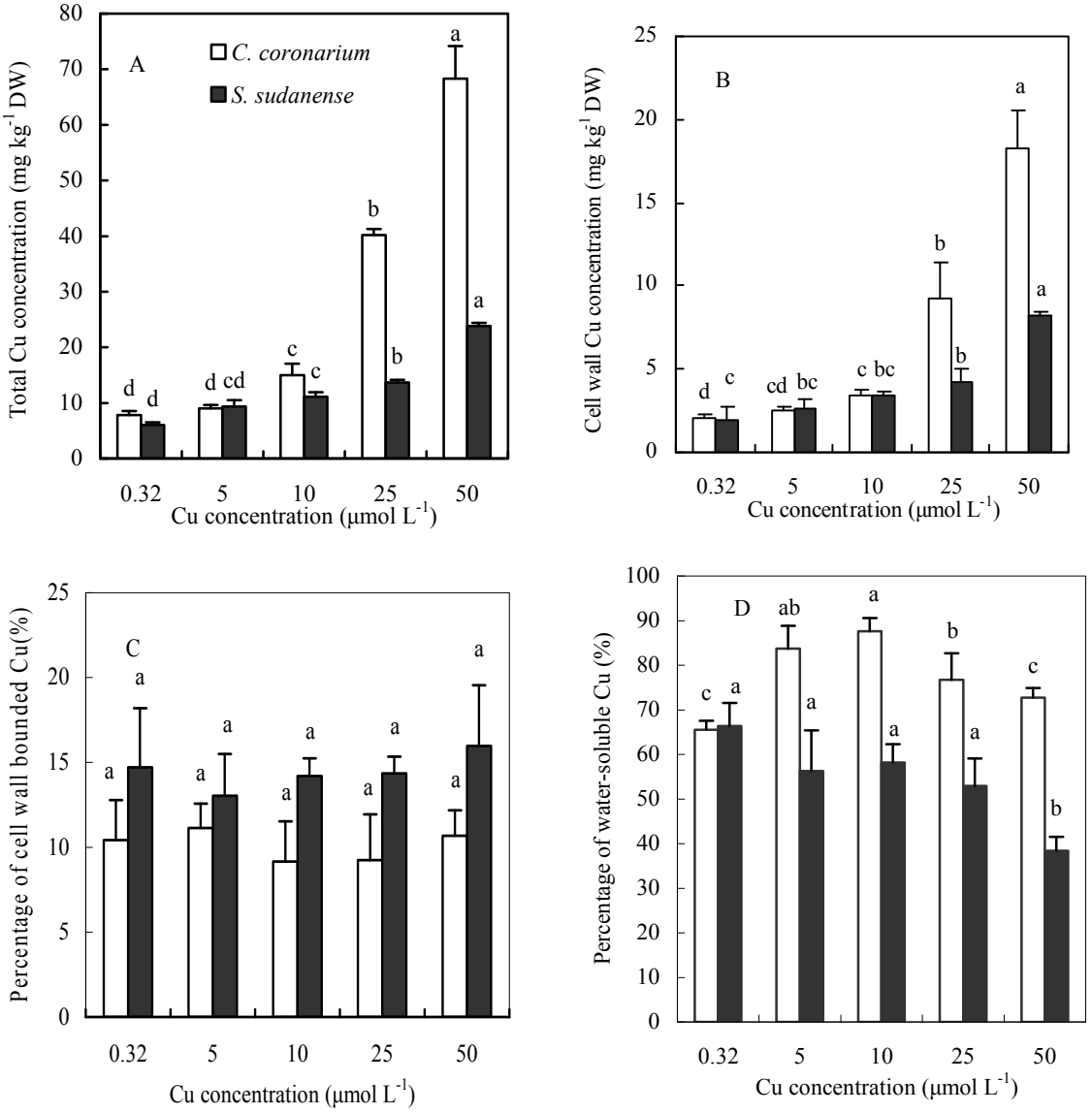

Fig. 1. The effects of various Cu treatments on the total Cu concentrations (A), cell wall Cu concentrations (B), the percentage of Cu on cell walls (C) and the percentage of water-soluble Cu (D) in the shoot of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

