Effect of heavy metals combined stress on growth and metals accumulation of three *Salix* species with different cutting position

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**ABSTRACT**

This study aimed to compare growth performance and heavy metal (HM) accumulation at different cutting positions of *Salix* species grown in multi-metal culture. Three *Salix* species stems cut at different positions (apical to basal) were grown hydroponically for four weeks. The plants were then treated for three weeks with 0, 5, 10, and 20 \( \mu \text{M} \) Cd, Cu, Pb, and Zn, resulting in total metal concentrations of 0, 20, 40, and 80 \( \mu \text{M} \). The growth parameters and HM content in shoots and initial cutting were measured. Results showed that, compared with *S. fragilis*, *S. matsudana* grew more poorly in uncontaminated condition but grew better and accumulated lower metal in shoots under mixed HM treatment. In addition, cuttings from apical parent stem position exhibited poorer growth performance before and after treatment, as well as greater metal content in shoots than base parts under the HM treatment. These results suggest that *S. matsudana* may undergo a special mechanism to hinder metals in the initial cutting, thus mitigating growth damage. The apical portion also showed poor resistance against the invasion of mixed HMs because of the immature structure. Therefore, in the selection of phytoremediation plants, metal accumulation ability is not proportional to growth performance.

**KEYWORDS**

cutting position; growth performance; phytoremediation

**INTRODUCTION**

Heavy metal (HM) pollution of the soil and aquatic environment has garnered considerable public concern for the last several years. Large areas of soil and water have been contaminated by multiple metals as a result of human activities, including mining, dumping of solid wastes, the disposal of sewage sludge, as well as rapid urban and industrial development (Nriagu and Pacyna 1988; Keller 2006; Van Nevel et al. 2007; Kumar 2013). Contamination is widespread and has become a major challenge for plants and other organisms worldwide (Ovečka and Takáč 2014). Owing to their non-degradable characteristics, HMs accumulate in the soil and remain for a long time. HMs can thereby be transferred throughout the ecosystem and pose a risk to human health through the food chain (Liu 2003; Zhuang et al. 2009; Hu et al. 2013; Mico et al. 2006).

Various physicochemical techniques, such as electrodialysis, leaching, stabilization and land filling, have been applied to remediate HM-contaminated soil (Bournonville et al. 2004; Pedersen, Ottosen, and Villumsen 2005; Ward, Bitton, and Townsend 2005; Bayat and Sari 2010). Immobilization and extraction using physicochemical techniques mostly exert a negative impact on biological activity, soil structure and fertility, and are often appropriate only for small areas with significant engineering costs (Pulford and Watson 2003; Al Chami et al. 2014). In contrast, phytoremediation, which involves a series of techniques using green plants to extract and remove pollutants from soils, is efficient, low-cost, visually unobtrusive, applicable to large areas, environmentally friendly, and has aesthetic value (Yamato, Yoshida, and Iwase 2008; Zhang, Lin, and Deng 2013; Sheoran, Sheoran, and Poonia 2010). Phytoextraction is a phytoremediation approach that relies on the removal of pollutants upon accumulation in above-ground plant parts; this process depends on the ability of plants to accumulate large quantities of contaminants in their parts (Terry and Banuelos 2000). In general, two kinds of plants can be used for HM phytoextraction, namely, hyperaccumulators and fast growing species. Hyperaccumulators are herbaceous plants, such as *Phalaris arundinacea*, *Phragmites australis* L., and *Typha domingensis*, which have been mostly identified by current research to possess high phytoextraction abilities (Marchand et al. 2014; Salem et al. 2014; Mufarrege et al. 2014). Fast growing species possess significantly higher total biomass production than hyperaccumulators; however, lower phytoextraction ability has been observed in certain cases (Syc et al. 2012).

