Rhizosphere characteristics, heavy metal accumulation and growth performance of two willow (*Salix* × *rubens*) clones

M. Vysloužilová[1, 2], M. Puschenreiter[2], G. Wieshammer[2], W.W. Wenzel[2]

[1]*Czech University of Agriculture in Prague, Czech Republic (Present address: Research Institute for Soil and Water Conservation, Prague, Czech Republic)*
[2]*Department of Forest and Soil Sciences, University of Natural Resources and Applied Life Sciences in Vienna, Austria*

ABSTRACT

High-biomass tree species holds promise for a clean up of metal contaminated soils. Root and fungal activities modify soil characteristics that are important factors for the phytoextraction process (metal availability and toxicity). In a rhizobox experiment, two clones of *Salix* × *rubens* derived from contaminated and non-contaminated sites were tested for growth performance and metal (Cd, Pb and Zn) accumulation on a polluted Calcaric Cambisol. The largest metal concentrations in leaves were 66.7 mg Cd/kg, 12.8 mg Pb/kg and 1090 mg Zn/kg. The results indicate that metal tolerance and accumulation of *S.* × *rubens* may be a constitutive rather than an adaptive property. Soil pH did not differ among rhizobox compartments. However, acid neutralization capacity was decreased in rhizosphere. DOC in rhizosphere was increased by 37% and seemed to enhance labile fraction of Pb and Zn, whereas Cd was not affected. The replenishment of labile metals from less labile soil fractions was efficient enough to almost compensate the plant uptake. *S.* × *rubens* can effectively induce chemical changes in the rhizosphere is very promising for a clean up of metal-polluted soils.

Keywords: willow; *Salix* × *rubens*; phytoextraction; heavy metals; Cd; Pb; Zn; rhizobox; mobilization; soil pH

Specific willow clones are able to accumulate substantial amounts of Cd and Zn in the aboveground biomass and have therefore been tested for phytoextraction of moderately contaminated soils (Greger and Landberg 1999, Rosselli et al. 2003, Vysloužilová et al. 2003). Significant differences in metal tolerance and accumulation were found among willow varieties and clones (Riddell-Black 1994, Punshon et al. 1995). The ability of willows to tolerate large concentrations of heavy metals in soil is indicated by their frequent appearance on contaminated sites such as mining areas or polluted dredged sediment disposal sites (Reimann et al. 2001, Pugh et al. 2002, Vandecasteele et al. 2002). Landberg and Greger (1994) reported that the accumulation or exclusion of heavy metals by willows did not depend on their tolerance. Punshon and Dickinson (1997) found that short-term single pre-treatment of willow clones did not increase their tolerance to heavy metals, whereas gradual cumulative doses of heavy metal resulted in reduced phytotoxicity and increased resistance, notably to Cd. Landberg and Greger (1996) investigated metal uptake and tolerance of *Salix* clones from unpolluted and polluted areas grown in hydroponics. Although the tolerance of the clones did not differ, there was a difference in accumulation and translocation of heavy metals. The clones derived from polluted areas showed larger metal accumulation in the roots and lower translocation into shoots compared to clones from unpolluted areas. Stolz and Greger (2002) observed differences in metal uptake and translocation properties of the same plant species (*Salix phylicifolia*, *S. borealis*, *Carex rostrata*, *Eriophorum angustifolium*, *Phragmites australis*), planted in hydroponics and soil (field-grown) because of the processes in the soil-root interface, i.e. in the rhizosphere. Despite the limitations of hydroponics and metal resistance tests, Watson et al. (2003) showed that the

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response of the same clones tested in hydroponic systems broadly corresponds to results from the field. Pulford and Watson (2003) concluded that the survival of trees (e.g. willows, poplars, birches) seems to be due to their tolerance, notably avoidance of highly contaminated substrate by roots or immobilization of heavy metals in the root.

Willows have naturally occurring associations with both endo- and ectomycorrhizal fungi (Lodge 1989). This symbiosis is advantageous for the plant as the fungi increases the nutrient acquisition by exploring the soil with hyphae. On the other hand, the fungi receives carbon products from the plant (Marschner 1995). However, the effects of mycorrhiza on the metal uptake by willows are largely unknown and appear to be metal and plant specific (Lasat 2002). Virtually, no information is available regarding changes in soluble and labile metal fractions and their chemical controls (pH, buffer power, DOC) in the rhizosphere and mycorrhizosphere of metal-accumulating willows.

The objectives of our study were thus the assessment of:

– chemical changes in the rhizosphere and mycorrhizosphere of *Salix* × *rubens* grown in contaminated soil in a rhizobox experiment;
– metal uptake and growth characteristics of clones derived from contaminated and non-contaminated sites.

