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Comparative Studies of Copper Tolerance and Uptake by Three Plant Species of the Genus Elsholtzia

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Abstract Solution culture experiments were conducted to investigate the effects of excessive Cu on the seed germination and growth of three plant species of the genus Elsholtzia (*Elsholtzia haichowensis*, *Elsholtzia aypriani* and *Elsholtzia ciliata*), and to compare Cu uptake and tolerance mechanisms of the three plants. The results showed that *E. haichowensis* had higher tolerance to excessive Cu than *E. aypriani* and *E. ciliata*, and that the adaptive Cu tolerance mechanism in *E. haichowensis* might involve the active participation of proteins.

Keywords *Elsholtzia* · Copper · tolerance · Accumulation

Copper is known to be an essential micronutrient for the development of plants and algae (Marschner, 1995). However, excessive Cu in the soil causes chlorosis of leaves, inhibits photosynthesis, affects nitrogen and protein metabolism, disturbs mineral uptake, and induces lipid peroxidation (Shen et al., 1998; Nielsen et al., 2003; Demirevska-Kepova et al., 2004). It is well known that plants have different resistances and very different capacities to accumulate heavy metals (Baker et al., 2000; Peng et al., 2006). Depending on the plant species, metal resistance may result from two basic strategies: (1) exclusion, whereby plants avoid excessive uptake and transport of metal ions from the roots to the shoots, and (2) accumulation and sequestration, whereby plants detoxify free metals by compartmentalization of metals in vacuoles (Baker, 1981).

Elsholtzia haichowensis Sun (*Elsholtzia splendens*) is an indicator plant with characteristics of fast growth and higher biomass. In solution experiments, the maximum Cu concentration in the shoots of *E. haichowensis* treated with 1,000 μmol/L Cu was 3,417 mg/kg (Yang et al., 2002). The closely related species, *Elsholtzia cypriani* and *Elsholtzia ciliata*, are found in the old mine waste in Xiangxi area of Hunan province, China. However, little information is available about the difference of tolerance of the three *Elsholtzia* species for copper.

This research was performed by studying *E. haichowensis*, *E. cypriani* and *E. ciliata* from highly polluted copper-smelter wasteland sites. The aim was to compare their capacities for accumulating Cu under controlled conditions. In addition, this study reports on the effect of excess Cu on seed germination, plant growth, and soluble protein content, as well as on the accumulation of nutritional elements.

Materials and Methods

Seeds of three plant species of the genus Elsholtzia were collected from Tongling city in Anhui province and Xiangxi areas of Hunan province, China. The seeds were washed twice with distilled water. Fifty to sixty seeds were sown on filter paper wetted with 2.0 ml dd H_2O or test solution in a 7.0-cm Petri dish. The seeds were incubated under controlled environment conditions (12 h day length with a photo flux density of 300 μmol supplied by fluorescent tubes, and 25/20°C day/night temperature). The germination percentage was recorded. The radicle and hypocotyl lengths were measured 10 days after treatment.

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Table 1 The effects of Cu treatment on seed germination, the radicle length, and the hypocotyl length of three plant species during seed germination

Elsholtzia species	Cu treatment (μmol/L)	Germination percentage (%)	Radicle length (cm)	Hypocotyl length (cm)
E. haichowensis	0.32	88.1 ± 4.5 a	3.21 ± 0.07 b	0.55 ± 0.05 a
	50	92.0 ± 1.9 a	3.01 ± 0.18 ab	0.68 ± 0.07 a
	100	89.2 ± 2.9 a	2.75 ± 0.16 a	0.73 ± 0.06 a
E. cypriani	0.32	60.7 ± 5.5 a	0.54 ± 0.02 b	0.54 ± 0.03 b
	50	72.2 ± 4.4 a	0 ± 0 a	0.37 ± 0.01 a
	100	67.0 ± 3.7 a	0 ± 0 a	0.28 ± 0.04 a
E. ciliata	0.32	72.7 ± 1.0 a	1.29 ± 0.11 b	0.89 ± 0.10 b
	50	78.4 ± 3.0 a	0.12 ± 0.01 a	0.68 ± 0.02 ab
	100	72.5 ± 4.8 a	0.09 ± 0 a	0.49 ± 0.06 a

