Influence of *Cadophora finlandica* and other microbial treatments on cadmium and zinc uptake in willows grown on polluted soil

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ABSTRACT

We conducted a pot experiment to evaluate the Cd and Zn accumulation in leaves and roots of *Salix smithiana* (BOKU-03DE-001) and *S. caprea* (BOKU-01AT-004) clones grown on a metal-contaminated soil as affected by native microbes extracted from the same experimental soil, and the fungus *Cadophora finlandica*. Plant biomass production of *S. smithiana* was decreased in all the treatments compared to the sterilized control. In contrast, *S. caprea* grew best on the non-sterilized soil. Similar effects were observed for plant Zn and Cd contents. Microbial treatments affected metal accumulation differently in the two *Salix* species. The effects of the microbial treatments on biomass and metal content of leaves were not related to the degree of mycorrhization. A comparison with literature data suggests that the plant response to microbial inoculation in terms of metal accumulation may depend on the plant-internal metal concentration. Our findings also illustrate a difficulty of successful rhizosphere management using metal-tolerant microbial isolates to further enhance the phytoextraction process.

Keywords: bacteria; metal; mycorrhiza; phytoextraction; tolerance; willow

The use of high-biomass willows that accumulate metals in shoot tissues appears to be a promising approach for phytoextraction of polluted soils (Eltrop et al. 1991, Robinson et al. 2000, Hammer et al. 2003, Pulford and Dickinson 2005). In previous pot experiments (Dos Santos Utmazian and Wenzel 2007) we identified *Salix smithiana* (BOKU 03 DE-001) and *S. caprea* (BOKU 01 AT-004) clones with excellent capacity to accumulate Cd and Zn in leaves. However, *S. smithiana* showed metal toxicity symptoms when grown on a highly polluted soil whereas *S. caprea* grew better and had a lower mortality rate. These results suggested that *S. smithiana* and *S. caprea* may have different resistance to high metal concentrations in soils.

Rhizosphere processes are involved in controlling metal bioavailability in soil and uptake by plants and could therefore play a key role in phytoremediation technologies (Wenzel et al. 2004). Natural associations between willow species and mycorrhizal fungi are well known (van der Heijden et al. 2001, Püttsepp et al. 2004, Trowbridge and Jumpponen 2004, Kahle et al. 2005), however, the effect of microbes on the metal uptake by willow and poplar species is still poorly understood (Vysloužilová et al. 2006). The number of published studies dealing with the effect of mycorrhization to willows on metal uptake in pot experiments (Enkhtuya et al. 2005, Sell et al. 2005, Baum et al. 2006) and field (Harris and Jurgensen 1977) is very

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limited. Mycorrhizal associations can enhance the metal availability (Davies et al. 2001), bind metals and limit their translocation to shoots (Brown and Wilkins 1985, Dehn and Schüepp 1989), and appear to partially protect plants against the toxicity of heavy metals (Leyval et al. 1997, Jentschke and Godbold 2000). Mycorrhiza are known to occur naturally and colonize plants grown in metal polluted soils (Harris and Jurgensen 1977, Hetrick et al. 1994, Galli et al. 1994, Weissenhorn et al. 1995, Sommer et al. 2002), suggesting tolerance of these fungi to high metal concentrations in soils. On the other hand, resistant bacterial isolates may also increase the metal uptake by plants due to an enhanced metal availability in soils (Whiting et al. 2001) and/or induce a positive effect on growth and plant development (Sizova et al. 2004).

Most metal tolerance tests with tree species grown in association with mycorrhizal fungi were performed with tree seedlings in *in-vitro* experiments (e.g., Adriaensen et al. 2004, Colpaert et al. 2004, Mleczko 2004). Results are still limited to a relatively small number of trees (*Pinus* sp., *Picea abies* and *Betula* sp.) and ectomycorrhizal fungal species (*Paxillus involutus*, *Pisolithus*, *Laccaria* spp., *Amanita muscaria*, *Suillus* sp. and *Scleroderma* spp.). To our knowledge, the only published pot experiments on microbial inoculation of a metal-accumulating, soil-grown willow species to study the effect on phytoextraction were done with *S. viminalis* (Sell et al. 2005), and very recently with *S.* × *dasyclados*, an hybrid *of S. caprea* × *S. cinerea* × *S. viminalis* (Baum et al. 2006). The study of Sell et al. (2005) showed enhanced metal transfer to leaves of *Populus canadensis* treated with the ectomycorrhizal fungus *Pisolithus tinctorius*, whereas none of the tested fungi affected metal accumulation in leaves of *S. viminalis* compared to the sterilized, non-treated control. Baum et al. (2006) reported that two isolates of the ectomycorrhizal fungus *Paxillus involutus* obtained from contaminated and non-contaminated sites affected the metal transfer from the soil to the host plant. The inoculation with the isolate from a contaminated site increased the phytoavailability of Cd, whereas the inoculation with both fungal strains increased the stem and root biomass, but had no effect on metal concentrations in the stems.

