



Variation in copper and zinc tolerance and accumulation in 12 willow clones: implications for phytoextraction*

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Abstract: Willows (*Salix* spp.) have shown high potential for the phytoextraction of heavy metals. This study compares variations in copper (Cu) and zinc (Zn) tolerance and accumulation potential among 12 willow clones grown in a nutrient solution treated with 50 µmol/L of Cu or Zn, respectively. The results showed differences in the tolerance and accumulation of Cu and Zn with respect to different species/clones. The biomass variation among clones in response to Cu or Zn exposure ranged from the stimulation of growth to inhibition, and all of the clones tested showed higher tolerance to Cu than to Zn. The clones exhibited less variation in Cu accumulation but larger variation in Zn accumulation. Based on translocation factors, it was found that most of the Cu was retained in the roots and that Zn was more mobile than Cu for all clones. It is concluded that most willow clones are good accumulators of Zn and Cu.

Key words: *Salix* spp., Copper, Zinc, Accumulation, Tolerance, Hydroponic screening

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1 Introduction

Soil and water contaminated with heavy metals have serious consequences for the environment and human health (Tanhan *et al.*, 2007). Copper (Cu) and zinc (Zn) are important environmental pollutants owing to their elevated concentrations and bioavailability in natural ecosystems (Kopponen *et al.*, 2001).

Cu and Zn contamination originates from mining, smelting, textile, microelectronic and hydrometallurgical industries, sewage sludge, fossil-fuel combustion, and agricultural sources (including fertilizers, manures, pesticides, and fungicides containing Cu or Zn) (Kopponen *et al.*, 2001; Paschke *et al.*, 2006). Cu and Zn are also essential elements in plant nutrition and development. However, the presence of Cu and Zn at high concentrations induces severe phytotoxicity, resulting in the reduction of plant growth, mineral nutrient uptake, and photosynthetic activity (Salemaa and Monni, 2003; Yan *et al.*, 2006; Borghi *et al.*, 2007; Disante *et al.*, 2010).

Phytoremediation is the process of using trees, shrubs, and grasses to de-contaminate soil and water, and is considered to be a promising, cost-effective

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solution to heavy-metal pollution (Licht and Isebrands, 2005; Vangronsveld *et al.*, 2009; Xing *et al.*, 2012; Lu *et al.*, 2013). Generally, phytoremediation and especially phytoextraction rely on plants known as hyperaccumulators; however, these are species that often have a low biomass and slow growth rate, leading to a long time periods required for metal removal (Tanhan *et al.*, 2007). Recently, willows have been shown to be good candidates for remediating metal-contaminated sites because of their rapid growth, large biomass, deep roots, and high accumulation capacity for heavy metals (Kuzovkina and Quigley, 2005; Kuzovkina and Volk, 2009). In the last decade, short-rotation willow coppices have provided an economic and practical solution for remediation as well as for bioenergy production (Rockwood *et al.*, 2004; Mirck *et al.*, 2005).

Apart from genetic modifications, increasing the metal bioaccumulation capacity of a plant is primarily accomplished through the artificial selection of *Salix* clones; the selection of appropriate species or clones is crucial for achieving successful phytoremediation (Zalesny *et al.*, 2007; Zalesny and Bauer, 2007a; 2007b). Numerous studies have revealed a remarkable genotypic variability of the genus *Salix* in its ability to tolerate/accumulate heavy metals (Wang and Greger, 2004; Dos Santos Utmazian *et al.*, 2007; Purdy and Smart, 2008; Zhivotovsky *et al.*, 2010). Most results of willow screening have shown that the tolerance and uptake of heavy metals depend strongly on species and clones (Punshon *et al.*, 1995; Dos Santos Utmazian *et al.*, 2007; Zacchini *et al.*, 2009; Zhivotovsky *et al.*, 2010). *Salix* comprises about 450 species as well as a large number of willow clones produced through breeding projects worldwide, which together provide a large number of plants to be screened for phytoremediation (Kuzovkina *et al.*, 2004; Kuzovkina and Quigley, 2005). Owing to the high genetic variability in *Salix*, the use of clone selection has been proposed to find those best suited for phytoremediation purposes (Zalesny *et al.*, 2007; Zalesny and Bauer, 2007b).

Hydroponic screening is the primary method for evaluating the tolerance and accumulation potential of heavy metals in *Salix* (Watson *et al.*, 1999; 2003). Many studies have investigated the metal accumulation potential of *Salix* to identify prospective phytoremediation species/clones through carefully con-

trolled hydroponic screenings (Punshon *et al.*, 1995; Watson *et al.*, 2003; Kuzovkina *et al.*, 2004; Wang and Greger, 2004; Dos Santos Utmazian *et al.*, 2007; Purdy and Smart, 2008; Mleczek *et al.*, 2010; Zhivotovsky *et al.*, 2010). As a rapid screening technology, hydroponic screening reduces the time and space required in comparison with other methods, and is especially suitable for high-throughput screening to evaluate large amounts of plant materials for phytoremediation (Dos Santos Utmazian *et al.*, 2007). However, short-term exposure to metals in a hydroponic system results in large quantities of metal accumulating in the roots, especially metals with low mobility in plants, as this leads to low translocation of metals from the roots to the shoots (Cosio *et al.*, 2006; Dos Santos Utmazian *et al.*, 2007; Zhivotovsky *et al.*, 2010). Consequently, metal accumulation potentials are easily underestimated in willows. Zhivotovsky *et al.* (2011) concluded that willows may need more time to adapt to a specific environmental contaminant in order to improve its transport to above-ground plant tissues.

To the best of our knowledge, most recently published research has focused on the toxicities of Cu and Zn in Salicaceae rather than their accumulation capacity, especially for Cu (Punshon *et al.*, 1995; Borghi *et al.*, 2007; 2008; Baud and Pezeshki, 2011), and therefore, the Cu accumulation capacity of willows is still poorly understood. In fact, the metal tolerance and accumulation capacity of a plant depend on several factors, including plant species, genotype, growth stage, treatment concentration, and exposure time (Monni *et al.*, 2000; Weng *et al.*, 2005; Maxted *et al.*, 2007; Dauthieu *et al.*, 2009; Colzi *et al.*, 2011).

In previous studies, the willows investigated were limited to European and North American species. *Salix* has a high genetic diversity worldwide, and China has a rich willow resource with 275 native species that have not yet been explored or utilized (Karp *et al.*, 2011). In this study, the willow clones tested belong to Asian species, and they are commonly planted in China for biomass production on account of high biomass productivity and fast growth rates. In order to overcome the drawbacks of hydroponic screening, we selected relatively large seedlings and used prolonged exposure time to Cu and Zn to evaluate their uptake and accumulation potential in 12 willow clones.

