

# Cadmium and copper uptake and accumulation by *Sesbania rostrata* seedling, a N-fixing annual plant: implications for the mechanism of heavy metal tolerance

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**Abstract** *Sesbania rostrata*, an annual tropical legume, has been found to be tolerant to heavy metals, with an unknown mechanism. It is a promising candidate species for revegetation at mine tailings. In this study, sequential extractions with five buffers and strong acids were used to extract various chemical forms of cadmium and copper in *S. rostrata*, with or without Cd or Cu treatments, so that the mechanisms of tolerance and detoxification could be inferred. Both metals had low transition rates from roots to the aboveground of *S. rostrata*. The transition ratio of Cd (4.00%) was higher than that of Cu (1.46%). The proportion of NaCl extracted Cd (mostly in protein-binding forms) increased drastically in Cd treated plants from being undetectable in untreated plants. This suggests that Cd induced biochemical processes producing protein-like phytochelatin that served as a major mechanism for the high Cd tolerance of *S. rostrata*. The case for Cu was quite different, indicating that the mechanism for metal tolerance in *S. rostrata* is metal-specific. The proportion of water-insoluble Cu (e.g. oxalate and phosphate) in roots increased significantly with Cu treatment, which partially explains the tolerance of *S. rostrata* to Cu. However, how *S. rostrata* copes with the high biotic activity of inorganic salts of Cu, which increased in all parts of the plant under Cu stress, is a question for future studies. *Sesbania rostrata* is among the very few N-fixing plants tolerant to heavy metals. This study provides evidence for the detoxification mechanism of metals in *Sesbania rostrata*.

**Keywords** *Sesbania rostrata*, phytoremediation, heavy metal tolerance, sequential extraction, chemical forms

## 1 Introduction

Heavy metal pollution in soil is a serious environmental problem. Traditional rehabilitation methods are often expensive, and may cause secondary pollution (Sekhar et al., 2003). Phytoremediation, on the other hand, is usually low-cost, simple to operate, and environmentally friendly; therefore, it has been receiving more and more attention in recent years. For a period of time, selection of hyperaccumulative plants has been the focus of phytoremediation. However, most selected hyperaccumulative plants have limitations, such as slow growth and low biomass, to remove heavy metals from polluted soils in a timely manner (Cunningham et al., 1995). It reportedly took *Thlaspi caeulescens*, a hyperaccumulator, 13–14 years to clean up a heavy metal contaminated experimental site due to its smaller biomass (Baker et al., 1994). For this reason, it is desirable to select species that are fast-growing and have larger biomass, while being tolerant to heavy metals. Moreover, heavy metal contaminated environments, such as mine tailings, are often nutrient poor, which further limits the successful establishment of plants (Bradshaw, 1987). It has been suggested to use the N-fixing legume-rhizobia symbiosis as a potential solution to overcome the lack of nitrogen in the environment (Archer et al., 1988).

*Sesbania rostrata* is a tropical legume which can form nitrogen-fixing nodules in both stems and roots when the plant is infected by *Azorhizobium caulinodans* (Dreyfus and Dommergues, 1981; Dreyfus et al., 1988). It grows fast, with the highest N-fixing ability among existing N-fixing symbioses, and it was found to contribute to improvements of soil nitrogen, soil organic matter, and productions of subsequent crops in many cases (Dreyfus et al., 1985; Manguiat et al., 1987; Pareek et al., 1990; Lahda et al., 1992; Bar et al., 2000). *S. rostrata* also has

relatively strong tolerance to heavy metals in the soil, which is quite unusual for N-fixing plants (Radziah and Shamsuddin, 1990; Yang et al., 1997; Ye et al., 2001). It forms nitrogen-fixing symbiotic nodules on stems with *A. caulinodans* which is tolerate to heavy metals (zheng et al., 2005), and can survive and reproduce on N-poor habitat in Pb/Zn mine tailings (Yang et al., 1997). Within 55 days after the 15-day-old seedlings were transplanted to the Pb/Zn mine tailings in Fankou, China, the plants accumulated  $3200 \text{ kg} \cdot \text{hm}^{-2}$  dry biomass and fixed  $69.4 \text{ kg} \cdot \text{hm}^{-2}$  nitrogen (Yang et al., 1997). Its high tolerances to toxic metals Pb and Cd are especially noticeable (Yang et al., 2004). *S. rostrata*, therefore, is an ideal candidate pioneer species for phytoremediation, immobilization, and revegetation in moderately polluted habitat at these mine tailings. However, its physical and biochemical responses to heavy metal stresses are unknown, and the mechanism of heavy metal tolerance in *S. rostrata* is yet to be clarified.

