REGULAR ARTICLE

Fungal community structure under goat willows (*Salix caprea* L.) growing at metal polluted site: the potential of screening in a model phytostabilisation study

**Marjana Regvar · Matevž Likar ·
Andrej Piltaver · Nives Kugonič · Jane E. Smith**

Abstract Goat willow (*Salix caprea* L.) was selected in a previous vegetation screening study as a potential candidate for the later-stage phytostabilisation efforts at a heavily metal polluted site in Slovenia. The aims of this study were to identify the fungi colonising roots of *S. caprea* along the gradient of vegetation succession and to estimate their colonisation levels in relation to metal pollution in order to reveal its mycorrhizal status at the site. Additionally the metal accumulation capacity of *S. caprea* and photosynthetic pigments were analysed as indications of its fitness at four differentially polluted plots. Despite high concentrations of leaf accumulated Cd, no significant differences in photosynthetic pigment concentrations were observed. The roots were colonised by arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (EM) fungi, and dark septate endophytes (DSE), with EM as the dominant type on all the plots. Molecular characterisation showed poor correlation of the root EM community with the above-ground sporocarp diversity. Members of Sordariaceae were the most frequent colonisers with an average colonisation of 21% of all root tips, followed by Thelephoraceae with 10%. DSE colonisation increased with increasing Pb concentrations and decreasing organic matter (OM).

M. Regvar (✉) · M. Likar
Department of Biology, Biotechnical Faculty,
University in Ljubljana,
Večna pot 111,
1000 Ljubljana, Slovenia
e-mail: marjana.regvar@bf.uni-lj.si

A. Piltaver
Institute for systematics of higher fungi,
Zofke Kvedrove 24,
1000 Ljubljana, Slovenia

N. Kugonič
ERICO Velenje,
Koroška 58,
SI-3322 Velenje, Slovenia

J. E. Smith
U.S. Department of Agriculture, Pacific Northwest
Research Station, Forestry Sciences Laboratory,
3200 SW Jefferson Way,
Corvallis, OR 97331, USA

Introduction

Representatives of *Salix* (willow) species are well known pioneer plants with high tolerance to excess heavy metal concentrations (van der Heijden 2001; Punshon and Dickinson 1997; Unterbrunner et al. 2007; Regvar et al. 2006). Once established, they help stabilize the soil surface, reduce soil wind and water erosion, while the demand of their perennial root

system for water decreases the risk of contaminant leaching (Sander and Ericsson 1998). Willows have been successfully used for the re-vegetation of heavily contaminated land (Dickinson 2000) and proved to be a useful part of a decontamination scheme of soils with increased Cd and Zn concentrations (Hammer et al. 2003; Unterbrunner et al. 2007). As such, willows are considered to be significant for future reforestation in phytoremediation efforts.

Toxicity of excess metals (Cd, Pb, Ni, Cu) affects plant metabolism, including synthesis of photosynthetic pigments due to metal binding to protein sulphydryl groups (Van Assche and Clijsters 1990), or by direct destruction of photosynthetic pigments through generation of highly active oxygen radicals (Pinto 2003). Resulting changes of photosynthetic properties can lead to severe physiological stress and changes in carbon allocation, with possible impact on below-ground fungal communities and their associations with plant roots (Smith and Read 2008).

Several studies have focused on benefits of mycorrhizal symbioses for the plant hosts growing on heavy metal enriched sites (Leyval et al. 1997; Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005), with the aim of possible biotechnological applications such as phytoremediation (Krupa and Kozdrój 2007). Willows are known to form mycorrhizal associations with both ectomycorrhizal (EM), and arbuscular mycorrhizal (AM) fungi (Kovács and Szigetvári 2002; Trowbridge and Jumpponen 2004; Cazares et al. 2005, Obase et al. 2007). Hosting different mycorrhizal types might be of functional importance for plant nutrition (Read and Perez-Moreno 2003) and has been shown to contribute to metal tolerance of host plants (Hartley et al. 1997; Vogel-Mikuš et al. 2006), as fungi can reduce the metal uptake by the plant by sequestration, extracellular precipitation and biosorption to the cell walls (Gadd 1993; Bellion et al. 2006).