MATERIAL AND METHODS

The experiment was conducted in a modified rhizobox (Figure 1) based on the design by Li et al. (1991). The plants grow in a central compartment (A) and both root and fungal activities create rhizosphere soil. The roots are restricted from growing into the adjacent compartment (B) by nylon membranes (2 µm mesh size), whereas the mycorrhizal hyphae can penetrate this membrane. The compartment with mycorrhizal hyphae is separated only by another nylon membrane with smaller mesh size (0.45 µm) from the neighboring soil interface compartment (C). This soil interface compartment has a width of 2 mm and is free of mycorrhiza but is influenced by its activities. This compartment is separated from the bulk soil (D) by another nylon membrane (0.45 µm mesh size). In the bulk soil compartment, the soil is influenced neither by root nor by mycorrhizal activities. The experimental soil (Calcaric Cambisol) was collected from a former Zn and Pb smelter area in Arnoldstein (Carinthia, Austria) and showed high concentrations of Zn, Cd and Pb. Soil characteristics are listed in Table 1. Field-moist soil was passed through a 2-mm sieve, homogenized and frozen in plastic bags at –18°C. Soil was thawn and equilibrated at the room temperature for 14 d before it was filled into rhizoboxes (200, 146, 18 and

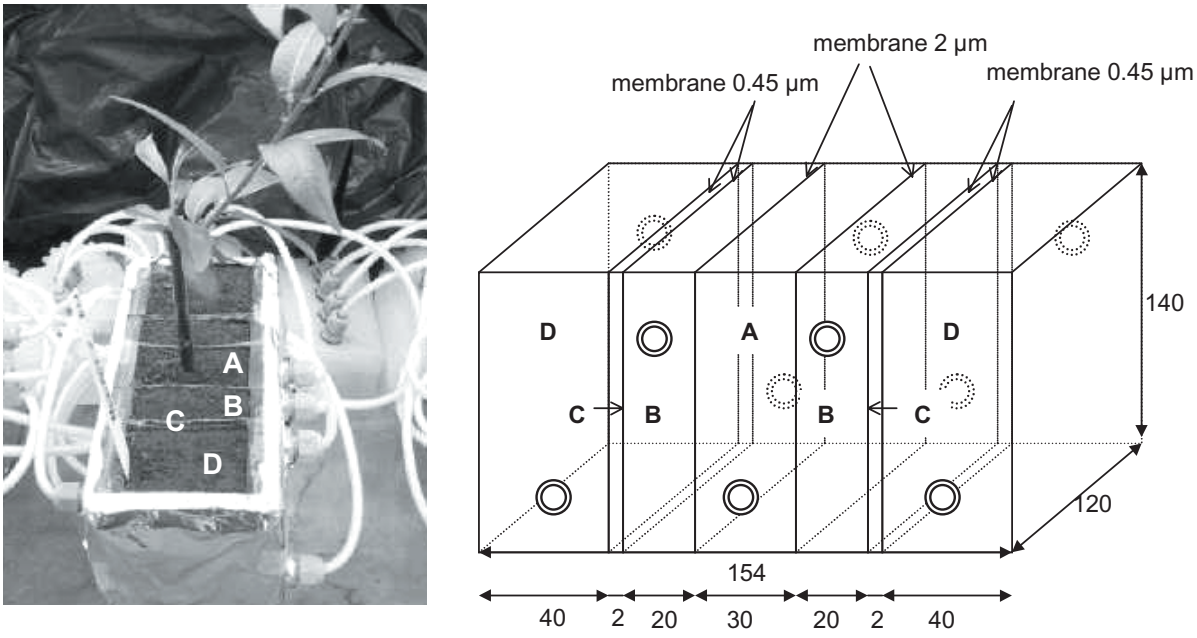

Figure 1. Design of rhizobox used in the experiment (all dimensions in mm); A – rhizosphere; B – mycorrhizosphere; C – 2 mm soil interface; D – bulk soil

Table 1. Characteristics of the experimental soil derived from a contaminated site in Arnoldstein (Carinthia, Southern Austria)

Parameter	Unit	
$pH_{(H2O)}$	–	6.41
CEC	mmol/kg	231
Sand	g/kg	668
Silt	g/kg	239
Clay	g/kg	92.5
$CaCO_3$	g/kg	44.3
Zn_{tot}	mg/kg	3440
Pb_{tot}	mg/kg	14 600
Cd_{tot}	mg/kg	76.6
Zn_{labile}	mg/kg	56.2
Pb_{labile}	mg/kg	23.4
Cd_{labile}	mg/kg	3.21

298 g soil per rhizosphere, mycorrhizosphere, 2-mm soil interface and bulk soil compartment, respectively).