The use of woody plant species for phytoremediation has been reported both in the laboratory (Zacchini et al. 2009; Evlard et al. 2014) and in field experiments (Syc et al. 2012; Hu et al. 2013). Willow (*Salix* spp.) has, in recent years, shown considerable potential as a suitable species for use in phytoremediation. Numerous clones are fast-growing and can be managed in a short rotation coppice system (Punshon and Dickinson 1997). However, different species of willow and different varieties within the same species vary considerably in...
terms of their tolerance to HMs and the amount of HMs taken up into the woody parts of the tree (Greger and Landberg 1999; Punshon and Dickinson 1997; Kuzovkina, Knee, and Quigley 2004; Evlard et al. 2014). Meanwhile, growth performance can be used to represent the ability of plants to tolerate contaminants, but whether this parameter can also reflect the contaminant accumulation ability of plants remains unclear. Willow cutting propagation is a rapid and efficient method that is often used to remediate contaminated soils. Cuttings derive from different positions of the female parent root and germinate at different times; moreover, their positions change depending on the willow varieties (Dickinson et al. 1994; Kuzovkina, Knee, and Quigley 2004; Punshon and Dickinson 1999). In an environment polluted by combined HMs, whether the resistance and metal absorption of initial cutting and shoot change with the cutting position of the maternal plant has been rarely reported.

Therefore, we selected three species of Salix, a fast-growing and widely cultivated genus in China. Cadmium, copper, lead, and zinc were used for simulating multi-metal pollution in hydroponic conditions, which is believed to be a comparatively simple and easy method relative to soil culture in selecting appropriate plants for phytoremediation (Iori, Zacchini, and Pietrini 2013). All four metals applied in this experiment are present in equimolar amounts, thus providing an equal metal concentration premise and a good approach toward estimating Salix metal selectivity. In addition, we designed a concentration gradient to investigate the effect of metal ion concentration on the biomass and tolerance ability of the Salix species. The number and longest length of the live shoots and roots were measured for plant growth comparison. These data, along with their changes, were used to define the growth condition in this experiment. In relation to these, we present the following hypotheses: (1) Salix species with good growth performance under HM pollution will possess strong HM accumulation ability, and (2) cuttings from the apical position of parent stems will present poorer growth before and after HM treatment, as well as poorer HM accumulation ability compared with basal cuttings.

Materials and methods

Plant materials and treatment

The plant materials of S. fragilis (Sf), S. matsudana (Sm), and S. babylonica (Sb) were collected in field plantations which is not contaminated by any heavy metals in Suqian, Jiangsu Province, China, on September 10, 2013. For each Salix species, original stems of a minimum height of 75 cm were cut into uniform 15 cm length cuttings. Three parallel white plastic boxes, each box containing 15 cuttings, were applied for treatment. Twelve plastic boxes with a size of 70 cm × 35 cm × 16 cm (length × width × height), with each box in triplicate with the control, were used in the experiment (Fig. S1). Woody cuttings were planted hydroponically and grown for seven weeks in white plastic boxes filled with Hoagland’s nutrient solution (Arnon and Hoagland 1940), which was replaced once a week. Cd(NO₃)₂, CuSO₄·5H₂O, ZnSO₄·7H₂O, and Pb(NO₃)₂ (0, 5, 10, and 20 μM) were mixed for preparing a multi-metal solution containing 0, 20, 40 and 80 μM total metals, which was added to Hoagland’s nutrient solution in the fourth week and were refreshed every week.

Cuttings were planted in a climate chamber under a photosynthetic photon flux density (PPFD) of 200 mol m⁻²s⁻¹, a photoperiod of 14 h, relative humidity of 60%, and at a temperature regime of 25°C/20°C.

Growth parameters and tolerance index (T)

During the experiment, the longest shoot length, live shoot number, and root were measured every week. At the end of the experiment, cuttings were harvested, washed thoroughly with running tap water followed by deionized water, and then divided into roots, initial cuttings, and shoots (Fig. S1). The fresh and dry weights (dried at 60°C for 48 h) of the roots, initial cuttings, and shoots were measured.

Tolerance index (Tᵢ) was determined to assess the ability of willow varieties to grow under HM treatments according to the following equation (Wilkins 1978):

\[ Tᵢ(\%) = \frac{D_W_m}{D_W_c} \times 100\%, \]

where \( D_W_m \) is the dry weight of plant parts grown in HM solution, and \( D_W_c \) is the dry weight of plant parts grown in the control solution.

HM Analysis and TF<sub>aerial</sub>

Dried plant samples (shoot, root and the whole initial cutting) were finely ground and sieved through a 1 mm nylon sieve for metal analysis. Samples (1.0 g) were digested in 5 mL of nitric acid and 0.5 mL hypochloric acid using a digestive apparatus (LNK-872, China). Samples were then heated on a digestive tube at 200°C for 2 h until all organic matter had decomposed. Digests were poured into plastic bottles and then supplemented with deionized water to 5 mL before analysis. Metal content was determined by using an inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 5300DV, PE, USA).