Healthy and uniform-sized seeds from the three plant species were chosen and sown in vermiculite for germination in a growth chamber with a photoperiod of 12 h light/12 h darkness and day/night temperature of 25/20°C. Seedlings were transferred to 2-L containers containing Hoagland solution two weeks after germination. Solution pH was adjusted to 5.4 ± 0.1 by 0.1 mol/L HCl or 0.1 mol/L NaOH. The nutrient solution was renewed every two days. After one month, copper ($CuSO_4 \cdot 5H_2O$) was added to the nutrient solution at concentrations of 0.32, 50, and 100 μmol/L. Every treatment had three replicates. Plants were harvested 10 days after treatment. After measurement of the length of the longest root, the fresh shoots and roots were washed twice and weighed. The plant samples were dried for 15 min at 105°C and for 24 h at 80°C in an oven, then dry weights were measured. The dried plant samples were completely digested with extra-pure-grade HNO_3 and $HClO_4$ (87:13, v/v). Elements such as Cu, Mg, Zn, Ca, and Fe concentrations were analyzed by flame atomic absorption spectrometer (AAS) (TAS-986). The detection limits for the AAS were as follows: Cu = 0.008, Mg = 0.001, Zn = 0.005, Ca = 0.009 and Fe = 0.006 mg/L. Phosphorus was analyzed spectrophotometrically following the procedure described by Shi et al. (1980). A certified standard reference material (SRM 1573a, tomato leaves) from the National Institute of Standards and Technology, USA, was used in the digestion and analysis. The recovery rates for elements in the standard reference material were 95–106%. Blanks were also used for background correction and other sources of error. Ten days after copper treatment, chlorophyll content was determined. Fresh leaves (0.2 g) were extracted with 10 ml of 95% ethanol as described by Li et al. (2000). Samples were analyzed using a UV-2450 Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Tokyo) at 665 and 649 nm. For the determination of soluble proteins, the frozen plant materials were homogenized in 100 mmol/L PBS (pH 7.0) buffer solution and centrifuged at 12000×g for 15 min at 4°C. Content of soluble protein was determined as described by Li et al. (2000). All chemicals were of analytical reagent grade.

The data were analyzed using one-way analysis of variation (ANOVA) using the SPSS version 10.0 statistical package. Means of treatments were compared using the least-significant difference (LSD) test at the significance level of $p < 0.05$.

Results and Discussion

After 10 days, Cu at 50 and 100 μmol/L had no significant effect on seed germination of the three plant species (Table 1). However, 50 and 100 μmol/L Cu significantly decreased the hypocotyl and radicle lengths of *E. cypriani* and *E. ciliata* compared to the corresponding controls except for the 50 μmol/L Cu treatment on the hypocotyl growth of *E. ciliata* (Table 1). For *E. haichowensis*, a significant decrease was found only in the length of radicle treated with 100 μmol/L Cu. No significant difference in the length of hypocotyl was observed between different Cu treatments. The results showed that *E. haichowensis* had a higher tolerance to excessive Cu than *E. cypriani* and *E. ciliata* during germination of their seeds. Previous studies indicated that *E. haichowensis* has high Cu tolerance and may accumulate large amounts of Cu in its aerial tissues (Lou et al., 2004). In the present study, *E. haichowensis* was found to be more tolerant to Cu than the closely related species *E. cypriani* and *E. ciliata* (Table 2). Copper at 50 μmol/L did not significantly inhibited shoot and root growth of *E. haichowensis* in comparison with the control. But for *E. cypriani* and *E. ciliata*, root elongation was nearly completely inhibited, and root and shoot dry matter yields were decreased by 48, 44% and 60, 34%, respectively, when the plants were exposed to 50 μmol/L Cu for 10 days. Jouili and Ferjani (2003) also reported that dry-matter production of sunflower seedling roots was significantly decreased by treat-