In addition, several studies have focused on benefits of mycorrhizal symbioses for the plant hosts growing on heavy metal enriched sites (Leyval et al. 1997; Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005), with the aim of possible biotechnological applications such as phytoremediation (Krupa and Kozdrój 2007). Extremely polluted sites are reported to have lower rates of mycorrhizal colonisation, fewer fungal propagules and lower fungal species diversity (Gadd 1993; Hartley et al.

1997; Leyval et al. 1997; Markkola et al. 2002). Because of the selection pressure of metal toxicity EM genotypes develop on metal enriched soils (Hartley et al. 1997; Leyval et al. 1997; Markkola et al. 2002; Colpaert et al. 2004; Adriaensen et al. 2005) with the ability to alleviate the effects of heavy metal toxicity for themselves and their host trees (Adriaensen et al. 2005, 2006; Krznaric et al. 2009), by providing a more balanced access to mineral nutrition (Marschner and Dell 1994). Furthermore, literature data (Kayama et al. 2006) suggest the adaptation to heavy metal enriched soils may depend upon the interaction of specialised genotypes of both host trees and EM fungi.

Willows are also known to be colonised by dark septate endophytes (DSE) (Kovács and Szigetvári 2002; Trowbridge and Jumpponen 2004) that are ubiquitous root-associated and possibly symbiotic fungi (Jumpponen 2001). Their prevalence in extreme habitats and their alleged role in protecting plants under abiotic stress (Haselwandter and Read 1982; Mullen et al. 1998; Ruotsalainen 2003) make the studies on DSE even more compelling.

The objectives of the present study were (1) to evaluate metal accumulation capacity and photosynthetic pigments, as indicators of stress, in *Salix caprea* (goat willow) from the plots with differing metal levels, in order to confirm its physiological suitability for phytostabilisation, (2) to identify fungal symbionts colonising *S. caprea* along the gradient of vegetation succession, and (3) to establish the extent of colonisation with different groups of fungal symbionts in relation to metal levels, as data from such screening studies are an important step in identification of potential fungal symbionts for their application in phytoremediation efforts. For this, *S. caprea* shrubs at four plots with differing soil metal levels were deliberately selected at the increasing distances from the lead smelter as the main pollution source (aerial pollution) and examined for metal accumulation capacity and colonisation with various fungal partners.

Materials and methods

Site description

The study site is located in Žerjav (northern Slovenia; 46°8′ N, 14°51′ E), between 500 m and 700 m above

sea level, close to a lead smelter facility. A profound disturbance with severe erosion on the most heavily affected sites is the result of lead mining activities initiated in the 15th cent. Four plots (P1-P4) of ca. 300 m² (Table 1) were deliberately selected along the gradient of vegetation succession (Regvar et al. 2006). Earlier stages of the succession are dominated by herbaceous perennials: *Minuartia gerardii* (Willd.) Hayek, *Sesleria caerulea* (L.) Ard., *Calamagrostis epigeios* (L.) Roth, *Calamagrostis varia* (Schrad.), *Thlaspi praecox* Wulfen and *Thymus serpyllum* agg. In the later succession stages broadleaf species such as *Salix* spp., *Acer* spp. and *Betula pendula* Roth. are found more abundantly.

The underlying bedrock on the plots is composed mainly of Triassic limestone and metalliferous dolomite (calamine). The soils are stony of a rendzina type, polluted with lead (Pb), cadmium (Cd) and zinc (Zn).