Here we report the results of a pot experiment aimed at evaluating metal tolerance and the accumulation of Cd and Zn in a *S. smithiana* and a *S. caprea* clone as affected by microbial treatments. The treatments include native microbes extracted from the experimental soil, the root-associated fungus *Cadophora finlandica* (Harrington and McNew 2003) and a combined microbial treatment. *Cadophora finlandica* can form ectomycorrhizal associations with *Picea rubens*, *Betula alleghaniensis* (Wilcox and Wang 1987), *S. viminalis*, *S. dasyclados* (Püttsepp et al. 2004), *P. tremula* and *S. caprea* (Sommer et al. 2002). *Cadophora finlandica* was isolated from a mycorrhizal root tip of an adult *Salix caprea* specimen growing natively in heavily metal-polluted soils from Arnoldstein (Austria). The isolation, genetic characterization and metal tolerance tests of the *C. finlandica* isolate used here are described.

MATERIAL AND METHODS

Isolation and characterization of *Cadophora finlandica*

For isolation of root-associated fungi from *S. caprea* from Arnoldstein, mycorrhizal root-tips were repeatedly washed in sterile distilled water + 0.1% Triton X-100. Washed root-tips were put on malt extract agar plates + 50 mg/l rose bengal + 30 mg/l streptomycin to suppress bacterial growth. Slow growing, non-sporulating fungi were chosen for further experiments. After three rounds of subculturing, 18 selected isolates were characterized by PCR-amplification and sequencing of the ITS-region with primers ITS1 and ITS4 (White et al. 1990). Two isolates (PRF14 Acc. Nr. DQ485203 and PRF15 Acc. Nr. DQ485204) showed 98% identity to *Phialophora finlandica* CBS444.86 (Acc. Nr. AF486119.1), which was later renamed to *Cadophora finlandica* CBS444.86 (Acc. Nr. AF486119.1) (Harrington and McNew 2003). The remaining isolates mainly belonged to the genera *Nectria* and *Verticillium*.

The resistance of the *C. finlandica* isolates PRF14 and PRF15 to Cd, Pb and Zn was tested on Malt extract agar (MEA). Metals were added as nitrate salts. Good growth was still observed at 200µM Cd, 6mM Pb and 1mM Zn. Although we could establish a close contact of the two *C. finlandica* isolates with willow roots, the mycorrhizal status of this association requires further work that is beyond the scope of the study presented here. As *C. finlandica* was shown to be a common root associated fungus in metal contaminated sites (Vrålstad et al. 2002), and in preliminary experiments the isolates PRF14 and 15 showed a positive effect on biomass production of *Salix* and *Populus* species in hydroponic cultures in the presence of increased

levels of heavy metals, *C. finlandica* PRF15 was chosen for the pot inoculation experiment. The *C. finlandica* isolate PRF15 was grown under sterile conditions on 500 ml perlite soaked with 250 ml Moser nutrient medium in a 1000-ml-Erlenmayer flask. Flasks were incubated for one month at room temperature without shaking, but regular agitation by hand for mixing was done. Perlite overgrown with fungal mycelium was directly used as an inoculum. Tolerance to heavy metals (Cd, Pb and Zn) of the isolate *C. finlandica* PRF15 was regularly tested under laboratory conditions on MEA and did not change during four years of subculturing.