2-years-old cuttings of two *Salix* × *rubens* (*S. alba* L. × *S. fragilis* L.) clones were sampled for this experiment. The clones were obtained from a contaminated site (Kutná Hora, clone C) and a non-contaminated site (Kuřivody, clone N) in the Czech Republic. The As, Cd, Cu, Pb and Zn contamination of the Kutná Hora site had been caused by mining activities for several centuries. The clone derived from Kutná Hora showed a large Cd and Zn uptake into the leaves (35 and 960 mg/kg d.w., respectively) in a previous screening of *Salix* spp. and *Populus* spp. grown in the area (Vysloužilová 2003). The clone from the site Kuřivody had recently been tested for phytoextraction efficiency in a pot experiment and showed a large Cd and Zn uptake and removal from contaminated soils (Vysloužilová et al. 2003).

Cuttings of both clones were pre-grown in quartz sand in a growth chamber (15/25°C day/night temperature, 16 h light period) until roots had developed (14 d). Five plants of each clone were treated with MycorTree^{TM}, a commercially available root dip for tree seedlings (Plant Health Care, Inc., Pittsburgh) containing spores of the ectomycorrhizal fungi *Pisolithus tinctorius* and rhizosphere bacteria (*Bacillus thuringiensis*, *B. megaterium*, *B. licheniformis*, *B. subtilis*, *B. polymyxa* and *Paenibacillus azotofixans*). Following the directions for use of the product, slurry was produced, where roots were dipped into until they were completely covered with the gel being then transferred into the soil. The rhizoboxes were made of transparent Perspex acrylic material, allowing an observation of root growth, and wrapped with aluminium foil during the experiment to avoid algae growth. In total, the experiment included 10 rhizoboxes with two *S.* × *rubens* clones in five replicates. The rhizoboxes were transferred to a greenhouse and plants were left to grow for two months (February/March) at about 20–25°C during the day and 16–18°C at night, with additional light to allow 16 h light periods. Soil compartments A, B, and D of the rhizoboxes were uniformly and continuously watered by the distilled water using irrigation wicks (Figure 1). The water content of the soil in the individual compartments (determined after harvest) was about 30% of the maximum water holding capacity (MWHC). Harvested plants were separated into roots, leaves and woody parts. Aboveground plant tissues were washed with distilled water and roots in 5mM $CaCl_2$ in an ultrasonic bath. All plant samples were dried until constant weight at 80°C. The weight of the biomass was recorded before grinding in a metal-free mill (IKA®-WERKE MF 10). The ground plant material (0.2 g samples) was digested in a closed high-pressure microwave system (mls 1200 mega, Milestone) using a mixture of H_2O_2/$HClO_4$/HNO_3 (0.5/1/6 ml).

A two-step sequential extraction procedure was performed on the soil of each rhizobox compartment. Fresh soil samples corresponding to 5 g of soil dried at 105°C were extracted for 2 hours using distilled water at a ratio of 1:5 (w/v). To obtain the labile metal fraction, the samples were extracted for two hours on an end-over-end shaker using 1M NH_4NO_3 at a soil-solution ratio of 1:2.5 (w/v). Soil of all compartments was analyzed for dissolved organic carbon (DOC) concentration in the water extracts using a total C/N analyzer (DIMA-TOC 100, Dimatec, Essen, Germany). Supernatants of all extracts were centrifuged (1700 g) and passed through 0.45-µm hydrophilic cellulose acetate membrane filters (Sartorius). Soil-water slurry at the soil-solution ratio of 1:2.5 (w/v) was measured for pH, using a combined pH electrode, and for electrical conductivity (EC), using a conductometer (WTW inoLab, Cond 740). For the determination of acid neutralization capacity, soil was equilibrated with 0, 20, 50, 100, and 200 mmol H^+ (added as HCl) per g soil (1:5, m/v) for 2 h before measuring pH.

Cd, Pb, Zn, Ca, K and Mg concentrations in extracts and digests were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Zeiss Plasmaquant 100). Chemical analyses were validated by using reference materials (Eurosoil 7; internal plant material standard) and Fisher's least significant difference (LSD) procedure (Statgraphics Plus 4.0) was used for the statistical evaluation of differences between treatments and soil compartments ($\alpha = 0.05$).