Soil analyses

Soil and plant material for the analyses were collected in spring and fall 2000. Soil samples were collected according to a standard procedure (DIN ISO 10381-1) at the section of a concentric circle around the node of coordinates, on six points. The samples were homogenized, dried at 36°C, ground in a ceramic mill, and passed through a 2-mm plastic sieve. Samples were ground further prior to metal analyses in an agate mill (10 min) and passed through a 150-µm sieve. Micro-wave assisted wet digestion of the samples was performed in aqua regia. After filtration, the contents of Cd and Pb were determined using simultaneous multi-element atomic absorption spectrometry (Perkin-Elmer SIMAA 6000). A standard

reference material (NIST SRM 2711, Montana Soil) was used for analytical quality assurance. Soil organic matter content was determined by the Walkey-Black method of wet combustion (Nelson and Sommers 1982). Soil pH was measured in a soil: KCl (1M) (1:5) suspension.

Root fungal colonisation

Root samples were collected under each plant (5 plot⁻¹) app. 20–30 cm from the stem of the shrub by following the root from the base of the stem to the root with unbranching last root branch, to assure their provenance. Approximately 10 g of terminal roots were collected at the depth 5–20 cm below the soil surface.

Root samples of *S. caprea* were cleaned of soil debris and examined with stereomicroscopy (Leica, MZ8, Wetzlar, Germany). Ectomycorrhizal root tips were assigned to individual morphotypes (A–G), distinguished by differences in colour, size, and the type of ramification (Agerer 1996–2006). The colonisation levels were estimated on a 6×6-cm grid (36 squares plant⁻¹, five plants plot⁻¹) and expressed for each individual EM morphotype as a percentage of all examined root tips.

For the assessment of AM and DSE colonisation the roots were cleared by autoclaving in 10% KOH for 15 min (0.1 MPa, 121°C) and stained in 0.05% trypan blue for 40 min (Phillips and Hayman 1970). The frequencies of DSE and AM colonisation (F%) were estimated on the same 1-cm fragments (20 fragments plant⁻¹, five plants plot⁻¹) (Zeiss, KF2, Oberkochen, Germany) according to Trouvelot et al. (1986). Non-septate hyphae with vesicles and only occasional arbuscules were counted as AM, whereas

Table 1 Plot description and soil metal concentrations (Mean± SE, n=6). Different letters show statistically significant difference at $p<0.05$, ANOVA, Duncan's post hoc test

Plot	P1	P2	P3	P4
Distance to smelter	50 m	250 m	500 m	800 m
Vegetation	loose herbaceous cover with individual *Salix* shrubs	grassland, individual *Salix* shrubs	grassland, individual shrubs and trees	complete perennial cover and with upper canopy of shrubs and trees
Soil metals				
Cd (mg/kg)	91±1b	121±2.5a	46±0.4c	44±0.4c
Pb (mg/kg)	51,400±205a	28,133±393b	7,157±21d	10,353±102c

inter/intracellular melanised hyphae with microsclerotia were recorded as DSE.

Plant analyses

For determination of plant accumulated metals and photosynthetic pigments, fully developed leaves of five plants per each plot were collected randomly (five samples plant^{-1} around the whole tree canopy). Subsamples were taken from each sample for both, photosynthetic pigments and element analysis. Determination of metals accumulated in *S. caprea* roots was done on the root samples used for estimation of EM colonisation.

For metal analysis, plant samples were dissolved in concentrated HNO_3 in a microwave oven (CEM MSP 1000, Matthews, NC, USA), filtrated, and determined according to the same protocol as soil samples. For determination of photosynthetic pigments, leaves were immediately frozen in liquid nitrogen and extracted in 100% cold (4°C) acetone according to the method of Lichtenthaler (1987).

PCR amplification and RFLP genotyping

Sporocarps were collected on all plots for identification and DNA analysis. The sporocarps were freeze-dried (Christ, Alpha 2–4, Osterode am Harz, Germany) and deep-frozen (−70°C). A small piece of sporocarp tissue at the junction of the stem (stipe) and cap (pileus) was used for DNA extraction.

Two root tips of each EM morphotype detected on a root system of each individual plant (102 samples in total) and two samples from each species found as sporocarps were selected for DNA extraction. The ITS region of the nrRNA gene was amplified (with 70% success rate) for molecular characterisation using restriction fragment length polymorphism (RFLP) and sequencing. DNA extraction and RFLP protocols followed those of Gardes and Bruns (1993).