Experimental soil and soil analysis

Soil material was collected from the A horizon of a Calcaric Cambisol near a former Zn and Pb smelter area in Arnoldstein (Carinthia, Austria). The mineral soil fraction (after removal of organic matter) contains (g/kg) 350 sand, 550 silt and 100 clay. The soil pH (H_2O) is 7.2 and the organic carbon content 24.6 g/kg, the C:N ratio 12.7. Due to emissions from the smelter, the soil contains (mg/kg) 32.7 Cd, 1760 Zn, 85.0 Cu, and 6560 Pb, measured after acid digestion ($HNO_3/HClO_4$). Chemical analyses followed standard procedures (Blum et al. 1996). Cadmium and Zn concentrations in soil were determined using the Flame Atomic Absorption Spectroscopy GF-AAS Perkin Elmer 2100. Chemical analyses were validated using reference materials. For the pot experiment, part of the soil material was sterilized by means of gamma-radiation (25.5 kGy/min).

Pot experiment

Field-moist soil was passed through a 2-mm sieve and homogenized. *Salix smithiana* clone BOKU 03 DE-001, a hybrid of *S. cinerea* × *S. viminalis,* was obtained from a nursery in Germany, and *S. caprea* clone BOKU 01 AT-004 came from a nursery in Austria. These clones were chosen as they showed unusually high accumulation of Cd and Zn in a previous pot experiment (Dos Santos Utmazian and Wenzel 2007). Non-sterilized willow cuttings were grown in perlite for approximately one month until sufficient roots and shoots of both clones were developed. Rooted cuttings with a shoot length of approximately 30 cm were planted in 8 kg pots filled with the experimental soil. Plants were kept randomly distributed in a greenhouse under controlled environmental conditions (Temperature: day 20°C, night 15°C, air moisture 60% and 16 hrs light period/day).

The following treatments were implemented: (a) sterilized control, (b) non-sterilized control, (c) *Cadophora finlandica*, (d) native soil microbes and (e) combined microbial treatment (*C. finlandica* + native microbes). All microbial treatments were applied to sterilized soil. Treatments (a) and (c) were replicated eight times, all other treatments were replicated five times. Plants were not fertilized during the experiment. To prevent a cross-contamination, the soil surface was covered with a black plastic sheet.

The mycorrhizal inoculum of *C. finlandica* isolate PRF15, overgrown in perlite as described before, was mixed with the soil (5 g/pot) before plants were transplanted. Both controls and the native soil microbes treatment received 5 g of sterilized inoculum per pot (Gazey et al. 1992).

The mix of native soil microbes was obtained from the experimental soil by extraction for 30 minutes using a soil-solution in the ratio of 1:10 (w/v). The soil slurry was then filtered through a nylon net (30 µm) to remove soil particles. The filtrate (50 ml/pot) was added directly to the roots when the willows were transplanted to the experimental soil. Other pots (both controls and *C. finlandica* treatment) received 50 ml of deionized water (Millipore ELIX 3 analytical grade, bacterial counts < 1 cfu/ml).

After a growth period of 11 weeks the degree of mycorrhization was assessed in each three replicates of the sterilized control and *C. finlandica* treatments. Root subsamples (1/3 of the total mass) were cleared and stained with blue ink (Vierheilig and Piche 1998) for the evaluation of the mycorrhizal colonization. The roots were examined in a compound microscope at × 100 magnification using a modification of the method of McGonigle et al. (1990). The degree of mycorrhization was calculated as the sum of roots with a typical fungal mantle and those showing colonization inside the root as a percentage of the total observed roots (no mycorrhiza + typical mantle + colonization inside + hyphae along the root). Because no mycorrhizal structures were observed at this harvest, the remaining plants, including the native microbial treatments and controls, were inoculated again as described above mixing the topsoil with the respective mycorrhizal and sterilized inoculum.

Root hair length was measured on at least 50 root segment intercepts at × 100 magnification. To

estimate the total root length, slides were made with root pieces preserved in ethanol. All root hair intersects with a grid line on one side of the root were counted at × 200 magnification. Total length of root was assessed using a line intercept method (Newman 1966). The proportion of the total length of root covered by root hairs was assessed by recording the absence or presence of root hairs on more than 100 root segment intercepts at × 25 magnification.