Translocation factor (TF) is traditionally used to describe the movement of the target within below-ground biomass to aerial biomass. However, to explore the distribution of HM on the ground, we modified TF as follows:

\[ TF_{aerial} = \frac{M_s}{M_w} \times 100\%, \]

where \( M_s \) is the HM content in the shoot, and \( M_w \) is HM content in the initial cutting.

Statistical analyses

Basic statistical analysis was carried out with one-way analysis of variance (ANOVA) and Fisher’s least significance difference (LSD) test using SPSS 17.0 (Chicago, IL, USA). Separation of means was performed at a significance level of \( p<0.05 \).
Results and discussion

Growth parameters, biomass production, and tolerance index \((t_i)\) of the Salix species

The growth parameters of the three Salix species within seven weeks are presented in Fig. 1. Sf sprouted earlier than Sm, which in turn, started earlier than Sb prior to HM treatment. However, the live shoot number of Sf showed a rapid decline, whereas Sb and Sm showed similarly slow declines after HM treatment (Fig. 1a). The length of the longest shoot increased significantly and did not show considerable difference among Salix species before HM treatment. In the fourth week, the species showed different shoot lengths and remained unchanged after treatment (Fig. 1b). The root numbers of Sb and Sf presented similar trends during the experiment, whereas that of Sm showed the minimum and the maximum root numbers before and after HM treatment, respectively (Fig. 1c). HMs exert a significant influence on the longest root lengths of both Sb and Sf but did not considerably affect Sm (Fig. 1d).

Cuttings of most Salix species showed better performance than other trees. This result can be attributed to the fact that the species can easily produce roots and sprouts within a short time period (Verwijst et al. 2012; Tognetti et al. 2014). As illustrated in Fig. 1, the growth of three Salix species showed the following order: \(\text{Sf} > \text{Sm} > \text{Sb}\) (except number of alive root) under control conditions (Fig. 1), indicating that Sf grew best in an uncontaminated environment compared with Sb and Sm. Moreover, other works also reported that different reduction rates in root and shoot growth parameters exist in various types of willows under HM pollution (Dickinson et al. 1994; Kuzovkina, Knee, and Quigley 2004; Punshon and Dickinson 1999). Fig. 1 shows that the growth of Sm was not optimal under the uncontaminated condition but was the best under HM treatment among the three Salix species (except for the length of the longest shoot, \(p < 0.05\)). This finding is consistent with the results reported by former studies (Jia et al. 2013; Yang et al. 2014). This phenomenon indicates that Sm may undergo a special mechanism to mitigate growth damage brought about by contamination.

The shoot and root biomass of three Salix species under different HM concentrations are presented in Table S1. The largest biomass values of the shoots and roots were recorded in Sf (3.62 and 0.43 g plant\(^{-1}\), respectively) under control conditions. This finding is consistent with a previous study, which reported that, among Salix clones, Sf showed the highest biomass production on control soil (Evlard et al. 2014). For Sm and Sb, no significant differences in the biomass values of the roots were observed.
between the control and 5 μM concentration treatments. Meanwhile, a significant decrease (p < 0.05) in root biomass was observed in Sf that was exposed to the same concentration compared with the control. Moreover, the root biomass values of Sm and Sb did not decrease significantly with the HM treatment change, unlike that of Sf. In terms of shoot biomass, those of all three Salix species were significantly reduced (p < 0.05) under mixed HM treatment in comparison with the control. However, no significant differences were observed in the shoot biomass values of Sm and Sb between the 5 and 10 μM concentration treatments, whereas a significant reduction (p < 0.05) in that of Sf was observed under the 10 μM concentration treatments. The sensitivity of Sf to pollutants has also been reported by Evlard et al. (2014). Compared with other Salix species, Sf produces significantly less biomass when exposed to pollutants (Evlard et al. 2014). In addition, we found that Sf was more susceptible to HM concentration compared with Sb and Sm, which showed no significant differences (p < 0.05) among three metal concentrations (except the shoot biomass of Sb in the 5 and 20 μM concentrations). A visual symptom of metal toxicity with the increase in metal concentration was noted on the shoot and root of Sf.