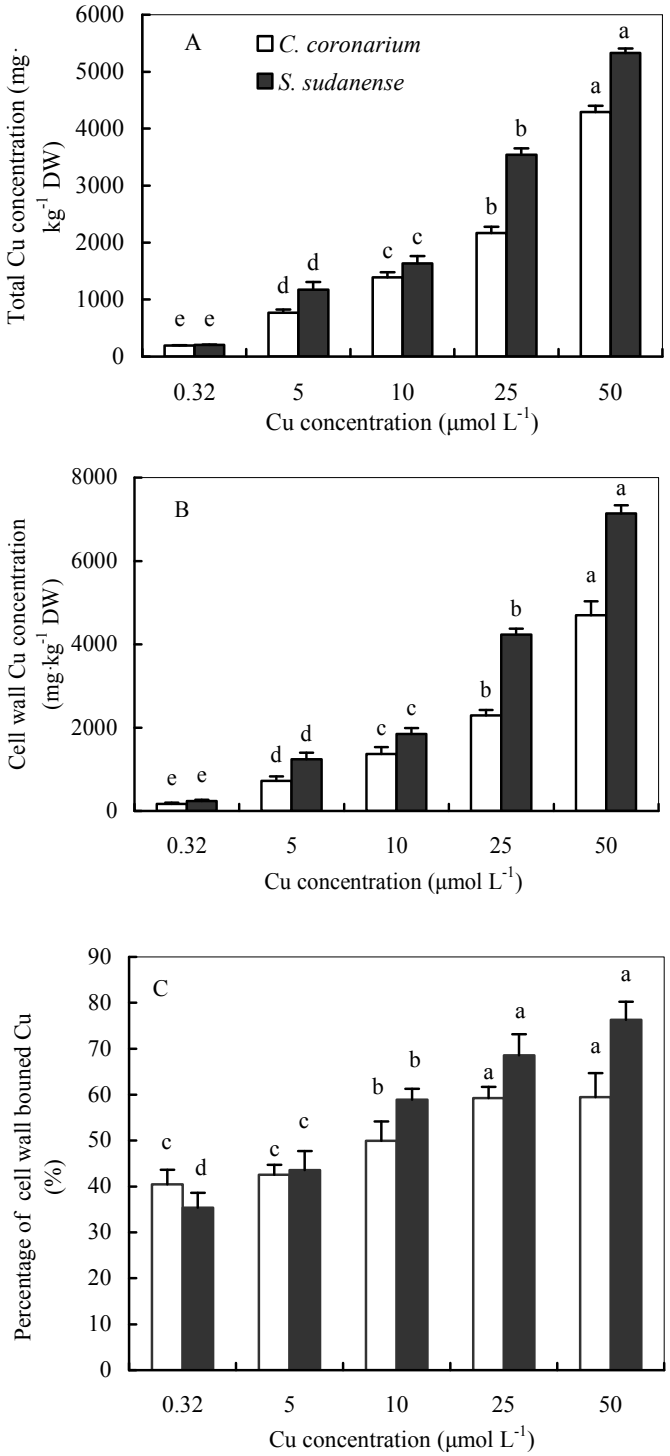

Fig. 2. The effects of various Cu treatments on the total Cu concentrations (A), cell wall Cu concentrations (B) and the percentage of Cu on cell walls (C) in the root of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

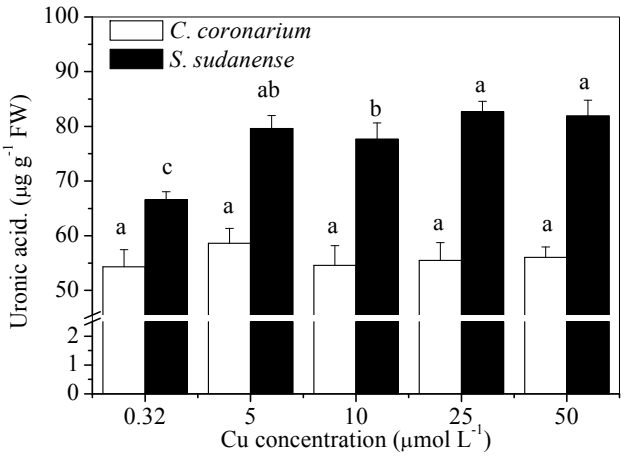

Fig. 3. The effects of various Cu treatments on the uronic acid content in the roots of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