Heavy metals in plants often exist with various complicated molecular structures. Those different chemical forms have distinctly varied moving efficiencies throughout plants. For example, water-soluble heavy metals, such as organic acid or inorganic acid salts like nitrates and chlorides, move much faster than those insoluble phosphates or the metals attached to cell walls. The former ones naturally lead to much higher toxicity to plants than the slow moving ones (Xu et al., 1991; Yang et al., 2000). Therefore, studies on the chemical forms of heavy metals in plants, and their association with the observed toxic effects, would have significant implications for the mechanisms of heavy metal tolerance of plants.

In this paper, we studied the chemical forms of Cd and Cu in the leaves, stems and roots of *S. rostrata*, with or without Cd or Cu treatments. We aimed to explore the mechanisms of tolerance of *S. rostrata* to heavy metals Cd and Cu.

## 2 Materials and methods

### 2.1 Experimental design and testing materials

*Sesbania rostrata* was a native species of West Africa and was later introduced to Japan for commercial planting as a green fertilizer. In this study, the seeds of *S. rostrata* were imported from Japan, and were then propagated in an experimental garden with potting soil in the Sun Yat-sen University, Guangzhou, China for three generations before the study. Seedlings of *S. rostrata* used in the experiment were grown in hydroponics with 1/2 Hoagland nutrient solution, and the nutrient solution was refreshed every 5 days. When the seedlings were 20 days old, 3 of them were randomly chosen to be treated with  $0.3 \text{ mmol} \cdot \text{L}^{-1}$   $\text{CdCl}_2$ , 3 seedlings were treated with  $0.3 \text{ mmol} \cdot \text{L}^{-1}$   $\text{CuSO}_4$  and 3 were not treated (control group). All seedlings were

harvested 5 days after the treatments. Fresh tissues of roots, stems and leaves were sampled for chemical tests. Root samples were first washed in  $10 \text{ mmol} \cdot \text{L}^{-1}$  EDTA-Na solution for 10 minutes in order to remove the metal ions attached to the root surface before the testing.

### 2.2 Chemical analyses of the forms of heavy metals in *S. rostrata*

The various forms of Cd and Cu in tissue samples (roots, stems and leaves) of treated and control groups were extracted sequentially with five extraction buffers, in the order of 80% ethanol, distilled water,  $1 \text{ mol} \cdot \text{L}^{-1}$  NaCl, 2% HAc, and  $0.6 \text{ mol} \cdot \text{L}^{-1}$  HCl (Xu et al., 1991). For each specific buffer, each tissue sample was extracted four times within 24 hours in a  $30^\circ\text{C}$  incubator, and the four extracts were merged to one, with the residue being extracted with the next buffer. The remaining residues after the sequential extractions were then digested with strong acids (at a ratio of  $\text{HNO}_3:\text{HClO}_4=3:1$ ) (Xu et al., 1991). Cd and Cu concentrations in these six chemical forms (in the treated and control groups) (Table 1) were analyzed with the AAS (Atomic Absorption Spectrometer) Analyst-100 of the Perkin Elmer (PE) Company. A Certified Reference Materials (CRM) of plants (GBW-07605, provided by the National Research Center for CRM, China) was used to control the precision of the analytical procedures. All the chemicals used in this study were of analytical grade.

**Table 1** Extraction buffers for different chemical forms of heavy metals

extraction buffers	chemical forms
80% ethanol	inorganic acid salts such as nitrate, chloride and chlorate
deionized water	water-soluble organic acid salts and dihydrogenphosphate, etc
$1 \text{ mol} \cdot \text{L}^{-1}$ NaCl	pectate, protein-binding or attached heavy metals, etc
2% HAc	water-insoluble phosphate or hydrogenphosphate, etc
$0.6 \text{ mol} \cdot \text{L}^{-1}$ HCl	oxalate, etc
residue	insoluble heavy metals, mostly silicate

## 3 Results

### 3.1 Concentrations and transition ratios

In the control group, the concentrations of Cd in different tissues varied slightly from each other ( $0.562 - 0.908 \mu\text{g} \cdot \text{g}^{-1}$ ), while those of Cu were significantly higher in leaves than in stems and roots, indicating leaves functioning as a Cu sink during photosynthesis (Table 2). With metal addition treatments, the concentrations of Cd were roots > stems > leaves with significant differences