Table 2 Net root elongation and dry weight of the shoots and roots of three plant species seedlings exposed to Cu for 10 days

Elsholtzia species	Cu treatment (μmol/L)	Root elongation (cm)	Dry biomass (mg/plant)	
			Shoots	Roots
E. haichowensis	0.32	5.65 ± 1.43 a	68.4 ± 5.7 b	20.7 ± 1.9 b
	50	5.46 ± 1.52 a	58.6 ± 4.9 ab	17.5 ± 1.1 ab
	100	2.91 ± 0.22 a	44.6 ± 0.8 a	15.6 ± 0.7 a
E. *cypriani*	0.32	9.29 ± 0.57 b	79.6 ± 3.4 b	18.7 ± 1.1 c
	50	0.03 ± 0.02 a	44.8 ± 2.9 a	9.7 ± 0.9 b
	100	0.07 ± 0.03 a	36.0 ± 3.0 a	6.5 ± 0.4 a
E. ciliata	0.32	3.17 ± 0.43 b	226.5 ± 7.8 b	63.0 ± 3.3 b
	50	0.12 ± 0.07 a	149.8 ± 14.8 a	25.0 ± 4.4 a
	100	0.06 ± 0.02 a	120.4 ± 14.2 a	17.8 ± 2.5 a

Table 3 Chlorophyll *a* and *b* contents in the leaves of the three plant species seedlings under different Cu concentrations

Elsholtzia species	Cu treatment (μmol/L)	Chl *a* content (mg/g FW)	Chl *b* content (mg/g FW)
E. haichowensis	0.32	1.692 ± 0.007 c	1.147 ± 0.066 c
	50	1.435 ± 0.091 b	0.557 ± 0.063 b
	100	0.834 ± 0.036 a	0.278 ± 0.010 a
E. cypriani	0.32	1.706 ± 0.014 b	1.032 ± 0.102 b
	50	1.628 ± 0.035 a	0.800 ± 0.068 ab
	100	1.632 ± 0.007 ab	0.785 ± 0.007 a
E. ciliata	0.32	1.633 ± 0.012 a	1.477 ± 0.042 a
	50	1.650 ± 0.021 a	1.343 ± 0.223 a
	100	1.611 ± 0.033 a	1.609 ± 0.137 a

ment with 50 μmol/L Cu for five days. The 3.6 μmol/L Cu treatment produced a marked reduction in elongation of wheat roots and shoots (Ciscato et al., 1997).

As a consequence of Cu treatment, *E. haichowensis* showed a marked reduction in chlorophyll *a* content (Table 3). Chlorophyll *b* content was also decreased by excess Cu in *E. haichowensis* and *E. cypriani*. In the former species the decrease was more pronounced: about 80% in *E. haichowensis* and 20% in *E. cypriani* for the 100 μmol/L Cu treatment. For *E. ciliata*, increasing the Cu supply did not have any significant effect on chlorophyll *a* and *b* content. The decrease in chlorophyll concentration may be the result of an inhibited biosynthesis of chlorophyll either through the direct inhibition an enzymatic step or through an induced Fe deficiency (Lou et al., 2004). For *E. cypriani* and *E. ciliata*, less change in chlorophyll content is more likely related to the reduction in shoot biomass on exposure to 50 and 100 μmol/L of Cu.