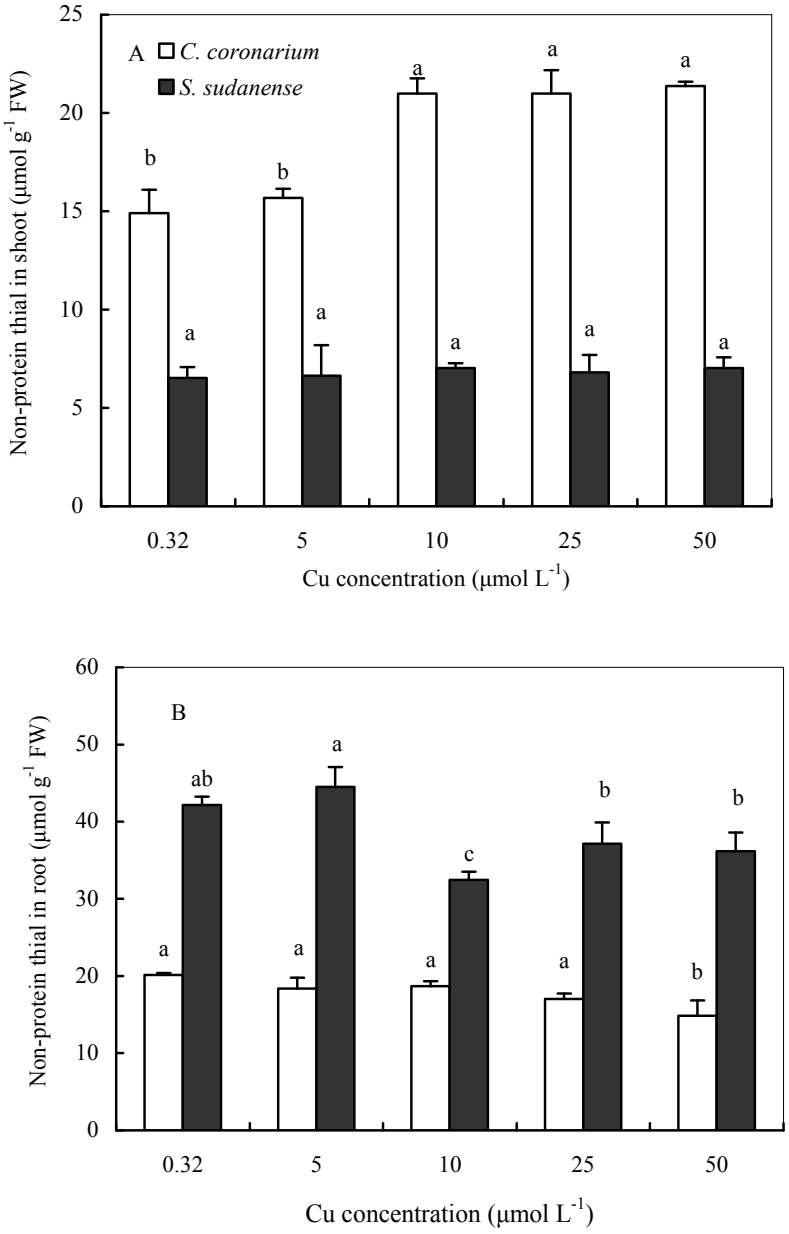

Fig. 4. The effects of various Cu treatments on the non-protein thials contents in the shoots (A) and roots (B) of *C. coronarium* and *S. sudanense*. Different letters over the column within the same plant indicate a significant difference at $P \leq 0.05$ according to the LSD tests.