Ti was calculated (Fig. 2) to investigate the effect of HM treatment on the ability of the Salix species to tolerate the mixed metals. The Ti,shoot (20 μM) of the three Salix species did not show much difference, whereas the Ti,root (20 μM) values of Sf, Sm, and Sb were found to be 56, 95, and 90, respectively. With the increase in total HM concentration, the Ti of each species displayed an obvious declining trend. Taking Sf for example, the Ti,shoot decreased from 81 to 43, and Ti,root decreased from 56 to 38. However, Sm still possessed the highest Ti among the three species, followed by Sb. This finding means that Sm and Sb possessed higher tolerance to HMs than Sf (p < 0.05). The results suggest that Sm may possess a strong metal tolerance to shield itself from HM toxicity or absorb HM selectivity to avoid toxicity under mixed HM stress.

**HM accumulation and above-ground TFaerial of the Salix species**

In the 20 μM mixed HM concentration, a significant difference (p < 0.05) was observed in the metal accumulation concentration of the Salix species (Fig. S2). Zn concentration in the initial cutting of Sf was higher than those of Sm and Sb (p < 0.05), whereas the concentrations of the three other metals were lower (p < 0.05). Sm showed lower metal concentration (Cd, Cu, Pb; p < 0.05) in the shoots. This finding is inconsistent with the superior growth performance (p < 0.05) of Sm compared with Sf (Fig. 1, Fig. S2, Table S1).

A larger TFaerial value translates to a higher HM content transported from the initial cutting to the shoot (Table 1). The TFaerial of HMs (except Zn) in the Salix species was of the same order of magnitude (Sf > Sm), consistent with the growth performance under control conditions and inconsistent with that after three weeks of metal treatment (Table 1, Fig. 1). Sm displayed the best growth performance but not the highest metal concentration, which may be due to its higher metal tolerance and stronger metal tolerance compared with Sf and Sb. The defensive ability mentioned in this paper involves the tendency of Sm compared with the other two Salix species, especially Sf, to hold back more metals in the initial cutting instead of transporting metals to shoots.

The greater HM accumulation in the initial cuttings than in shoots is attributed to the direct contact between cuttings and metal solution in hydroponics (Kuzovkina et al. 2004). In

| Salix species   | Cd       | Cu       | Pb       | Zn       |
|----------------|----------|----------|----------|----------|
| S. fragilis    | 49.1 ± 8.6c | 133.6 ± 16.6b | 79.3 ± 19.7c | 77.8 ± 6.5a |
| S. matsudana   | 19.4 ± 1.5a | 62.8 ± 6.8a | 25.6 ± 9.1a | 118.3 ± 17.8b |
| S. babylonica  | 37.9 ± 3.8b | 70.8 ± 2.9a | 50.4 ± 3.2b | 114.6 ± 6.9b |
addition, metals absorbed by roots would be transported to shoots through the initial cuttings, thus enabling these initial cuttings to hold back specific amounts of metals to protect the shoot from metal toxicity. The contents of Cd and Pb in the initial cuttings were higher than in the shoot in all three *Salix* species (TF_{aerial} < 100, p < 0.05), whereas the content of Zn in shoots of Sf was higher than those of Sm and Sb initial cuttings (TF_{aerial} > 100, p < 0.05). This phenomenon may be explained by the transportation of Zn to shoots in Sm and Sb as an essential element, the concentration of which did not reach the toxicity concentration range (Evlard *et al.* 2014). The measured metals concentrations were surprisingly lower in our experiment compared with the result of Dos Santos Utmazian and Wenzel (2007), which is 4–6 g kg⁻¹ Cd and 100–500 g kg⁻¹ Zn in leaves of the three *Salix* species under metals cocktail treatment. The dissimilarity may be attributed to the uniformed concentration of metals and the multiple contaminated environments that we created. Ion competition and the metabolic response of plants caused by the complex environments may lead to the decrease in metal concentration in willows. Moreover, the low metal concentration in willows observed in our experiment may also be partly attributed to the short-term exposure to metals in our hydroponic study.