Shoots and roots of *E. haichowensis* had significantly higher concentrations of Cu than *E. cypriani* and *E. ciliata* (Fig. 1). The concentrations of Cu in the shoots of *E. cypriani* and *E. ciliata* exposed to 0.32 μmol/L Cu were within normal range of 5 to 30 mg/kg (Kabata-Pendias and Pendias, 1992). For *E. haichowensis*, even with low Cu supply (0.32 μmol/L), the Cu concentration in shoots was still considerably higher than normal values and reached levels that are toxic to many other species. With increasing Cu level in the nutrient solution, there was a progressive increase in Cu concentrations in the shoots and roots of the three plant species, except in the shoots of *E. cypriani*. The maximum Cu concentrations in the shoots and roots were 217 and 13,170 mg/kg dry weight (DW), respectively, when the plants of *E. haichowensis* were grown in the 100 μmol/L Cu treatment. For *E. cypriani*, 100 μmol/L Cu resulted in severe toxicity and decreased Cu uptake by shoots. At all the levels of Cu treatments, Cu concentrations were substantially higher in the roots than those in the shoots of the three species. Yang et al. (2002) reported that *E. haichowensis* shoots accumulated > 1,000 μg Cu/g DW under hydroponics and suggested that it is a Cu hyperaccumulator. Recently, it has been demonstrated that *E. haichowensis* was not a Cu hyperaccumulator, but a Cu excluder (Lou et al., 2004). It is assumed that its roots may play an important role in Cu resistance, because Cu concentration in the roots has been reported to be much higher than that in the shoots, and most of this Cu is bound to root cell walls, accounting for more than 60% of the total concentration (Lou et al., 2004). This is further supported by the current study, which showed that the roots accumulated 39–61 times more Cu than the shoots under excess

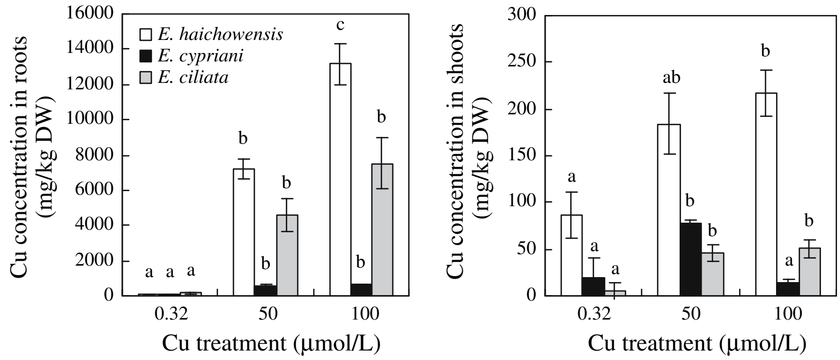

Fig. 1 Copper concentrations (mg/kg DW) in the roots and shoots of three plant species seedlings exposed to Cu for 10 days

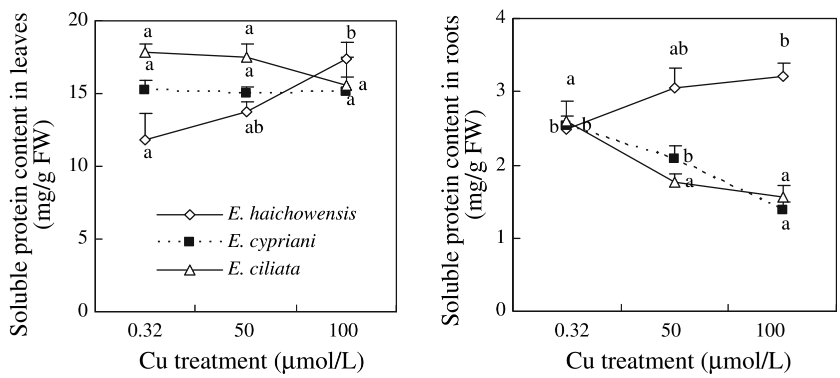

Fig. 2 The contents of soluble protein in the leaves and roots of the three plant species seedlings under different Cu concentrations

Table 4 Nutrient element concentrations in the roots and shoots of three plant species seedlings exposed to Cu for 10 days