Plant harvest and analysis

After a growth period of 19 weeks, all plants were harvested and separated into roots, old shoots, new shoots and leaves. Plant samples were washed in an ultrasonic bath with de-ionised water. Roots were also exposed for five minutes to a 0.05M $CaCl_2$ solution in the ultrasonic bath to remove metals from the apparent free space of the root tissues. All plant tissues were dried to a constant weight at 80°C and the dry weight was recorded before they were ground in a metal-free mill (IKA®-WERKE MF 10). Aliquots (0.2 g) of ground root and leave material were digested using a mixture of HNO_3/$HClO_4$ in an open digestion system (Velp Scientifica DK Heating Digester).

Cadmium and Zn concentrations in digests were determined using the Flame Atomic Absorption Spectroscopy (GF-AAS Perkin Elmer 2100). Chemical analyses were validated by blanks and reference materials.

The leaf:root ratio was calculated for each treatment according to: metal concentration (mg/kg) in leaves/metal concentration root. The total metal content was calculated for each treatment by multiplying the dry weight of leaves (kg) by the metal concentration in leaves (mg/kg). Bioconcentration factors (BCF) were calculated according to: metal concentration in leaves (mg/kg)/total metal concentration in soil (mg/kg).

All data were statistically treated using a two-way analysis of variance (ANOVA) for each measured variable with the fixed factors "clone" and "treatments" and the interaction between them. If required, variables were transformed to obtain normal distribution of the residuals using square-root transformation (leaf biomass, total Zn leaf content and mycorrhization degree) or log-10-transformation (Cd in roots, root biomass, total Cd and Zn content in roots and Cd BCF). Treatment means were compared using the least significant difference test (LSD). Differences were considered as significant at $P < 0.05$. All statistics were computed using the SPSS (SPSS Program 12.0.1 © Inc., 1989–2003).

RESULTS AND DISCUSSION

Biomass production

Root biomass of *S. smithiana* and *S. caprea* was significantly ($P < 0.05$) decreased in all microbial treatments compared to the sterilized control, with no difference between the microbial treatments. Compared to the sterilized control, root biomass of both willows was significantly lower in the two treatments containing *C. finlandica* (Figure 1).

Several *S. smithiana* plants, independent of the treatment, showed some marks of intercostal stippling, tip and spot necroses along edges few days after the plants were transplanted to the polluted soil; it was already observed in a previous experiment (Dos Santos Utmazian and Wenzel 2007). This clone also developed symptoms similar to Fe chlorosis, maybe due to Zn (Bergmann 1993) and Pb toxicity and/or Cd and Zn translocation to the leaves (Vollenweider and Günthardt-Goerg 2005). *Salix caprea* showed an overall better vitality and less symptoms of Fe chlorosis throughout the experiment.

Metal concentrations and contents in leaves and roots, leaf:root ratio

Zinc concentrations in leaves and roots were similar for both willow species, whereas Cd concentrations in leaves of *S. caprea* were somewhat smaller than in *S. smithiana*. Cadmium concentrations in roots of both willows were significantly increased in the combined microbial treatment compared to the non-sterilized control and to the *C. finlandica* treatment. Compared to the sterilized control, we found significantly increased Cd and Zn concentrations in leaves of both willows in the combined microbial treatment. Zinc concentrations in roots were significantly increased in the combined microbial treatment compared to the non-sterilized control, sterilized and the native microbe treatment (Figure 2). Sterilization increased metal contents (mg/plant) in leaves of *S. smithiana* compared to the non-sterilized control but the opposite effect was observed for *S. caprea* (data not shown). As the metal concentrations in leaves were significantly increased only in the combined microbial treatment, the differences in metal con-