2 Materials and methods

2.1 Plant materials and growth

The 12 willow clones (Table 1) used in this study were obtained from the Jiangsu Academy of Forestry, Nanjing (33°31' N, 118°47' E), China. They were maintained in a nursery in the Experimental Station of Agriculture of Zhejiang University, Hangzhou (30°16' N, 120°12' E), China. One-year-old hardwood stems from each clone were collected from the nursery and prepared in uniformly sized 10-cm long cuttings, which were mounted with styrofoam in plastic buckets containing a nutrient solution. Plants were pre-grown in the nutrient solution for 42 d. The composition of the nutrient solution was as follows: 1 mmol/L Ca(NO₃)₂, 1.25 mmol/L KNO₃, 0.5 mmol/L MgSO₄, 0.5 mmol/L NH₄H₂PO₄, and the micronutrients including 25 μmol/L FeEDTA, 23.1 μmol/L H₃BO₃, 0.4 μmol/L ZnCl₂, 0.18 μmol/L CuCl₂, 4.57 μmol/L MnCl₂, and 0.06 μmol/L Na₂MoO₄, as described by Watson *et al.* (2003). When the cuttings were well developed, they were used to evaluate metal tolerance and accumulation potential. In this study, according to our preliminary investigation on metal exposure concentrations, treatments containing 50 μmol/L Cu or 50 μmol/L Zn (added as CuSO₄ or ZnSO₄) were performed. The controls were maintained in the above nutrient solution.

Table 1 *Salix* clones tested

Entry No.	Clone	Species/hybrid
1	SB7	<i>Salix babylonica</i>
2	SM16	<i>S. matsudana</i>
3	SM30	<i>S. matsudana</i>
4	SI63	<i>S. integra</i>
5	J9-6	<i>S. integra</i> × <i>S. suchowensis</i>
6	SI646	<i>S. integra</i>
7	J795	<i>S. matsudana</i> × <i>S. alba</i>
8	J844	<i>S. baylonica</i> × <i>S. alba</i>
9	J1011	<i>S. baylonica</i> × <i>S. alba</i>
10	J1052	<i>S. suchowensis</i> × <i>S. leucopithecia</i>
11	SI102-1	<i>S. integra</i>
12	SI102-2	<i>S. integra</i>

All treatments were performed in triplicate, and each treatment had three seedlings. The pH of the nutrient solution was maintained at 5.5–6.0 using

H₂SO₄/NaOH. Solutions were aerated using a pump throughout the experiment and replaced weekly. The experiment was a randomized complete block design, and all plastic buckets were randomly arranged inside a greenhouse. The greenhouse conditions were 30/18 °C day/night temperature and a relative humidity of 70%–80%.

2.2 Plant samples and analysis

Cu and Zn exposure lasted for 50 d. After this time, all plants were harvested and separated into leaves, stems, and roots. The roots were rinsed carefully in 10 mmol/L ethylenediaminetetraacetic acid (EDTA)-Na₂ for 15 min to remove the adsorbed metals from root surfaces, and then washed in double-deionized water. Plant materials were dried at 70 °C for 48 h in a forced-air oven, and the dry weights (DWs) were recorded. The dried material was ground to a fine powder in a mill (Retsch 2M 200, Germany), and approximately 0.2 g of the plant sample was digested using a HNO₃:HClO₄ solution (4:1, v/v) in a Hot Block (ED36, LabTech, Germany). Cu and Zn concentrations in the extracts were measured using atomic absorption spectrometry (Analyst 800, PerkinElmer Inc., USA). Chemical analyses were validated by blanks and reference materials.

2.3 Calculation and statistical analysis

Following Deng *et al.* (2007), tolerance indexes (TIs) were calculated based on the dry shoot biomass (including leaf and stem) or root biomass treated relative to the controls, i.e., $TI = DW_{\text{treated plant}} / DW_{\text{control}}$. The metal concentration in the shoots (combined leaves and stems) was calculated according to the following equation: metal concentration in shoots = (stem biomass × stem metal concentration + leaf biomass × leaf metal concentration) / (stem biomass + leaf biomass). Following Deng *et al.* (2007), the translocation factors (TFs) of metals from roots to leaves or shoots were calculated as follow: $TF = \text{leaf or shoot metal concentration} / \text{root metal concentration}$. According to Dos Santos Utmazian *et al.* (2007), the total metal content (μg/plant) in the shoots was calculated by multiplying the shoot biomass (DW) by the metal concentration in the shoots.

The main purpose of the experiments was to examine clonal differences under Cu or Zn exposure. To analyze the differences in the treatments and

clones simultaneously, treatment and clone effects were determined by two-way analyses of variance (ANOVA) followed by Tukey-HSD test ($P < 0.05$). All statistics were performed using SPSS software (Version 16.0, SPSS Inc., Chicago, USA).

3 Results

3.1 Biomass production and tolerance

Biomass accumulation and the relative growth of shoots and roots are important parameters for characterizing metal tolerance.

The variation of the DW of tissue biomass for clones exposed to Cu treatments (Fig. 1) shows that

Cu depressed leaf biomass production in five clones (clones SM30, SI63, J1011, SI102-2, and J795). In contrast, Cu treatment had little effect on the leaf biomasses of clones J9-6, SI646, J1052, and SI102-1, and it enhanced the leaf biomasses of clones SB7, SM16, and J844 by 13%, 94%, and 35%, respectively, compared with the control (Fig. 1a). Fig. 1b shows the enhanced stem biomasses of eight of the treated clones compared with the control. Cu excess, however, reduced the stem biomasses of clones J795, J1011, J1052, and SI102-2. Other than for clone SI102-2, Cu stimulated root biomass growths of all clones compared with the controls (Fig. 1c). Figs. 1a and 1b also show that the leaves and stems were more sensitive to Cu than the roots.