**Table 2** Concentration and transition of Cd and Cu in roots, stems and leaves of *Sesbania rostrata*

	Cd concentration/ $\mu\text{g}\cdot\text{g}^{-1}$ FW					Cu concentration/ $\mu\text{g}\cdot\text{g}^{-1}$ FW				
	control		Cd treated		fold of increase <sup>c</sup>	control		Cu treated		fold of increase <sup>c</sup>
	C <sup>a</sup>	95%CI <sup>b</sup>	C <sup>a</sup>	95%CI <sup>b</sup>		C <sup>a</sup>	95%CI <sup>b</sup>	C <sup>a</sup>	95%CI <sup>b</sup>	
root	0.908	0.118–1.698	398.679	243.178–554.180	438.1	0.971	0.510–1.432	423.375	293.924–552.826	435.0
stem	0.562	0.309–0.814	15.947	12.446–19.448	27.4	1.110	0.840–1.380	6.171	2.917–9.425	4.6
leaf	0.778	0.476–1.078	1.336	0.598–2.074	0.7	2.645	1.748–3.542	5.923	2.568–9.278	1.0

<sup>a</sup>C: average concentration ( $\mu\text{g}\cdot\text{g}^{-1}$  FW) of Cd or Cu; <sup>b</sup>95% CI (Confidence interval): average concentration  $\pm$  1.96 $\times$ SD(standard deviation); <sup>c</sup>Fold of increase: (average concentration of treated/average concentration of control) - 1.

among different tissues, while those of Cu were roots > stems and leaves. Compared to the control group, Cd and Cu concentrations in roots increased 438.1 fold and 435 fold, respectively; those in stems increased 27.4 fold and 4.6 fold, respectively (Table 2). There were no significant differences in leaf Cd and Cu concentrations between the control and metal treated groups. Both metals had low transition rates from roots to aboveground. However, the transition ratio of Cd was higher than that of Cu (Table 2).

### 3.2 Percentage distribution of different forms of Cd in roots, stems and leaves

In the control group, 72.1% of Cd existed in roots as insoluble phosphate (HAc extracted). Other chemical forms accounted for a small percentage. In stems, Cd mostly existed as an insoluble residue (41.6%), as an H<sub>2</sub>O extractable form (32.7%) and as an HAc extractable form (15.8%) (Fig. 1b). The distribution pattern in leaves was similar to that in stems. Neither in the roots nor in the stems or leaves was there any detectable level of NaCl extractable Cd (Fig. 2).

In the Cd treated group, the percentage distribution of various chemical forms of Cd changed drastically (Fig. 1a). In roots, NaCl extracted Cd accounted for 51%, in contrast to the undetectable level in the control group. HAc extracted Cd accounted for 40.6%, still quite high. Other forms took up very small percentages. In stems, the pattern was very similar to that in roots. NaCl extracted Cd accounted for 56.6%, and HAc extracted Cd 16.2%. H<sub>2</sub>O extracted and ethanol extracted Cd accounted for 13.7% and 12.1%, respectively. In leaves, HCl extract Cd was not detectable. H<sub>2</sub>O extracted Cd took up a very small percentage. The ethanol, NaCl, and HAc extracted Cd and Cd in the residue accounted for 32.2%, 26.6%, 13.1% and 24.5%, respectively, quite balanced.

Overall, the percentage of residue Cd was roots > leaves > stems, whereas all the other forms were roots  $\geq$  stems > leaves. The biggest difference in chemical forms of Cd was that a very high amount of NaCl extracted Cd (over 50% in roots and stems) appeared after the metal treatment, in contrast to non-detectability in the control group.