RESULTS AND DISCUSSION

S. × *rubens* from the non-contaminated site Kuřivody (clone N) showed an overall better vitality (no signs of toxicity) and higher biomass production than the clone from the contaminated site Kutná Hora (clone C). Significant differences were found for leaf and root production (Figure 2). The clone N achieved larger shoot (+40%) and root (+60%) production compared to the clone C calculated for biomass production. The clone C showed signs of chlorosis probably due to Fe-deficiency. Iron deficiency can be induced by an excess of other metals such as Zn or Cd in the soil solution, depressing the Fe uptake by plant roots due to competition (Alcantara et al. 1994). Fontes and Cox (1998) found that Fe-deficiency negatively influenced the growth of plants exposed to high Zn concentrations. These results show, that willows, that are expected to be adaptable to high metal concentrations in the soil, do not necessarily grow better in metal contaminated soils than willows that derive originally from non-contaminated sites.

The Cd concentrations were larger in the shoot and root tissues of the clone N, whereas the differences in Pb and Zn concentrations were not significant between the clones (Figure 2). Our study shows that the willows from the contaminated site clearly suffered from large metal concentrations in the experimental soil as indicated by leaf chlorosis and yield reduction. Heavy metal concentrations in both clones were well above phytotoxicity values of 10 (Cd), 500 (Zn) and 20 (Pb) mg/kg for normal plants (Sauerbeck 1989). The comparison of metal

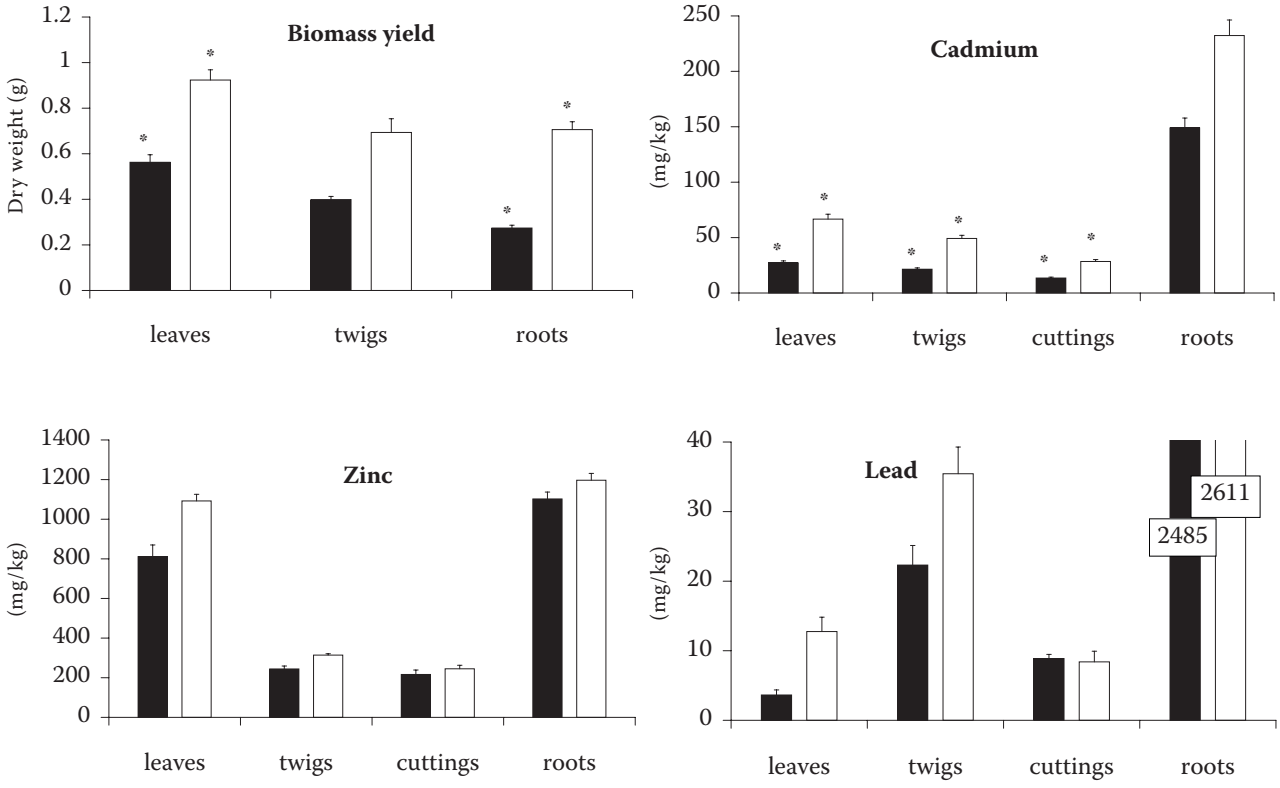

Figure 2. The biomass production (g dry weight per pot) and Cd, Pb and Zn concentration (mg/kg dry weight) in tissues of *S.* × *rubens* from contaminated (clone C, black bars) and non-contaminated sites (clone N, white bars); error bars represent standard error of the mean ($n = 5$); significant differences ($P > 0.05$) are indicated by asterisks