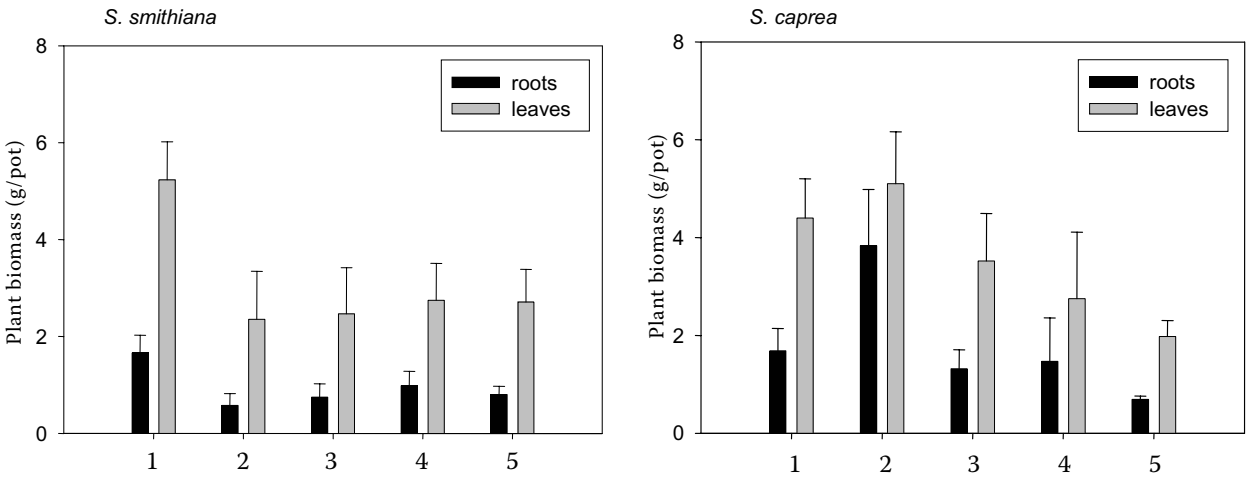

Figure 1. Shoots (leaves + twigs) and root biomass production (g/pot) of *S. smithiana* and *S. caprea*. Error bars represent standard error of the mean (n = 5)

x-axis: 1 – sterilized control, 2 – non-sterilized control, 3 – *Cadophora finlandica*, 4 – native microbes, 5 – *C. finlandica* + native microbes

tents are largely explained by the variation in biomass (Figure 1). The metal contents were generally smaller in the microbial treatments compared to the sterilized control, which can also be explained by the variation in biomass (Figure 1).

The leaf:root ratios of metal concentrations ranged from 1.41 to 2.84 for Cd and from 3.19 to 5.52 for Zn (Table 1). *Salix caprea* showed significantly enhanced translocation of Cd to leaves in the non-sterilized compared to the sterilized control.

Bioconcentration (BCF) factors varied between 8.13 and 13.6 for Cd and from 0.95 to 1.38 for Zn (Figure 2). The largest BCF for Zn was observed in the combined microbial treatments (1.31 for *S. smithiana* and 1.38 for *S. caprea*), with significant differences compared to the two control treatments. The largest metal BCF for Cd were found for *S. smithiana* in the combined microbial treatment (13.6) and for *S. caprea* in the native microbial treatment (10.5). We hypothesize that the presence of native microbes in the treatments showing the largest BCF for Cd indicates that bacterial exudates (e.g. siderophores) may have enhanced metal availability to the willows (Whiting et al. 2001, Abou-Shanab et al. 2003). However, this requires further investigations.

Root hair length and degree of mycorrhization

The root hair length varied between the willow clones and microbial treatments and was generally larger in the microbial treatments than in the sterilized controls (Table 1). The exception was *S. smithiana* in the microbial treatment. The specific root length (data not shown) was generally larger in the microbial treatments compared to the sterilized controls.

The mid-term sampling after 11 weeks showed no mycorrhizal associations in the sterilized controls and in the *C. finlandica* treatment. After 19 weeks, roots of all plants were colonized including the sterilized controls. The highest degree of mycorrhization was observed in the *C. finlandica* treatments for *S. smithiana* (25%) and *S. caprea* (21%), with significant difference compared to the two sterilized control and native microbe treatments. The lowest colonization (< 9%) was observed for *S. smithiana* in the sterilized control and in the native microbe treatment whereas for *S. caprea* it was found in the sterilized control and in the combined microbial treatment. Whether increased colonization rates are caused by mycorrhiza formation of *C. finlandica* PRF15 or by indirect promotion of mycorrhiza formation by other fungi is not known.