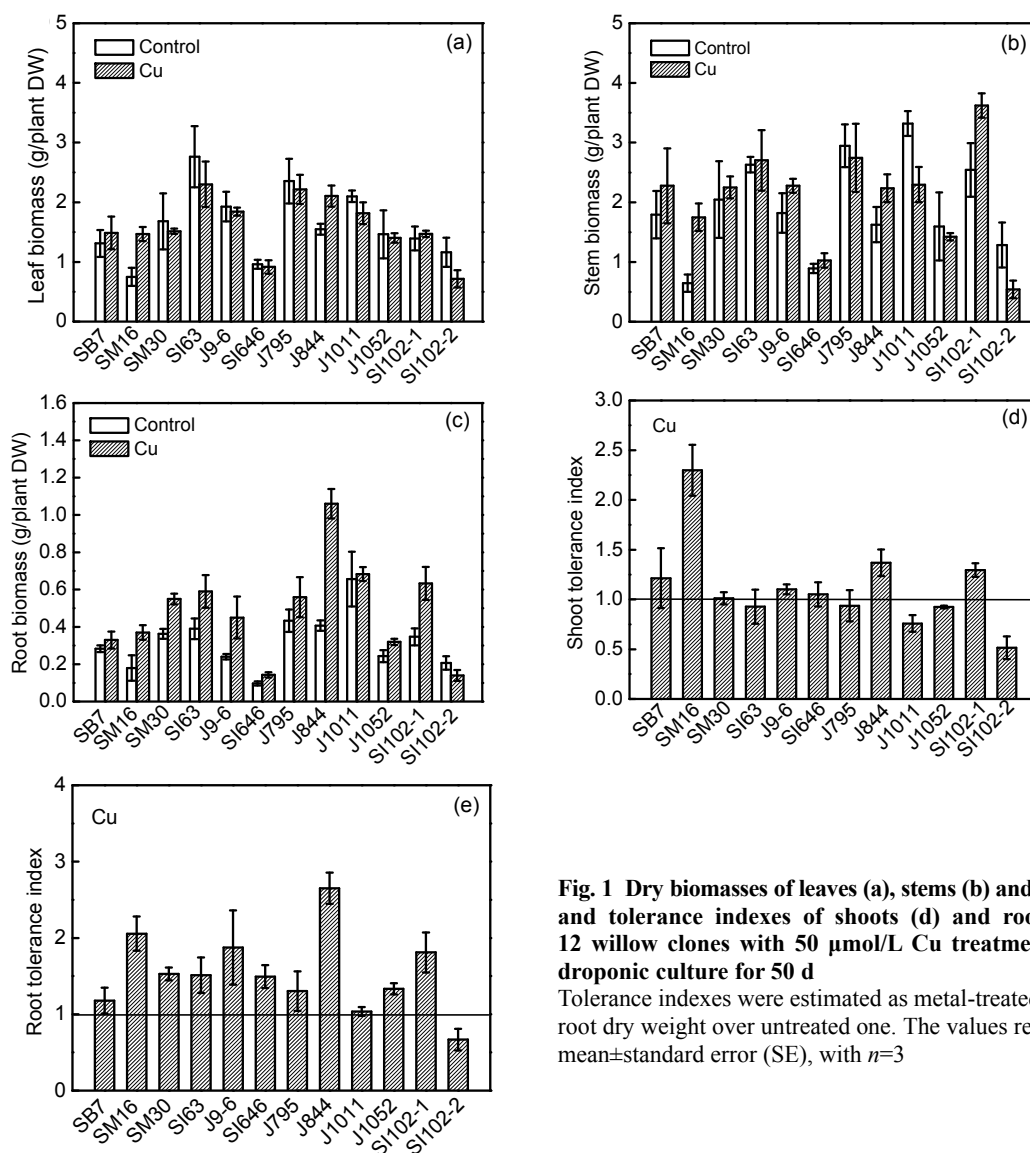


Fig. 1 Dry biomasses of leaves (a), stems (b) and roots (c), and tolerance indexes of shoots (d) and roots (e) of 12 willow clones with 50 $\mu\text{mol/L}$ Cu treatment in hydroponic culture for 50 d. Tolerance indexes were estimated as metal-treated shoot or root dry weight over untreated one. The values represented mean \pm standard error (SE), with $n=3$

TIs were expressed as the relative weights of shoots and roots. The Cu TIs based on shoot biomass ranged from 0.51 to 2.29, and seven out of twelve clones showed high tolerance to Cu ($TI > 1$), while clones J1011 and SI102 were more sensitive to Cu (Fig. 1d). The Cu TIs based on root biomass ranged from 0.66 to 2.05 among clones, and root TIs of eleven clones were > 1 (Fig. 1e).

The growth response of the clones to excess Zn is also presented in Fig. 2. The foliar biomass accumulation of most clones decreased in response to excess Zn. In contrast, Zn treatment had little inhibitory effect on the leaf biomasses of clones SM16 and J844. However, Zn treatment increased the leaf biomasses of clones SB7 and SI63 compared with the control (Fig. 2a). Exposure to excess Zn reduced the

stem biomasses of six clones, and clone SI102-1 showed the largest reduction (56%) compared with the control. In contrast, the stem biomass accumulation of clone SI63, J844, or J1052 was not affected by Zn excess, and Zn exposure significantly increased the stem biomasses of clones SB7 and SM30 compared with the control (Fig. 2b). Excess Zn strongly inhibited the root biomass productions of seven clones; clones SM16, SI646, J844, and J1052 were not affected. However, clone SB7 had a larger root biomass than the control plants (Fig. 2c).

The TIs to Zn treatment were calculated as for Cu. The Zn TIs based on shoot biomass ranged from 0.57 to 1.67 (Fig. 2d), and the root-based TIs ranged from 0.43 to 1.32 among clones (Fig. 2e). Considering both shoot- and root-based TIs (Figs. 2d and 2e),