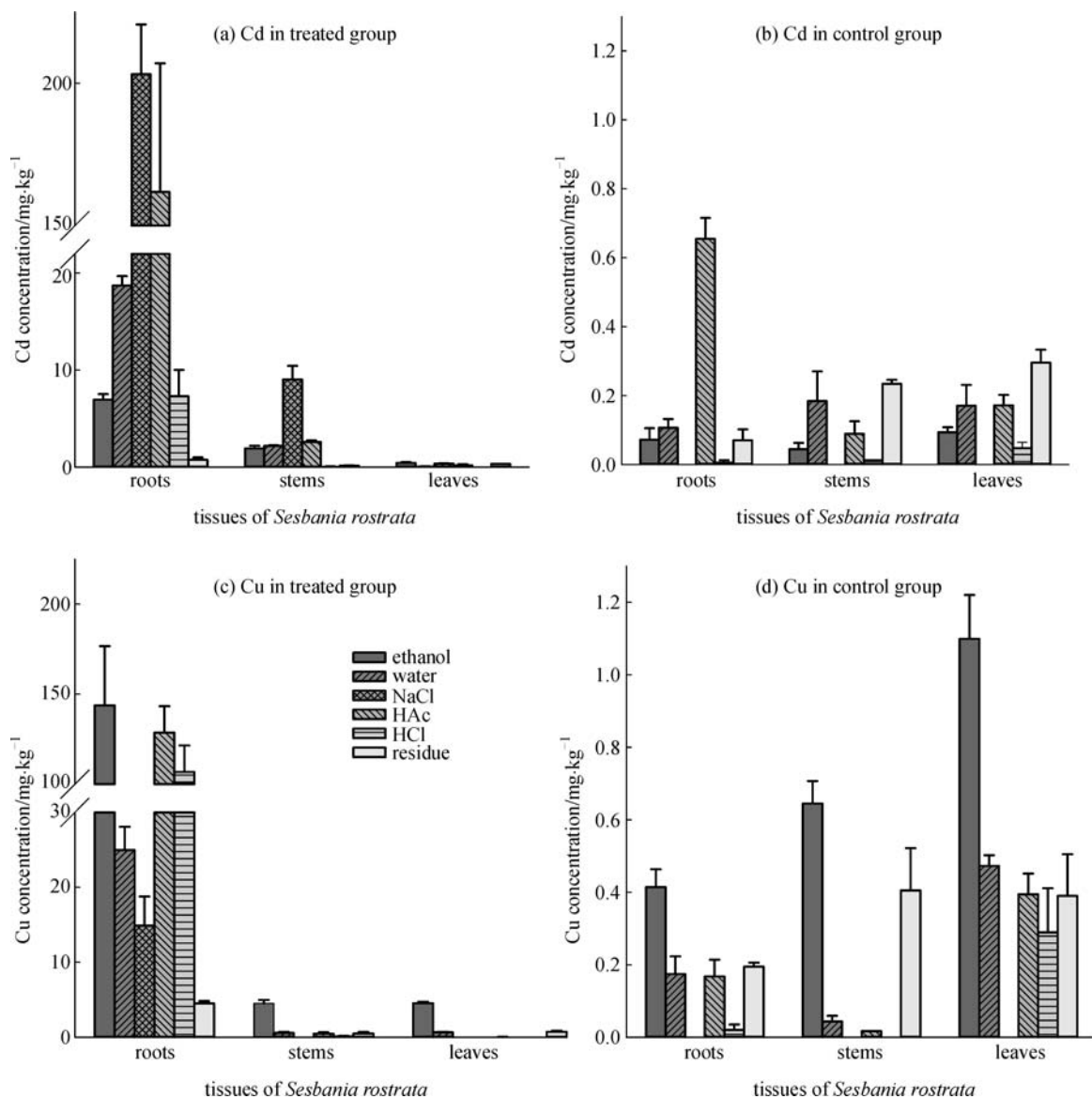
### 3.3 Percentage distribution of different forms of Cu in roots, stems and leaves

In the control group (Fig. 1d), Cu in roots mostly existed in ethanol extracted form (42.7%). The residue Cu, H<sub>2</sub>O and HAc extracted Cu in roots accounted for 20.0%, 18.0% and 17.3%, respectively. In stems, Cu mostly existed in ethanol extracted and residue forms, together taking up 94.6%. The percentage distribution of Cu in leaves was similar to that in roots (Fig. 3). Cu mostly existed in ethanol extracted form (41.6%). The H<sub>2</sub>O and HAc extracted Cu and residue Cu accounted for 17.9%, 14.9% and 14.7%, respectively. The HCl extract Cu also accounted for 10.9%. Except for the residue Cu and NaCl extracted Cu, all the other forms had higher concentrations in leaves than in roots and stems. Neither in roots nor in stems or leaves were the levels of NaCl extracted Cu detectable.