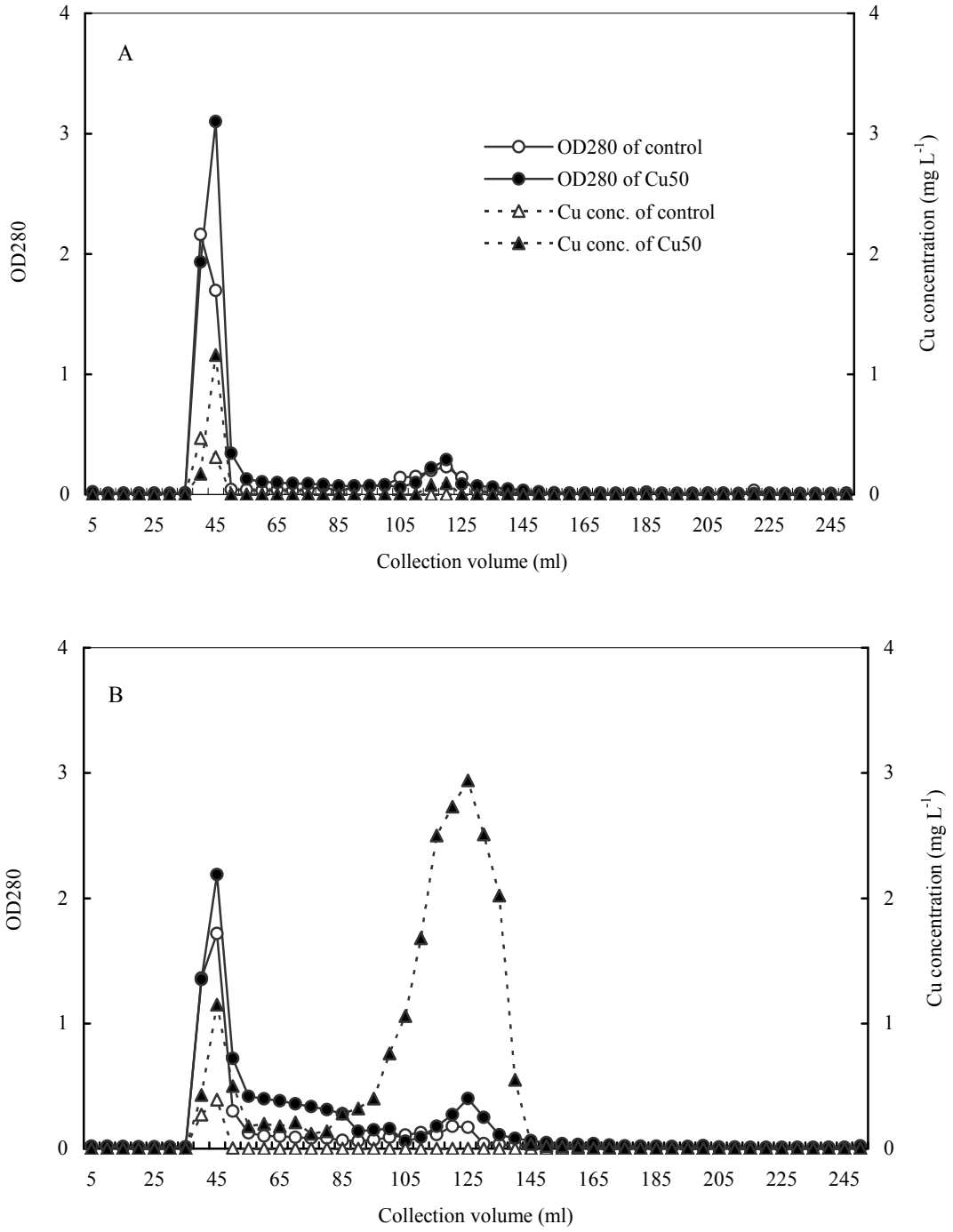

Fig. 5. Gel filtration of Cu-binding protein and Cu concentrations in the shoots (A) and roots (B) of *C. coronarium*. treated with 0.32 μmol L^{-1} (control) or 50 μmol L^{-1} Cu (Cu50) on Sephadex G-50 in different collection volume. (OD280 represents the absorbance of 280 nm).