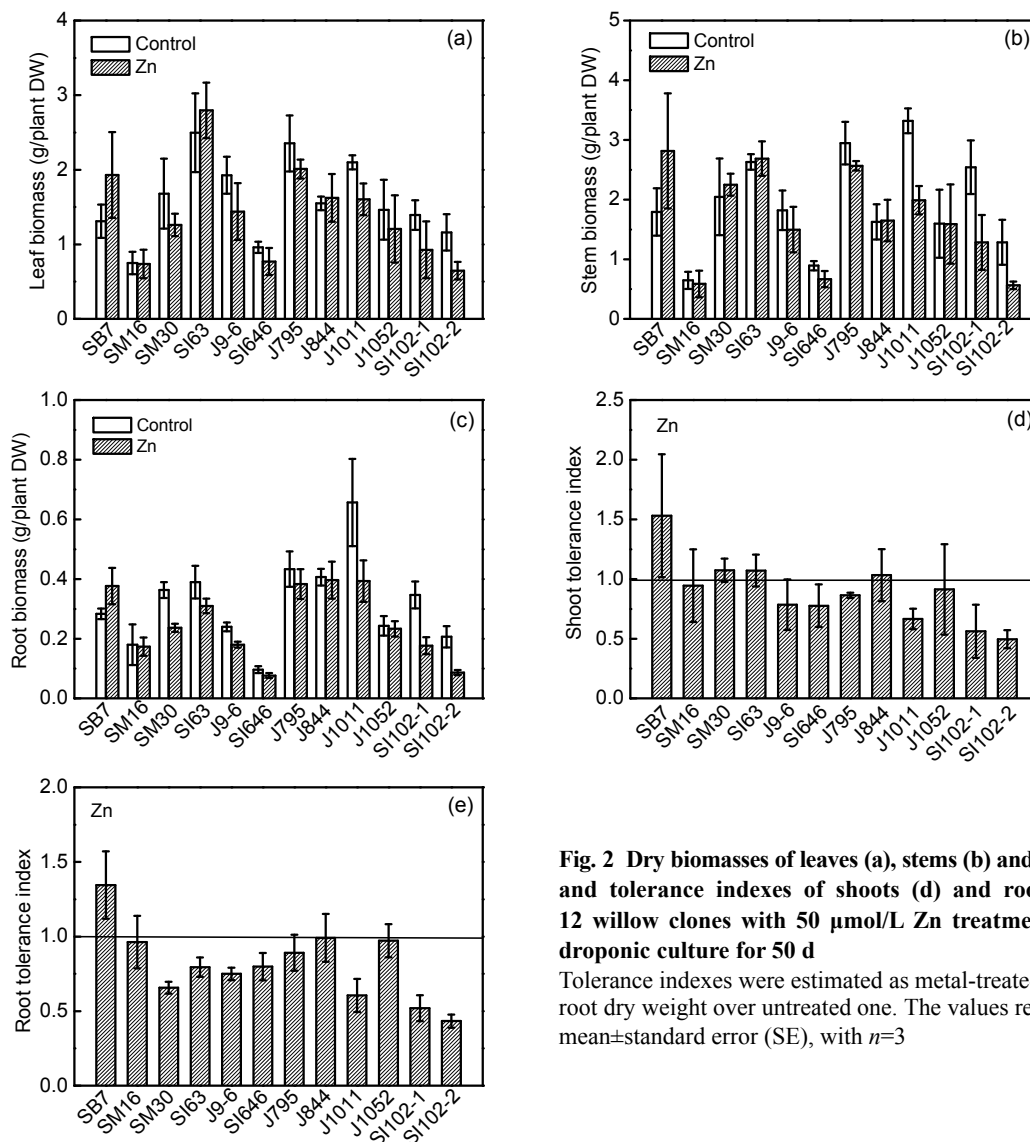


Fig. 2 Dry biomasses of leaves (a), stems (b) and roots (c), and tolerance indexes of shoots (d) and roots (e) of 12 willow clones with 50 $\mu\text{mol/L}$ Zn treatment in hydroponic culture for 50 d

Tolerance indexes were estimated as metal-treated shoot or root dry weight over untreated one. The values represented mean \pm standard error (SE), with $n=3$

clones SB7 and J844 showed high tolerances to Zn ($TIs > 1$), and seven clones showed medium tolerances ($0.7 < TIs < 1$); clones J1011, SI102-1, and SI102-2 were more sensitive to Zn ($TIs < 0.7$) than the other clones.

3.2 Cu and Zn accumulation

The accumulation and translocation of Cu in willow clones are shown in Fig. 3. The Cu concentration in the roots always exceeded that in the stems or leaves. The Cu concentration in the leaves ranged from 391.1 (J844) to 660.83 $\mu\text{g/g DW}$ (SB7) under Cu exposure, varying by a factor of 1.6 among the clones (Fig. 3a). The Cu concentration in the stems ranged from 417.5 (SM30) to 786.2 $\mu\text{g/g DW}$

(SI102-2) under conditions of excess Cu, varying by a factor of 1.8 among clones (Fig. 3b). The Cu concentration in the roots ranged from 2065.3 $\mu\text{g/g DW}$ for SI102-1 to 2876.2 $\mu\text{g/g DW}$ for SB7 under Cu treatment, varying by a factor of 1.4 (Fig. 3c). The TFs for Cu in the leaves ranged from 0.17 to 0.27 under Cu exposure (Fig. 3d), and similarly, the TFs for Cu in the shoots (leaves and stems) ranged from 0.21 to 0.27 (Fig. 3e). The TFs for Zn from the roots to the stems and leaves were much greater than those for Cu.

The accumulation and translocation of Zn in willow clones are shown in Fig. 4. The Zn concentration was higher in the roots than in the leaves and stems for eight clones; however, four clones showed