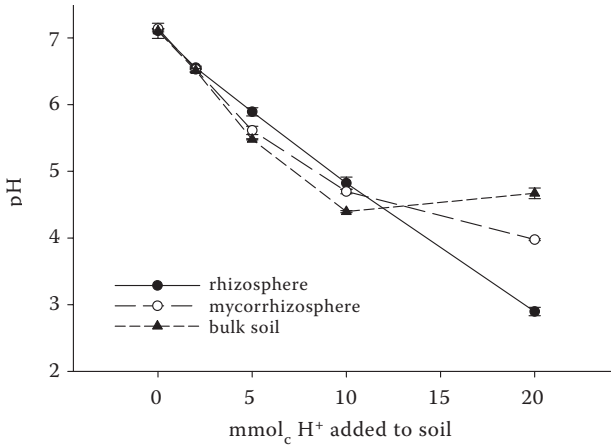

Figure 3. Acid neutralization capacity expressed by H^+ titration curves of soil from individual rhizobox compartments of clone N

concentrations in leaves, twigs, cuttings and roots showed that Cd and Zn were transferred from the root into the shoot, whereas Pb transport from root to shoot was restricted. Shoot Pb concentrations represented only 2% of those in the roots, which is in accordance to the results of Stolz and Greger (2002) and Pugh et al. (2002). The low Pb mobility in plants (Borůvka et al. 2001) is also confirmed by larger Pb concentrations in twigs than in leaves. Twigs as well as cuttings had small concentrations of Cd and Zn (Figure 2). Cadmium transfer from the roots to the leaves of both *S.* × *rubens* clones was not as efficient as for Zn. The translocation factor (concentration ratio leaves/root) was 0.24 for Cd and 0.82 for Zn. This confirms the results from a previous pot experiment (Vysloužilová et al. 2003). The fact that the clone N had larger metal concentrations in the tissues suggests that metal accumulation in willows is rather a constitutive than an adaptive property. A recent study by Watson et al. (2003) supports this hypothesis. On the contrary, concentrations of macro-elements such Ca, K and Mg were found to be lower in tissues of the clone N (17.8, 16.5 and 7.70 g/kg of dry leaves, respectively) probably due to a dilution effect associated with the higher biomass production compared to the clone C (25.3, 24.2 and 5.90 g/kg of dry leaves, respectively).

The variance of pH values within the four soil compartments was only marginal for both clones. However, we found a change of the acid neutralisation capacity in the rhizosphere soil compartments. This is obvious from the H^+ titration curve (Figure 3) which describes the neutralization capacity of soil collected from rhizoboxes planted with clone N. Protons released by roots may not change pH values in a well-buffered soil (Fitz et al. 2003), as it was used here, but the acid neutralization capacity of rhizosphere soil was decreased compared to bulk soil after addition of 20 mmol$_c$ H^+/kg. The change in the mycorrhizosphere was less pronounced than in the rhizosphere. From these results we conclude that the acid neutralisation capacity was decreased both by root and fungal activities.

Indeed, the largest concentration of dissolved organic carbon (DOC) was determined in the rhizosphere of both clones. Figure 4 shows DOC in the soil of individual compartments relative to DOC in bulk soil (100%). Although no significant differences could be found among the other com-