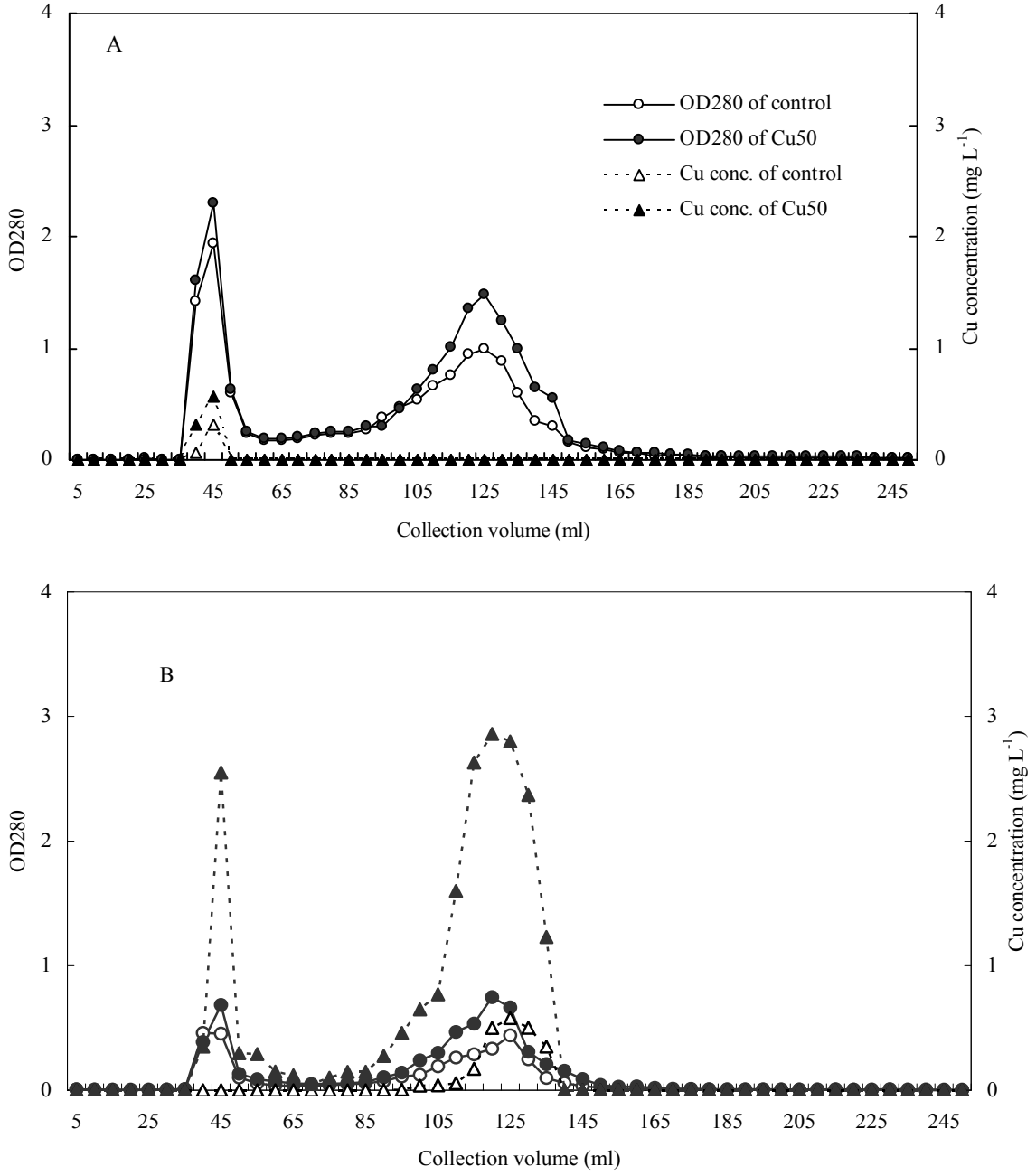

Fig. 6. Gel filtration of Cu-binding protein and Cu concentrations in the shoots (A) and roots (B) of *S. sudanense* treated with 0.32 μmol L^{-1} (control) or 50 μmol L^{-1} Cu (Cu50) on Sephadex G-50 in different collection volume. (OD280 represents the absorbance of 280 nm).