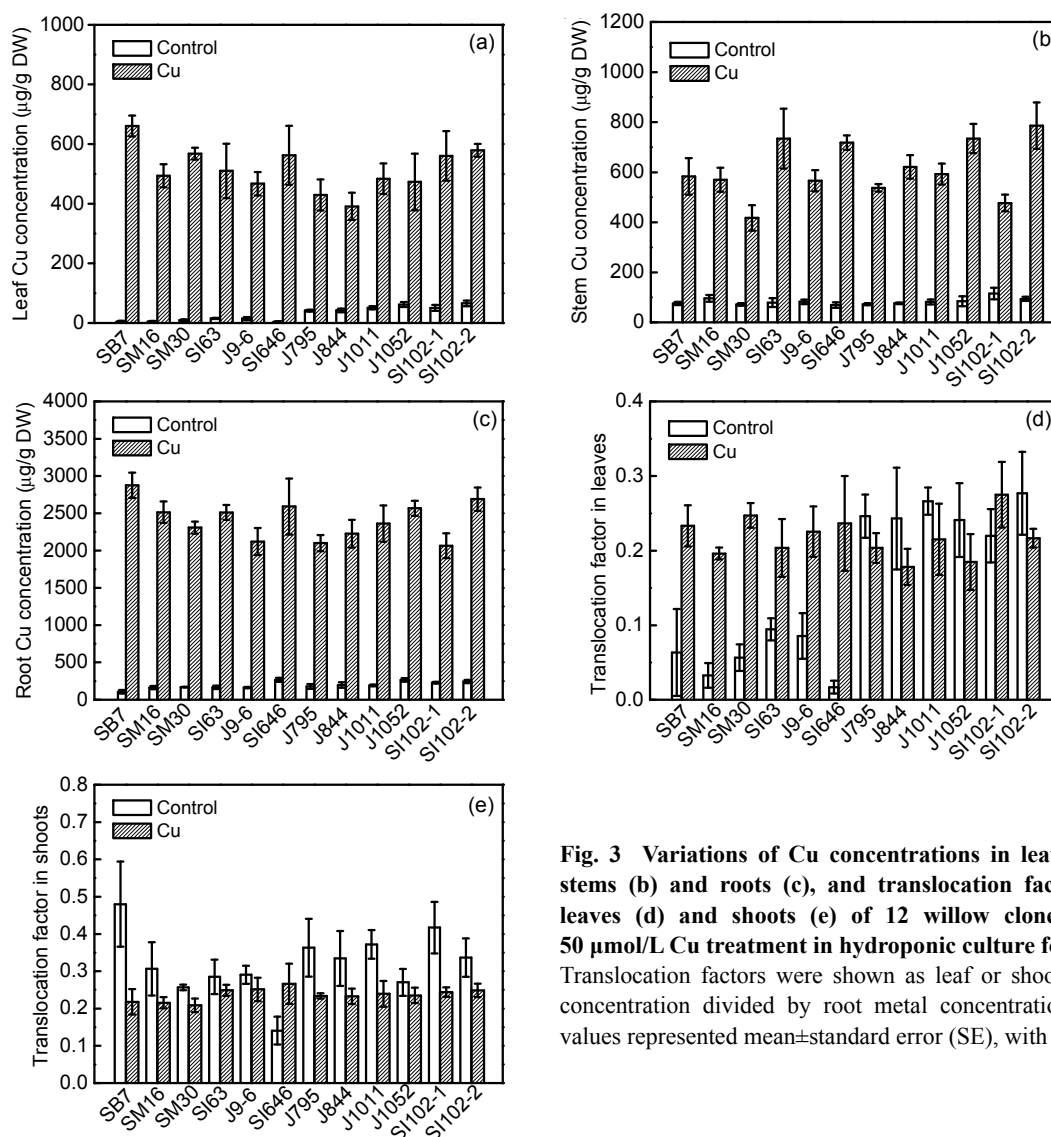


Fig. 3 Variations of Cu concentrations in leaves (a), stems (b) and roots (c), and translocation factors in leaves (d) and shoots (e) of 12 willow clones with 50 $\mu\text{mol/L}$ Cu treatment in hydroponic culture for 50 d. Translocation factors were shown as leaf or shoot metal concentration divided by root metal concentration. The values represented mean \pm standard error (SE), with $n=3$

higher Zn concentrations in the leaves than in the stems and roots, i.e., clones J795, J844, J1011, and J1052 under the Zn treatment. The Zn concentration in the leaves ranged from 607.1 (SI646) to 1218.8 $\mu\text{g/g}$ DW (clone J1011) under exposure to Zn, varying by a factor of 2 among clones (Fig. 4a). The Zn concentration in the stems ranged from 258.8 $\mu\text{g/g}$ DW for J844 to 1455.5 $\mu\text{g/g}$ DW for SM30, varying by a factor of 5.6 (Fig. 4b). The Zn concentration in the roots ranged from 575.8 (J844) to 2052.8 $\mu\text{g/g}$ DW (SM30), varying by a factor of 3.6 (Fig. 4c).

The leaf TFs for Zn ranged from 0.4 to 1.81, and leaf TFs for six clones were close to or >1 under Zn exposure (Fig. 4d). The shoot TFs for Zn ranged from 0.39 to 1.12, and there were four clones with TFs near to or larger than 1 (Fig. 4e). Combining leaf TFs with

shoot TFs, clones J795, J844, J1011, and J1052 showed higher Zn transfer capacities than the other clones.

3.3 Cu and Zn phytoextraction potentials

The metal contents in shoot tissues (combined stems and leaves) were calculated by multiplying the DWs of plant parts by the metal concentrations in the corresponding parts. The Cu and Zn contents of the shoots varied significantly among clones. Fig. 5a shows Cu contents in the shoots, ranging from 814.5 (SI102-2) to 3083.0 $\mu\text{g/plant}$ (SI63) under Cu treatment, thus displaying a 3.8-fold variation among all clones. The shoot Zn contents varied between 677.6 (SI646) and 4497.7 $\mu\text{g/plant}$ (SM30) under Zn excess, showing a 6.6-fold variation (Fig. 5b).