We found no relation between the degree of mycorrhization and the biomass or metal concentrations in the two investigated willows. Similarly, Sell et al. (2005) found no effect of ectomycorrhizal treatments on root and leaf biomass production of *S. viminalis* and *Populus canadensis* compared to the sterilized control. Takács et al. (2005) found no correlation between the root colonization (0 to 63%) with arbuscular mycorrhiza and biomass

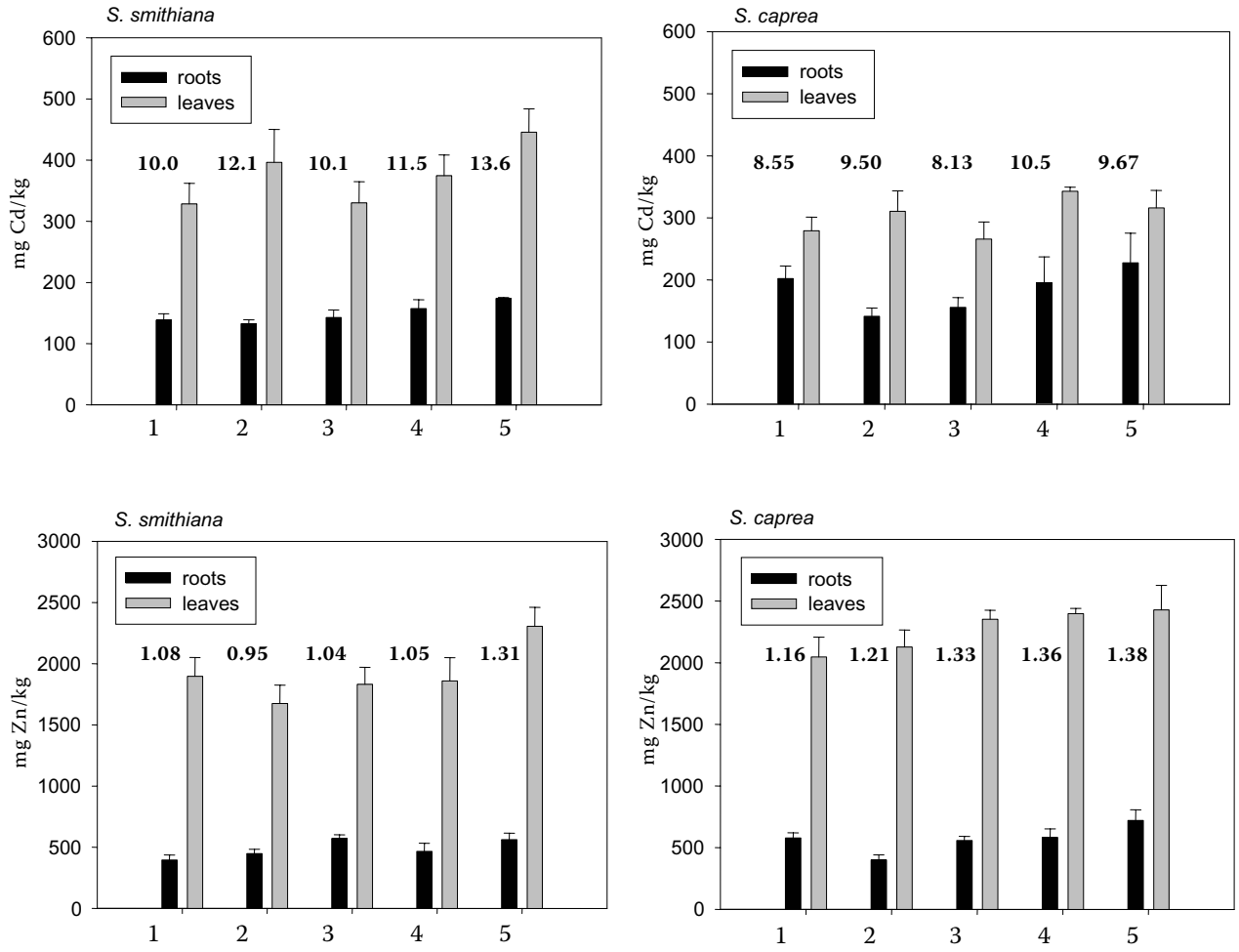

Figure 2. Concentrations of Cd and Zn (mg/kg) in dry plant tissues of *S. smithiana* and *S. caprea* after 19 weeks. Error bars represent standard error of the mean (n = 5). Numbers above bars represent the BCF: metal concentration in leaves (mg/kg)/total metal concentration in soil (mg/kg)

x-axis: 1 – sterilized control, 2 – non-sterilized control, 3 – *Cadophora finlandica*, 4 – native microbes, 5 – *C. finlandica* + native microbes