**Growth parameters of different cutting position**

To compare the effects of different cutting positions on the growth performance among *Salix* species, the longest length and live number of shoots and roots were measured (Fig. 3). The length of the longest shoot of the three *Salix* species with different original positions showed a decrease trend from base to apex. For example, the shoot lengths of Sf were 16.2 (base) and 11.7 cm (apex), whereas the difference between the apex and base of the shoot length was 4 cm in Sm and Sb. The difference between the base and apex was also reflected in the longest root length of the *Salix* species. Moreover, we observed an evident difference, in which the initial cutting with the basal position possessed more shoots and roots than the apex. In addition to selecting willow as a candidate for phytoremediation, we should consider the species, as well as the cutting position.

In our study (Fig. 3, Table S2), cuttings derived from the basal position along the parent stem displayed better growth performance than cuttings derived from apical parts. This phenomenon was predictable and also reported in Schefflera arboricola, Dalbergia melanoxylon, and poplar clones (Hansen 1986; Amri *et al.* 2010; Schroeder and Walker 1990). However, Verwijst *et al.* (2012) reported the opposite result. Meanwhile, Verwijst *et al.* (2014) reported that cuttings from the apical position grew better than other parts in the same size of cutting diameter. In the cutting propagation of willow, cutting size (length and diameter) exerted a positive effect on growth performance (Burgess, Hendrickson, and Roy 1990; Rossi 1999; Carpenter, Pezeshki, and Shields 2008). The discrepancy between our study and the works of Verwijst *et al.* (2012, 2014) may be caused by a slight difference in cutting diameter.

The difference in cutting growth between the fourth (before treatment) and seventh week (treatment for three weeks) were determined to assess the effect of HM on the cutting positions (Table S2). The cuttings from the base showed a decline in the number of shoots and roots, but to a lesser extent than that of the cuttings from the apex (p < 0.05). The basal cuttings of the three *Salix* species displayed a slight increase in shoot length, whereas the change in apical cuttings did not present a unified trend. The apical cuttings of Sm showed an increase, whereas those of Sf and Sb showed a slight decrease in shoot length. In addition, the root lengths of Sf and Sm showed a difference between apical and basal cuttings (p < 0.05), whereas that of Sb did not present positional difference. The change in growth parameters can thus be used to appraise the growth of plants.
under adverse conditions. The cuttings from basal position possessed a smaller decrement in growth parameter in comparison with cuttings from the apical position regardless of Salix species.

**HM content in different cutting positions**

HM content in plants whose initial cutting is from different parent stem positions of the three Salix species are shown in Table 2. The metal content displayed an increasing trend from basal cutting to apical cutting. This trend is completely opposite to that shown by growth performance. As indicated in Table 2, the plant whose initial cutting is from the apical position accumulated more metals than those from the basal position in most cases ($p < 0.05$), because the structure and function of apical cutting were not sufficiently developed to resist damage from mixed HM stress. As a result, the growth and defense ability of the species were weak, leading to extensive incursion of HM into the plant. So as to supplement the growth defect of less inherent nutrients, the plant whose initial cutting is from the apical position absorbed nutrition and water actively along with HMs. Thus, apical cuttings accumulated more HMs than the basal parts. The results suggest that a direct relationship does not exist between growth performance and accumulation ability of cuttings from different parent stem positions.

**Conclusion**

In our experiment, *S. fragilis* showed a greater number and longer length of roots and shoots in an uncontaminated environment. However, *S. fragilis* proved to be more susceptible to HM pollution, with higher $T_{Fr,env}$ (except Zn) in comparison with *S. matsudana* and *S. babylonica*. In addition, *S. matsudana* displayed minimal damage, with a larger number and longer length of roots and shoots after three weeks of HM treatment, but presented not highest metal concentration in the initial cutting and shoots compared with *S. fragilis* and *S. babylonica*. These results suggested that the *Salix* species, which exhibited good growth performance under HM pollution, did not possess a strong HMs accumulation ability. Moreover, cuttings from the apical position of the parent stem possessed higher HM content in shoots, but with more damage as well as fewer number and shorter length of roots and shoots than cuttings from the basal position before and after HM treatment. The results also indicated that cuttings from the apical position of the parent stem exhibited poorer growth performance before and after HM treatment and showed higher HM accumulation ability compared with the basal cuttings.

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