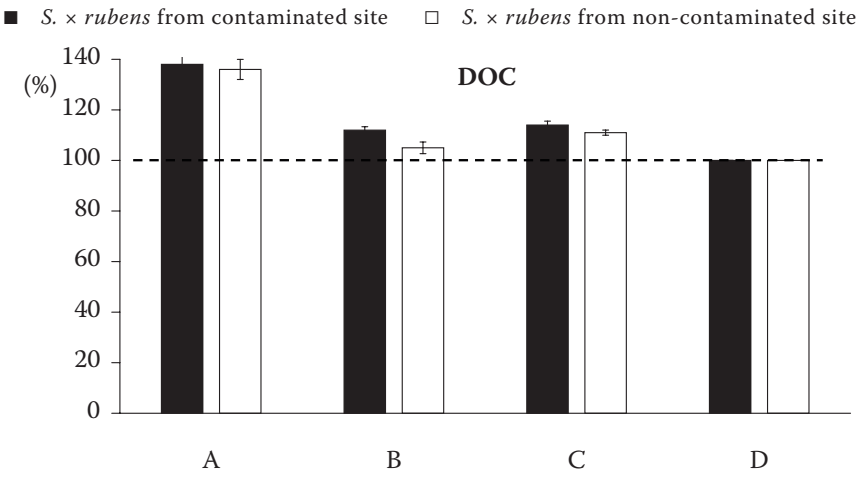

Figure 4. Relative amount of dissolved organic carbon in the rhizobox soil compartments (A – rhizosphere; B – mycorrhizosphere; C – 2 mm soil interface; D – bulk soil = 100%); error bars represent standard error of the mean (n = 5)

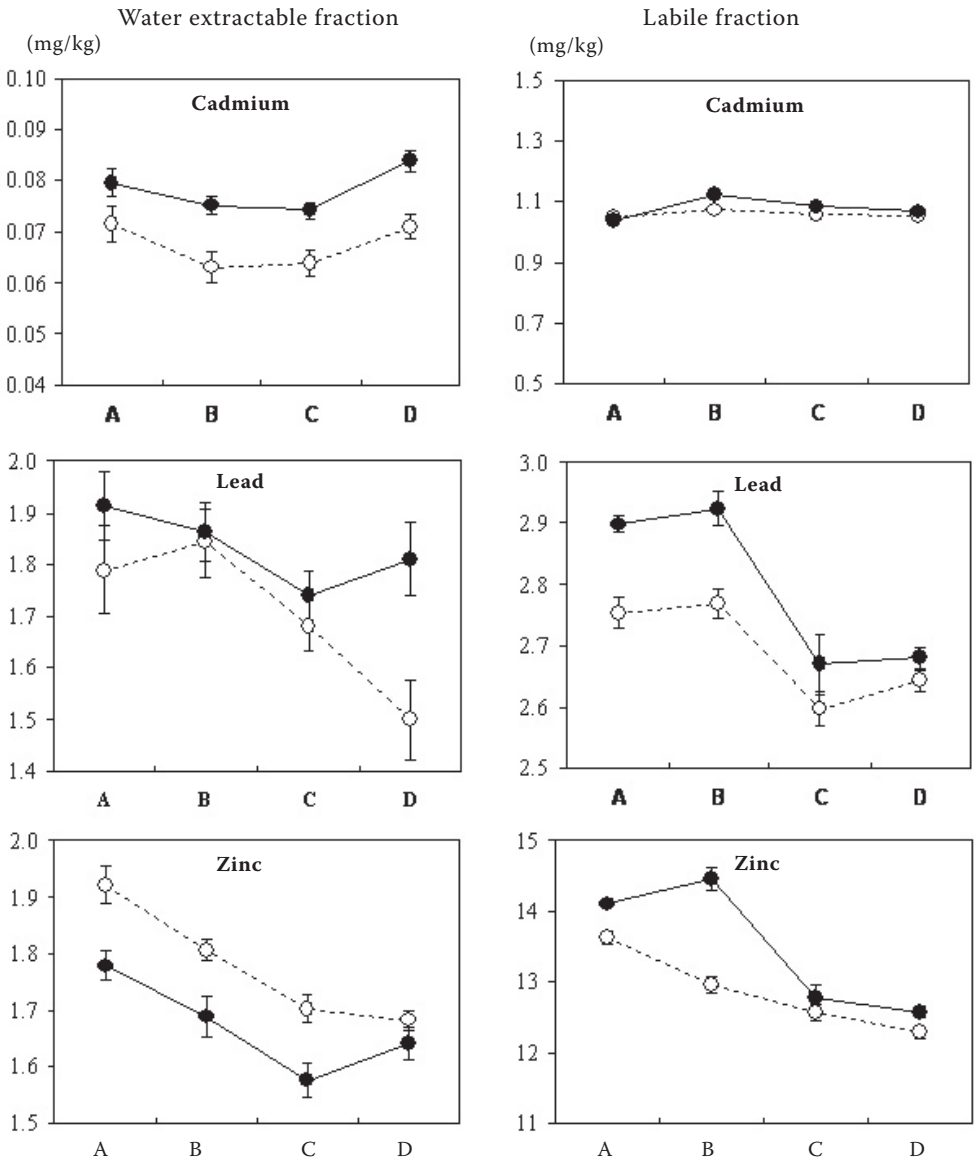

Figure 5. Cd, Pb and Zn concentrations (mg/kg) in soil extractable by water and 1M NH_4NO_3 (labile) from soil (A – rhizosphere; B – mycorrhizosphere; C – 2 mm soil interface; D – bulk soil) for *S.* × *rubens* clone C (white points) and N (black points); error bars represent standard error of the mean (n = 5)

partments, the highest relative amount (approximately 137%) of DOC was found in the rhizosphere of both clones. The larger concentration of DOC in the rhizosphere of both *S.* × *rubens* clones might be caused by root exudates, such as organic acids, sugars, and amino acids (Marschner 1995). The enhanced DOC in the rhizosphere compartment might contribute to an increased phytoavailability of heavy metals (Wenzel et al. 2003).