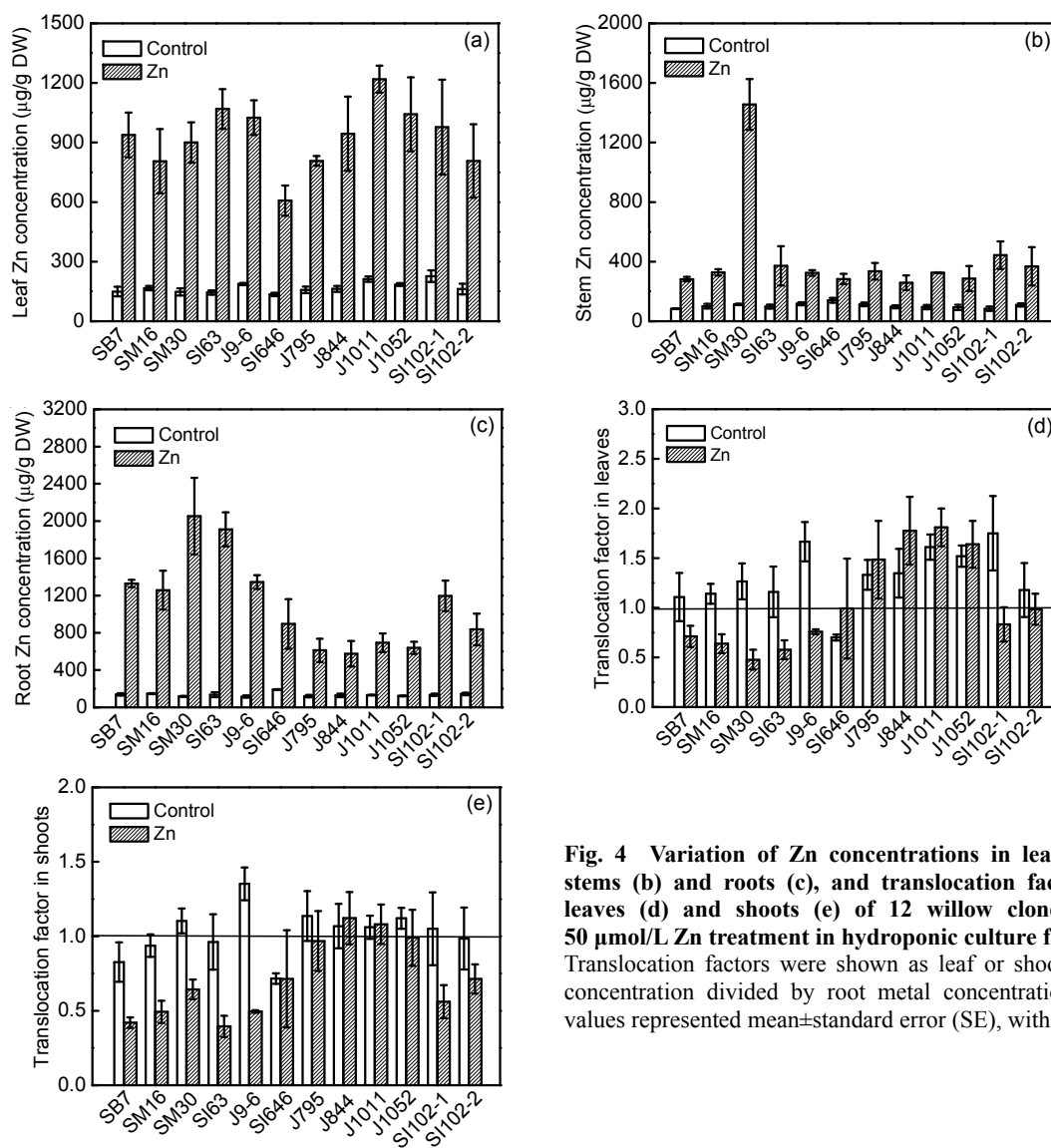


Fig. 4 Variation of Zn concentrations in leaves (a), stems (b) and roots (c), and translocation factors in leaves (d) and shoots (e) of 12 willow clones with 50 $\mu\text{mol/L}$ Zn treatment in hydroponic culture for 50 d. Translocation factors were shown as leaf or shoot metal concentration divided by root metal concentration. The values represented mean \pm standard error (SE), with $n=3$

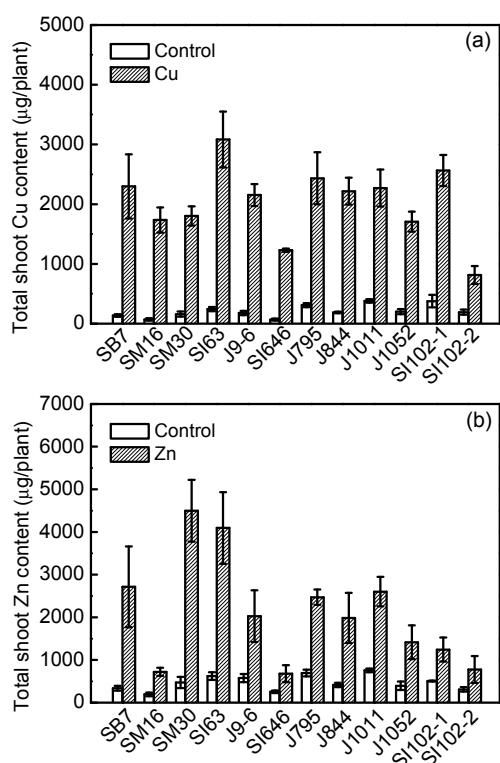


Fig. 5 Total phytoextraction Cu (a) or Zn (b) in the shoots of 12 willow clones with 50 $\mu\text{mol/L}$ Cu or Zn treatments in hydroponic culture for 50 d

The values represented mean \pm standard error (SE), with $n=3$

4 Discussion

4.1 Variation in plant growth and tolerance

No symptoms of necrosis or chlorosis were found in the leaves, suggesting that the levels of Cu and Zn in this study were not severely phytotoxic to the willow clones. The results showed wide variations in biomass accumulation in response to Cu and Zn, ranging from causing an increase, decrease, to having no significant effect. These results are consistent with previous findings, which illustrated high genetic variability in the tolerance of the Salicaceae family toward heavy metals (Punshon *et al.*, 1995; Dos Santos Utmazian *et al.*, 2007; Zacchini *et al.*, 2009; Zhivotovsky *et al.*, 2010).

Our data showed that the response to Cu exposure depended on the plant tissues. Cu treatment inhibited leaf biomass production in most clones, whereas it stimulated stem biomass production. Other than for SI102-2, Cu was shown to stimulate root growth in the treated willow clones compared with

controls. These results are different from those reported by Punshon *et al.* (1995). We calculated the TI based on the relative growths of the shoots and roots, and the results suggest that the clones tested have a relatively high tolerance to Cu, except for clones J1011 and SI102-2, and both the shoot and root TIs of seven clones were found to be >1 . Indeed, as an essential microelement, the present study showed that Cu enhanced plant growth under the current Cu treatment concentration. Therefore, under the current experimental conditions, willows can be described as Cu-tolerant plants, similar to the results reported by Borghi *et al.* (2007) on poplars.

The effect on growth in response to Zn was also highly dependent on the species and clones. Excess Zn resulted in a decrease in the biomass of most clones. Unlike Cu, the roots were more sensitive to toxic Zn concentrations than the shoots. Clone SB7 had the highest tolerance to Zn, and in fact, it strongly stimulated the growth of this clone, indicating a hypertolerance to the 50 $\mu\text{mol/L}$ Zn treatment.

Our results also indicated that Zn toxicity was higher compared with Cu toxicity; similar results have also been reported for some Mediterranean crops by López-González *et al.* (1999). Clones SB7, SM16, and J844 showed a significantly higher tolerance to both Cu and Zn than the other clones, while clones J1011 and SI102-2 were found to be more sensitive to both Cu and Zn than other clones.

4.2 Variation in Cu and Zn accumulation

The present data suggest that there are small variations in Cu accumulation among clones, and in contrast, a comparatively wide variation in Zn concentration. These results are in agreement with previous observations, confirming the genetic variation of metal accumulation in *Salix* (Punshon *et al.*, 1995; Dos Santos Utmazian *et al.*, 2007; Purdy and Smart, 2008; Zacchini *et al.*, 2009; Zhivotovsky *et al.*, 2010).