production in different poplar species grown on polluted and unpolluted soils. However, Baum et al. (2006) found an increase of shoot and root biomass production in *S. dasyclados* after inoculation with two isolates of *Paxilus involutus*, whereas our study showed a decrease of shoot and root biomass production of *S. smithiana* in the *C. finlandica* treatment. In the microbial treatments, we found no or only marginally increased metal concentrations in leaves of *S. caprea* and *S. smithiana* compared to the sterilized control. Sell et al. (2005) report no or marginally decreased Cd concentrations in leaves of *S. viminalis*, but enhanced Cd concentrations in leaves of *P. canadensis* in treatments with the ectomycorrhizal fungi *P. involutus* and *Pisolithus tinctorius*, compared to the sterilized control. Baum et al. (2006) found no significant effect of mycorrhizal treatment on metal concentration in leaves. It is interesting to note that in contrast to *P. canadensis* the above mentioned willows are all metal accumulator species, with the metal accumulation potential decreasing in the order *S. smithiana* > *S. caprea* > *S. dasyclados* (Baum et al. 2006) >> *S. viminalis* (Sell et al. 2005). Moreover, the study of Sell et al. (2005) was conducted on a soil with < 2 mg/kg total Cd, compared to > 30 mg Cd/kg in the experimental soil presented here. We hypothesize that the much larger internal metal concentrations present in the accumulator species, particularly when grown on highly contaminated soil, induce interactions with the inoculated microbial isolates or populations that lead to decreased metal transfer into the plant and to leaves. In our study, metal content in

Table 1. Translocation factors (TF = leaves: roots), root length per plant, and degree of mycorrhization of *S. smithiana* and *S. caprea* grown on a heavily polluted soil (Standard error, SE; n = 5)

Microbial treatment	Leaf:root				Root hair length		Mycorrhization	
	Cd	SE	Zn	SE	m/plant	SE	%	SE
S. smithiana								
Sterilized control	2.46	0.39	5.11	0.72	1343	441	8.82	4.04
Non-sterilized control	2.84	0.42	3.97	0.60	2255	763	16.0	6.48
Cadophora finlandica	2.48	0.43	3.19	0.14	1849	315	24.9	9.14
Native microbes	2.39	0.10	4.27	0.76	1273	150	7.00	4.19
C. finlandica + native microbes	2.57	0.26	4.35	0.77	2112	825	23.7	3.70
S. caprea								
Sterilized control	1.41	0.08	3.57	0.27	1239	268	8.94	2.73
Non-sterilized control	2.30	0.37	5.52	0.63	1739	510	11.7	2.65
Cadophora finlandica	1.74	0.17	4.28	0.30	2021	485	20.6	4.79
Native microbes	1.97	0.37	4.29	0.50	2005	248	11.6	2.86
C. finlandica + native microbes	1.43	0.27	3.43	0.22	1563	435	7.49	2.94

leaves was decreased through lower leaf biomass production in response to microbial treatments. However, metal uptake and transfer in willow or poplar clones with low metal accumulation potential could be enhanced by microbial treatments to previously sterilized soil, especially at lower contamination levels. This would be in line with the enhanced Cd accumulation in leaves of *P. canadensis* reported by Sell et al. (2005) and the hypothesis of Wilkins (1991) that in different circumstances mycorrhizal fungi are able either to increase or to decrease the supply of an ion to their higher-plant hosts. Also Ma et al. (2006) reported that arbuscular mycorrhiza infection stimulated uptake of metals by plants when soil metal concentrations were low, but decreased uptake when metal concentration were high. At relatively low plant-internal metal concentrations, some species may establish microbial associations in their rhizosphere that enhance the metal transfer into the plant.

This hypothesis needs further investigation, which should include also microbial treatments to non-sterilized soils to account for the real world conditions relevant for the application of microbial treatments in phytoextraction in the field.

The two closely related willow species used in our study responded differently to sterilization compared to the non-sterilized control. This illustrates the complex nature of interactions between plants and associated microorganisms in the rhizosphere even under controlled conditions and the potential limitations of direct management of rhizosphere microbes in phytoextraction under field conditions.

Further research is needed to study the influence of *C. finlandica* on metal uptake by plants as this fungus seems to be commonly associated with root in metal contaminated sites. Future experiments should also address microbial inoculation to metal-accumulating willow species in non-sterile soil to better link research results to real world phytoextraction technology.

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