The 1M NH_4NO_3 extraction revealed that labile Zn and Pb were enhanced in the rhizosphere and mycorrhizosphere of both *S.* × *rubens* clones, while concentrations of labile Cd did not show significant changes among the four rhizobox compartments (Figure 5). The increase of labile Zn and Pb in the rhizosphere and mycorrhizosphere may be related to mobilization effects, caused by activities of plant roots and mycorrhizal fungi. The results show that the mobilization of Pb and Zn was realized without pH changes in the soil. Increased DOC concentrations in the rhizosphere compartment may have induced mobilization of Zn and Pb. For the mycorrhizosphere compartment, no corresponding DOC increase was found, but the increase of labile Zn and Pb may be due to a change of quality of exudates. Since organic acids comprise a significant part of root exudates (Marschner 1995), ligand-induced co-dissolution

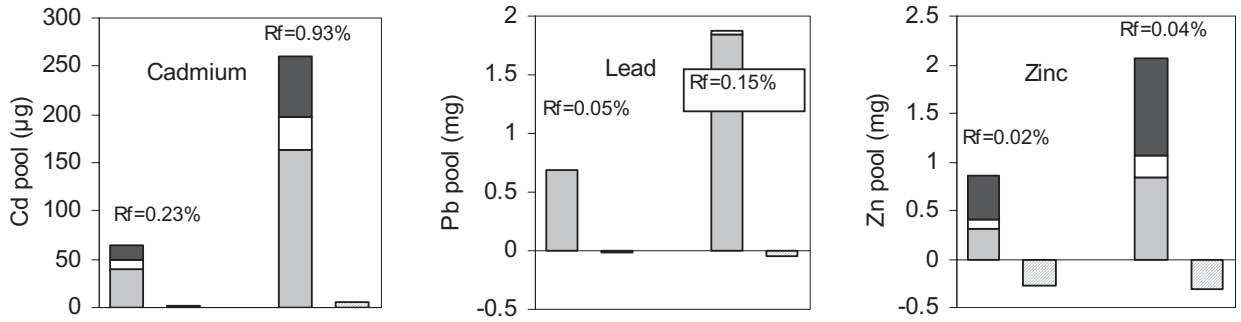

Figure 6. Amount of Cd, Pb and Zn in harvested biomass (grey column roots, white twigs and black leaves) of clone C and N; Rf expresses the amount of elements accumulated by biomass related to its total concentration in soil; hatched bars express differences between labile metal pools in the soil of central compartment and bulk soil at the end of the experiment; positive values indicate a decrease, negative values an increase in the central compartment (rhizosphere) compared to bulk soil

of metals may contribute to increased labile metal fractions. Mobilization of Pb and Zn, determined by 1M NH_4NO_3 extraction, in root and hyphae vicinity compared to bulk soil were found to be larger for clone N (8.7% and 12.6%) compared to clone C (5.3% and 8.5%). This might be caused by significantly higher root biomass. These differences in Pb and Zn mobilization between the two clones were less evident in the water extract. However, water extraction of soil showed the same trend of metal mobilization in rhizosphere and mycorrhizosphere compared to bulk soil as in 1M NH_4NO_3 extract of soil (Figure 5).

Labile fractions of metals did not differ (Cd) or even increase (Pb and Zn) in the rhizosphere and mycorrhizosphere despite the large metal removal by willows. In the harvested biomass, 0.07 mg Cd, 0.69 mg Pb and 0.86 mg Zn were removed by clone C and 0.26 mg Cd, 1.88 mg Pb and 2.07 mg Zn were removed by clone N (Figure 6). Related to total heavy metal concentrations in soil these amounts represent 0.23%, 0.05% and 0.02% for clone C and 0.93%, 0.15% and 0.04% Cd, Pb and Zn for clone N, respectively. Larger metal removal was obtained for clone N due to larger metal concentrations in its tissues and higher yield of biomass.