In the Cu treated group, the percentage distribution of various chemical forms of Cu also changed drastically (Fig. 1c). NaCl extracted Cu appeared in roots with a small percentage (3.5%), and was still not detectable in stems or leaves. In roots of plants exposed to Cu, Cu mostly existed in ethanol extracted (33.9%), HAc extracted (30.4%), and HCl extracted (25.2%) forms. In stems and leaves, Cu mostly existed in ethanol extracted form (72.9% and 76.5%, respectively). Overall, the absolute concentrations of all forms of Cu in roots were far greater than those aboveground. Between stems and leaves, HAc and HCl extracted Cu had higher concentrations in stems, and ethanol and H<sub>2</sub>O extracted Cu and residue Cu had higher concentrations in leaves.

## 4 Discussion

Plants need traces of certain heavy metals, such as Cu, Fe, Mn, and Zn, to grow normally. These metals are involved in many metabolic processes in plants. However, damage would be done once their concentrations in plants pass a certain threshold. Other heavy metals, such as Cd, Cr, and Hg, are not needed by plants. These elements would impact the plants negatively even at tiny amounts. In the



**Fig. 1** Concentrations of different Cd and Cu forms in roots, stems and leaves of *Sesbania rostrata* of the treated groups (a, c) and the control groups (b, d). Each bar represents the average concentration + 1SD (standard deviation).

evolutionary process, various mechanisms of heavy metal tolerance have evolved in plants, such as sequestration (Cosio et al., 2004) or reduced uptake (Baker, 1981; Tyler, 1989; Cho et al., 2003), compartmentation (Brooks et al., 1981; Salt et al., 1995; Cho et al., 2003), and chelating with metal-binding peptides and proteins (Cobbett, 2000; Hall, 2002; Ederli et al., 2004). Even the same plant species may have diverse types of mechanisms to tolerate one specific heavy metal element (Hall, 2002; Cho et al., 2003).

Tolerance of some plant species to Cd can be induced by Cd, by producing secondary metabolic products: S-based phytochelatins (PCs). These phytochelatins bind with Cd through S-bases, and then transport Cd to vacuoles where they are stored, consequently leading to detoxification of Cd in plants (Rauser, 1995; Souza and Rauser, 2003). In

other species, organic acids with low molecular weights, such as oxalic acid, citrate acid, and malic acid, may bind with metals for detoxification (Rauser, 1999). The reaction of *S. rostrata* when exposed to Cd seemed to be associated with the mechanism of detoxification with phytochelatins. Without Cd treatment, the NaCl extracted Cd was not detectable in any part of *S. rostrata*. After being exposed to Cd, the most important form of Cd became that which was NaCl extracted (in both roots and stems exceeding 50%).

Although we do not know the exact ligand, such results strongly suggest that large molecules of proteins, which can be extracted by NaCl solution, were probably induced with Cd treatment and led to detoxification through chelating Cd. If this is true, it is possible to find the corresponding genes in *S. rostrata* responsible for such a

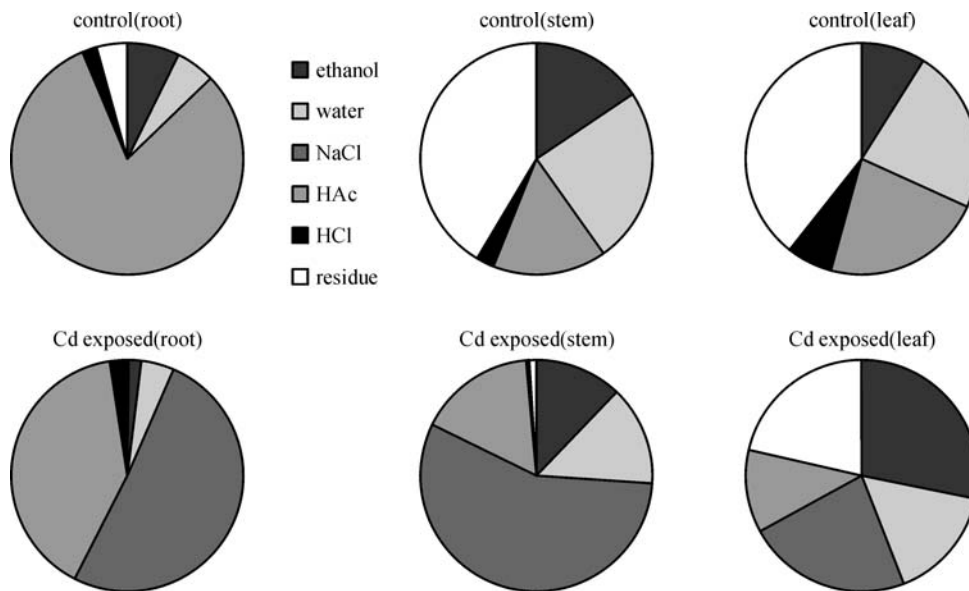


Fig. 2 Percentage distribution of different Cd forms in roots, stems and leaves of *Sesbania rostrata* in the control group and Cd treated group