The values presented in Fig. 3 indicate that clones accumulated higher percentages of Cu in the roots than in aerial parts, which is in agreement with previous reports (Ali *et al.*, 2003; Kuzovkina *et al.*, 2004; Borghi *et al.*, 2007; 2008; Castiglione *et al.*, 2009). The distribution pattern of Cu in plant parts was ranked in the order of roots>stems>leaves. Our results showed relatively high levels of Cu in the above-ground parts, especially in the aerial stems;

similar results were reported by Punshon and Dickinson (1997). To the best of our knowledge, however, our results indicate that the Cu accumulation ability of the clones investigated here is far higher than those reported in other studies of the Salicaceae family. Comparing this with previous results, Punshon *et al.* (1995) found that the Cu concentration for willows was in the range of 9.5–63.0 µg/g DW in the leaves and 10–47 µg/g DW in the stems; according to Ali *et al.* (2003), the Cu concentration of *Salix acmophylla* was 10.8–126.3 µg/g DW in leaves, 25.3–203.7 µg/g DW in stems, and 24.8–624.4 µg/g DW in roots. Punshon and Dickinson (1997) reported that *Salix fragilis* accumulated Cu >300 µg/g DW in leaves, 25–150 µg/g DW in stems, and 300–1200 µg/g DW in roots. However, under some experimental conditions, relatively high Cu concentrations in aerial parts of the Salicaceae family were also recorded. For example, Turchi *et al.* (2012) reported that Cu accumulation in *Populus alba* ranged from 128.87 to 312.92 µg/g DW in the leaves, and from 1401.66 to 1598.92 µg/g DW in the roots; Komárek *et al.* (2010) found high Cu concentrations (377 µg/g DW) in the leaves of poplars grown in soil; Castiglione *et al.* (2009) also showed that *P. alba* accumulated Cu >400 µg/g DW in leaves, >100 µg/g DW in stems, and >500 µg/g DW in roots. Therefore, owing to the different experimental conditions, these results are difficult to compare.

There seems to be considerable variation in reports on the uptake and accumulation of Cu. For some plants, although Cu is a micronutrient, many studies do not show high translocation from the roots to above-ground organs. For the Cu concentration in the roots, our results were similar to other reports on poplar, but in the shoots, our results were far higher than those in the poplars reported by Borghi *et al.* (2007; 2008). Under hydroponic conditions, Cu noticeably accumulated in the roots and there was almost no transport of Cu from the roots to the shoots (Borghi *et al.*, 2007; 2008). Borghi *et al.* (2007) found that the Cu concentration in the leaves and stems was only about 10 µg/g DW, while the root Cu concentration reached 2000–4000 µg/g DW; similarly, Borghi *et al.* (2008) noted that the average Cu concentration was 5.9–8.8 µg/g DW in the leaves and stems, whereas the root Cu concentration reached 6×10^5 – 1.4×10^6 µg/g DW. Guerra *et al.* (2009) found the Cu concentration in the leaves of *Populus deltoides* to be

close to 50 µg/g DW, while the Cu concentration in the roots ranged from 5000 to 6000 µg/g DW. Indeed, these studies on poplar using the hydroponic system evaluated Cu toxicity tolerance rather than Cu accumulation potential. These findings suggest that almost no Cu accumulation occurred in the shoot tissues compared with the roots, i.e., that Cu absorption was largely confined to the roots, and therefore, the findings may lead to an underestimate of the Cu accumulation capacity in aerial parts compared with experiments using soils (Castiglione *et al.*, 2009; Komárek *et al.*, 2010; Turchi *et al.*, 2012).

However, other studies have indicated considerable Cu accumulation and translocation to above-ground organs of woody plants, showing higher accumulation of Cu in aerial parts. For example, Monni *et al.* (2000) reported that the shrub, *Empetrum nigrum*, accumulated 690 µg/g Cu in leaves and 3038 µg/g in stems; Sahi *et al.* (2007) found that the shrub, *Sesbania drummondii*, accumulated 1282 µg Cu/g DW in shoots, and 27440 µg/g DW in roots. These different results may be due to the different species/genotypes, treatment concentrations, culture conditions, and exposure time.

In fact, Cu accumulation ability depends on many factors. For example, Cu accumulation increased with seedling age (Monni *et al.*, 2000); the optimum growth stage of the plants enhanced the maximum Cu uptake on account of a much higher transpiration rate, which enhances metal uptake (Arduini *et al.*, 2006; Mleczek *et al.*, 2010; Weng *et al.*, 2005). Colzi *et al.* (2012) reported that Cu accumulation in the apoplast and symplast of roots was higher in one-month-old plants than in two-month-old plants. Culture conditions and exposure time can also affect metal uptake in plants. In particular, short-term exposure in hydroponic systems may not be sufficient for metal accumulation to occur in aerial parts of willows, leading to higher metal concentrations in the roots (Cosio *et al.*, 2006; Dos Santos Utmazian *et al.*, 2007).

Consequently, under the experimental conditions in this study, genotype, seedling size, growth stage, treatment concentration, and exposure time might enhance Cu accumulation in the aerial parts of the willow clones. Relatively large seedlings and a good growth stage increased metabolic activity, leading to absorption and transport of larger amounts

of Cu (Weng *et al.*, 2005; Arduini *et al.*, 2006; Dauthieu *et al.*, 2009).

The TF of plants is an important parameter for evaluating the potential for phytoremediation by harvesting above-ground parts of the plants (Deng *et al.*, 2007). The TF of Cu was lower than that of Zn, ranging from 0.17 to 0.27 in leaves and from 0.14 to 0.47 in shoots. Low values of leaf and shoot TFs in the Cu treatments suggest that all clones had a poorer ability to translocate Cu from the roots to the leaves/shoots (including stems). This low mobility of Cu in the Salicaceae family has also been confirmed in previous studies (Punshon *et al.*, 1995; Kuzovkina *et al.*, 2004; Castiglione *et al.*, 2009). The low translocation of Cu to the shoots is probably due to the roots having a strong affinity for Cu (Sahi *et al.*, 2007), as Cu is confined to the root surface or is adsorbed by the apoplast (Kuzovkina *et al.*, 2004); further, Cu binds to the carboxylic groups of pectins and polygalacturonic acids in the roots (Borghi *et al.*, 2007).

For most willow clones, the largest Zn accumulation was found in the roots, followed by the leaves and stems, but in some clones, higher percentages of Zn were found in the leaves rather than in the roots. Under the experimental conditions of this study, Zn concentrations in the leaves were close to or larger than 1000 $\mu\text{g/g}$ DW; clone SM30 accumulated the highest concentration of Zn in the stems, reaching 1455 $\mu\text{g/g}$ DW. The findings demonstrated that all of the clones had a high phytoextraction potential for Zn. The Zn accumulation capacity in this study is similar to those in other reports. For example, the largest Zn concentration (3180 $\mu\text{g/g}$ DW) in leaves was detected in a *Salix smithiana* clone grown in soil containing 955 mg/kg Zn (Dos Santos Utmazian *et al.*, 2007). Wieshammer *et al.* (2007) reported large Zn concentrations (3300 $\mu\text{g/g}$ DW) in the leaves of *Salix* × *smithiana*. Castiglione *et al.* (2009) found that the Zn concentrations of *P. alba* were >2500 $\mu\text{g/g}$ DW in leaves, >700 $\mu\text{g/g}$ DW in stems, and close to 1200 $\mu\text{g/g}$ DW in roots.