Removal of all metals by both clones is much larger than differences between labile (NH_4NO_3-extracted) metal pool in the soil of the central compartment and bulk soil at the end of the experiment. It indicates a mobilization of metals during the growth of plants from less mobile soil fractions. The replenishment of labile metals from less mobile fractions was efficient enough to almost compensate the plant uptake, which suggests that plant and fungal activities could mobilise significant amounts of heavy metals from less soluble fractions, which is also indicated by the increased amounts of DOC compared to bulk soil (Figure 4). Similarly, Puschenreiter et al. (2003) detected almost no changes of labile fraction in soil for hyperaccumulator *Thlaspi caerulescens* grown in a similar soil derived from the site Arnoldstein.

The results of this experiment reveal changes of metal biogeochemistry in each compartment of the rhizobox and different effects for the individual heavy metals. It seems that the amount of Cd taken up by willow roots was re-supplied in the rhizosphere and mycorrhizosphere from the bulk soil or from less available metal fractions in soil. Mobilization of Pb and Zn by root and hyphae activities was larger than the amount of metals taken up by willows. As a consequence, labile and water-soluble fractions of both Pb and Zn increased in the rhizosphere and mycorrhizosphere.

The typical depletion of labile K in the rhizosphere (Marschner 1995, Figure 7) can be explained by the replenishment from the soil solid phase subsequently to the uptake in *S.* × *rubens*. On the contrary, extractable Ca and Mg are often found to accumulate in the rhizosphere, as the amounts taken up by the plant are lower than their mass flow to the roots (Marschner 1995). Results of the water extractable K, Ca and Mg show the same trend of depletion and accumulation in the rhizosphere and mycorrhizosphere (data not shown). Root and mycorrhizal fungi activities of both clones had the same effect on the fate of elements in the rhizosphere and mycorrhizosphere.

The results of this experiment indicate that the metal accumulation potential of *S.* × *rubens* is a constitutive property. As presented here, wil-

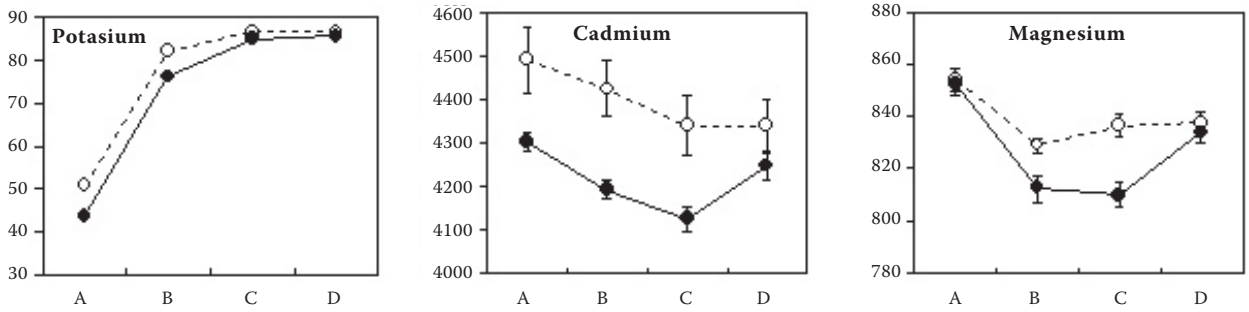

Figure 7. Labile K, Ca and Mg (mg/kg) extractable by 1M NH_4NO_3 from soil (A – rhizosphere, B – mycorrhizosphere, C – 2 mm soil interface and D – bulk soil) for clone C (white points) and clone N (black points); error bars represent standard error of the mean (n = 5)

lows originally derived from non-contaminated sites can even show a better growth and a larger metal accumulation than those derived from contaminated areas, when grown in metal polluted soils. An increased concentration of dissolved organic carbon in the rhizosphere together with increased concentrations of labile metals suggests the involvement of root and/or fungal exudates in the mobilization of contaminants. Further research is needed to investigate the role of exudates in the mobilization of metals in the rhizosphere of willows. A better understanding of relations between exudates and the mobilization of metals in the vicinity of roots might contribute to an increased phytoextraction potential of fast-growing and high-biomass woody plants. To conclude, due to the growth performance, heavy metal accumulation and mobilization, *S.* × *rubens* can be considered as a valuable plant for phytoextraction of metal-polluted soils.

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Corresponding author:

Prof. Walter W. Wenzel, University of Natural Resources and Applied Life Sciences, Department of Forest and Soil Sciences, Peter Jordan Strasse 82, A-1190 Vienna, Austria
phone: + 431 476 543 125, fax: + 431 476 543 130, e-mail: walter.wenzel@boku.ac.at