Elsholtzia species	Cu treatment (μmol/L)	Mg (μg/g DW)		P (μg/g DW)		Zn (μg/g DW)		Ca (mg/g DW)		Fe (μg/g DW)	
		Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots
E. haichowensis	0.32	1318 a	768 a	896 a	2845 a	55 c	111 b	39 a	6.4 a	208 b	3834 a
	50	1397 a	728 a	792 a	2512 a	44 b	74 a	42 a	7.9 ab	106 b	3664 a
	100	1120 a	1170 a	826 a	3040 a	35 a	67 a	46 a	9.9 b	31 a	3652 a
E. cypriani	0.32	703 a	835 a	684 a	2173 a	60 b	89 a	82 c	24.4 a	121 b	3440 a
	50	551 a	293 b	700 a	1915 a	39 a	86 a	58 a	33.8 ab	139 b	2999 a
	100	634 a	198 b	679 a	1534 a	42 a	88 a	69 b	55.8 b	59 a	3608 a
E. ciliata	0.32	597 a	729 b	880 a	2363 b	48 b	91 a	75 b	24.8 a	180 b	1406 a
	50	523 a	370 a	723 a	1686 a	29 a	78 a	66 ab	51.3 b	112 a	2200 ab
	100	544 a	335 a	644 a	2065 ab	34 a	70 a	63 a	50.2 b	124 a	3070 b

Cu supply. It is well known that some tolerant plant species can hold heavy metals in their cell walls, preventing the formation of heavy-metal complexes with biological macromolecules in the plasma membrane (Lou et al., 2004). It has been suggested that protein thiols may be involved in the adaptive tolerance mechanisms in response to Cu toxicity in *E. haichowensis*, and they were assumed to have a more pronounced role in the leaves than in the roots (Qian et al., 2005). The concentrations of soluble proteins were significantly higher in the leaves of the three plant species than in the roots at all the levels of Cu supply, including the control. Increasing Cu from 0.32 to 100 μmol/L resulted in a progressive increase of the soluble protein concentrations in the leaves and roots of *E. haichowensis* (Fig. 2). In contrast, there was no significant increment in the concentrations of soluble proteins in the leaves of *E. cypriani* and *E. ciliata*. In the roots of *E. cypriani* and *E. ciliata*, the concentrations of soluble proteins significantly decreased with increasing Cu supply in the nutrient solution. Excessive Cu might affect nitrogen and protein metabolism in plants and decrease protein contents in plants (Jouili and Ferjani, 2003). This reduction of soluble protein content might be due to the ability of Cu to interfere with thiol groups of a wide range of enzymes and cause protein degradation (Jouili and Ferjani, 2003). It has been reported that Cu can inhibit the uptake of essential elements, inducing nutrient deficiencies (Lin et al., 1994). The Cu-induced modulation of element composition varied between plant species and organs (Table 4). Compared to the control, the 50 and 100 μmol/L Cu treatment had no significant effect on the concentrations of P in the shoots and roots of the three species, but significantly decreased the concentrations of Zn and Fe in shoots. The decrease was most pronounced for Fe concentration in *E. haichowensis* shoots. Moreover, exposure of *E. cypriani* and *E. ciliata* plants to excess Cu resulted in a significant decrease in shoot Ca concentration. Excess Cu treatments also significantly decreased Mg concentration in the roots of *E. cypriani* and *E. ciliata*. For *E. haichowensis*, plants exposed to excess Cu did not show a significant decrease in the concentrations of Ca and Mg in the shoots and roots. *E. haichowensis* plants accumulated more Mg compared to the other two plant species. This enabled a higher concentration of Mg in chloroplast, which in turn could alleviate Cu toxicity. It is known that chloroplasts are the main site for Cu accumulation in plant leaves (Fernandes et al., 1991). Interactions between elements are often complex and depen on plant species, the element concentration, and the pH of the growth medium.

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