High values of leaf and shoot TFs for the Zn treatments suggest that most willow clones possessed a good ability to translocate Zn from their roots to their stems/leaves. Clones J795, J844, J1011, and J1052 had a greater tendency for Zn translocation from their roots to their shoots than other clones, demonstrated by their shoot TFs being >1. Although

clone SM30 accumulated 1455 $\mu\text{g Zn/g DW}$ in above-ground parts, which exceeded the Zn hyperaccumulator threshold, the shoot TFs did not exceed 1.0; hence, clone SM30 did not behave as a Zn hyperaccumulator. Previous studies have shown that Zn is easily translocated to the leaves of willow (Dos Santos Utmazian *et al.*, 2007).

In this study, nearly all willow clones exhibited the largest amount of Cu accumulation in their roots. In contrast, the concentration of Zn in aerial parts was higher than that of Cu. Cu was poorly translocated to the aerial parts of the willow clones, and thus, Zn is more mobile than Cu within a plant, this result being similar to the finding in *Chromolaena odorata* (Tanhan *et al.*, 2007). As shown in Figs. 3 and 4, most clones tested did not show a common uptake pattern for the two metals; these results indicated absorptions of Cu and Zn by willows via independent uptake mechanisms, as has been demonstrated for other plants (Ait Ali *et al.*, 2004).

4.3 Variation in phytoextraction potential

Tissue concentrations should not be used to evaluate the phytoremediation ability of species, because they do not take into consideration the whole plant biomass and metal concentration in the growth media (Ait Ali *et al.*, 2002). The phytoextraction efficiency of plants depends on the amount of heavy metals accumulated in the above-ground plant parts and the plant yields (Deng *et al.*, 2007). The total uptake of metals by the plants was calculated by multiplying the metal concentrations in each plant organ by the corresponding dry mass (Dos Santos Utmazian *et al.*, 2007).

Despite low TFs of Cu in willow clones, the present results show that willows have relatively high phytoextraction potential for Cu owing to its high biomass production. Clone SI63 showed the highest phytoextraction potential for Cu (3083.00 $\mu\text{g/plant DW}$ in shoots), while clone SI102-2 showed the lowest shoot Cu content (814.59 $\mu\text{g/plant DW}$); the shoot Cu content in SI63 was about 4-fold higher than that in SI102-2 under the same Cu treatment. This was because clone SI63 had a larger biomass than other clones and a relatively high Cu accumulation capacity in its aerial parts. In contrast, despite the high concentration in its shoots, SI102-2 showed a poor phytoextraction capacity for Cu owing to its low biomass.

Most clones produced more root biomass and a large proportion of Cu retention in roots exposed to excess Cu; hence, these results indicate that willows could be a good candidate for the phytostabilization of Cu.

The highest Zn content (4497.7 $\mu\text{g}/\text{plant DW}$) in the shoots was recorded in clone SM30 exposed to excess Zn, while the lowest Zn content (677.6 $\mu\text{g}/\text{plant DW}$) was found in clone SI102-2; the variation between clones SM30 and SI646 was 6.6-fold. Based on the total Zn content in its shoots, clone SM30 had the highest Zn phytoextraction potential, while SI646 had low phytoextraction potential, owing to the poor growth performance of SI646 under the Zn treatment. Hence, these results also confirm that the selection and improvement of willow clones for phytoextraction should focus on biomass production.

5 Conclusions

The results of this study demonstrated the variation among willow clones with respect to Cu/Zn tolerance and accumulation capacity. The willow clones investigated were less sensitive to Cu than to Zn, showing high resistance to 50 $\mu\text{mol}/\text{L}$ of Cu. Although Cu translocation to above-ground plant parts was low, willows showed good Cu phytoextraction potential owing to the large biomass and relatively high Cu concentration in shoots. Zn was more easily translocated to the above-ground tissues and mainly accumulated in the stems, thus showing higher phytoextraction potential than Cu. Therefore, based on relatively high Cu and Zn contents in the above-ground parts, our study showed that most willow clones are good accumulators for Zn and Cu. This study also highlights that the genetic variations in *Salix* can be exploited to improve their phytoremediation efficiency by selection of the most appropriate species/clones.

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Compliance with ethics guidelines

Wei-dong YANG, Yu-yan WANG, Feng-liang ZHAO, Zhe-li DING, Xin-cheng ZHANG, Zhi-qiang ZHU, and Xiao-e YANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要:

本文题目: 基于植物提取应用评价柳树无性系对铜锌耐性与富集的变异

Variation in copper and zinc tolerance and accumulation in 12 willow clones: implications for phytoextraction

研究目的: 柳树 (*Salix* spp.) 已广泛用于修复重金属污染, 而且修复效率与树种及无性系有关。目前涉及柳树研究主要为铜 (Cu) 和锌 (Zn) 的毒性效应, 而不是富集能力。本研究以中国常见的柳树无性系评价对 Cu 和 Zn 耐性与富集能力。

创新要点: 目前大多数研究仍集中 Cu 和 Zn 对柳树的毒性效应, 尤其是 Cu, 对它们富集能力仍没有评价。中国为柳树的主要分布区。本文以 12 种优良的常见柳树无性系, 通过温室营养液法评价对 Cu 和 Zn 耐性及富集潜力差异。利用柳树无性系的耐性与富集变异, 旨在选择高耐性与高富集的无性系, 提高环境修复效率和加快修复进程。

研究方法: 选择高生物量 12 种柳树无性系, 采用温室营养液培养法添加 Cu 或 Zn 处理, 测定生物量参数并计算耐性指数, 估计耐性差异; 用原子吸收法测定组织中金属浓度并计算金属位移系数, 评价富集差异。

重要结论: 无性系对 Cu 和 Zn 耐性显著不同, 生物量变化从无影响、抑制到促进生长。无性系对 Cu 耐性高于对 Zn 耐性, 无性系对 Zn 富集变异幅度大于对 Cu 富集变异幅度。Cu 主要富集于根部, 而 Zn 较易运输到地上部分。总之测定的大部分柳树无性系为 Cu 与 Zn 高富集植物, 显示出较高的植物提取能力。

关键词组: 柳树; 铜; 锌; 耐性与富集; 水培筛选