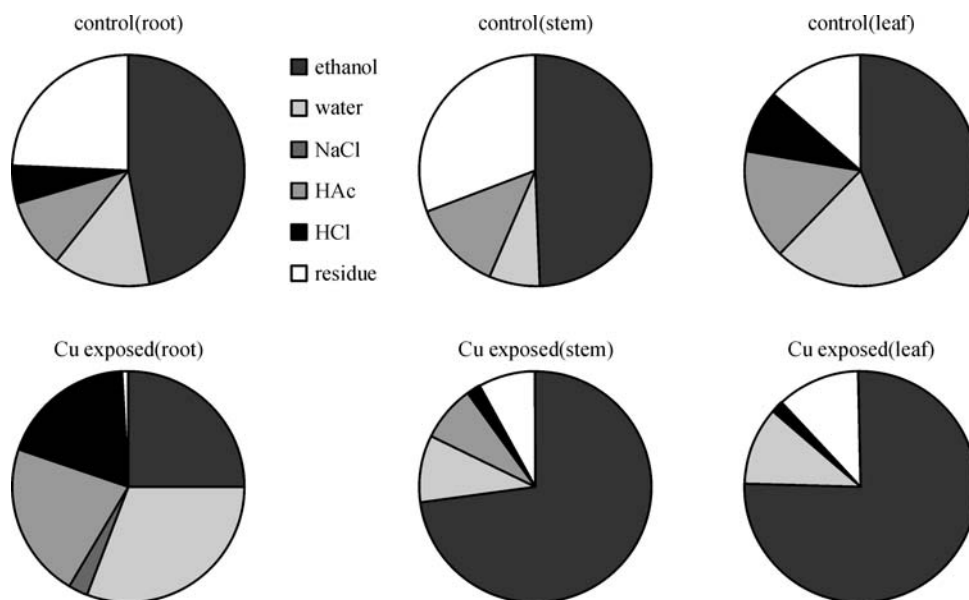


Fig. 3 Percentage distribution of different Cu forms in roots, stems and leaves of *Sesbania rostrata* in the control group and Cu treated group

mechanism (Ha et al., 1999; Vatamaniuk et al., 1999; Oven et al., 2002). Such an investigation is currently ongoing in our lab.

Without exposure to Cd, Cd in *S. rostrata* mostly exists as phosphate, which is insoluble in water. This itself appears to be a mechanism of detoxification, but it may be not sufficient when the concentration of Cd is high. It takes a certain amount of extra energy to initiate the production of phytochelatin (Gao et al., 2001), which are probably more efficient for detoxification. Therefore, the Cd concentration in plant tissues needs to pass a certain threshold to trigger a series of physiological and biochemical reactions.

The case of Cu was quite different, suggesting that the tolerance of *S. rostrata* to heavy metals is highly metal-specific (Macnair, 1993). Although exposure to Cu induced NaCl extractable Cu in roots, the percentage was very low (3.5%). NaCl extractable Cu remained undetectable in stems and leaves. Therefore, *S. rostrata* is unlikely to use the same detoxification mechanism with phytochelatin for Cu as it does for Cd.

In the roots of *S. rostrata* exposed to Cu, compared to those untreated, HCl extracted Cu increased from non-detectable to 25.2%; and HAc extracted Cu increased from 17.3% to 30.4%. HCl extracts oxalates, and HAc extracts water-insoluble phosphates, both of which can be effective

in detoxification. However, such changes were not found in the stems nor leaves. On the contrary, the percentage of ethanol extracted Cu increased significantly after exposure to Cu. Ethanol extractable Cu is mostly in highly soluble nitrates, chlorides and chlorates, which should be very mobile and active inside plant tissues. Such a pattern arouses questions on the actual tolerance of *S. rostrata* to Cu. Although *S. rostrata* may exclude excess Cu in vacuoles for detoxification, such a mechanism or other alternatives are yet to be verified.

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