PCR conditions for ITS1F-ITS4 (White et al. 1990; Gardes and Bruns 1993) primer pairs were 1 min at 94°C, followed by 35 cycles of 35 s denaturation at 94°C, followed by 53 s annealing at 55°C, and 30 s of elongation at 72°C. Time of elongation step was increased for 5 s per cycle. A final extension was performed at 72°C for 10 min. The PCR reaction mixture (25 µl) contained: 2.5 µl 10× PCR buffer, 2.5 mM $MgCl_2$, 200 µM concentrations of each

nucleotide, 500 nM concentrations of each primer, 0.75 U of *Taq* polymerase (Promega, Madison, WI, USA), and 12.5 µl of 100-fold diluted template.

ITS-RFLP patterns were produced using restriction enzymes *Hinf*I and *Mbo*I (New England BioLabs, Beverly, MA, USA). If two root tips of the same morphotype yielded two different RFLP patterns, additional root tips (up to eight) were analysed in order to achieve reliable estimates of RFLP pattern diversity. After the analysis, colonisation levels were assigned to every RFLP pattern. Because the majority of fungal species from roots remained unknown, the ITS region was sequenced for phylogenetic analysis.

Sequencing and sequence analysis

Prior to sequencing, the amplicons were cleaned and ligated into pGEMT-Easy vector (Promega, Madison, WI, USA). The recombinant vector was used for transforming cells of *E. coli* JM109. The transformants were plated on LB agar plates containing 50 µg/ml of ampicilin and X-Gal/IPTG. Screening for recombinant cells was carried out with blue/white selection. Three clones of each amplicon were used for sequencing. Prior to cycle-sequencing reactions with T7/SP6 primers, the presence of inserts in the vectors was confirmed with colony PCR using T7 and SP6 primers. Sequences are available at the GenBank (accession numbers AY916066-AY916071 and AY916073-AY916077). The sequences were subjected to GenBank searches, using the default option of gapped-BLAST (Altschul et al. 1997) and aligned with the closest matches and additional representatives of the groups from the GenBank. Neighbor-joining analysis was performed using MEGA4 (Kumar et al. 2008). The robustness of the internal branches was assayed by bootstrap analysis (1,000 runs). After the analysis, colonisation levels were assigned to the corresponding fungal sequences.

Statistical analysis

Data were analysed with Statistica® (Statsoft, Inc.). One-way ANOVA with Duncan's post hoc test was used for evaluation of differences in measured soil and plant parameters as well as in colonisation levels of AM, DSE and EM among different plots.

Translocation factors (TF=C_{shoot}/C_{root}) were calculated to quantify root to shoot translocation and

bioaccumulation factors (BAF=C_{shoot}/C_{soil}) were calculated to quantify the accumulation of the individual metal relatively to the soil concentrations.

We used Mantel test and ordination to evaluate the relationships among colonisation levels, RFLP pattern and sporocarp data with plant EM community composition and available environmental factors (Cd, Pb, organic matter, pH). Mantel test was performed using Vegan v1.15 library in R v2.7.2 and Bray-Curtis distance matrices. Tests were run with 10,000 permutations.

To evaluate colonisation levels and RFLP data sets, we used an indirect gradient analysis. Community data were first ordinated using nonmetric multidimensional scaling (MDS) (Kruskal 1964a, b) in R v2.7.2 (metaMDS, 20 iterations) and then the relationship of data with environmental gradients was estimated using *evfit* function of Vegan library (1,000 permutations).

Results

Soil properties, metal accumulation and physiological properties of *S. caprea*

Higest soil Cd concentrations were found on P2, whereas Pb was most enriched on P1 and decreasing toward P4 (Table 1). Average pH of the soil at individual plots was 6.8±0.2, while organic matter increased with closing of vegetation cover from 3.2% at P1 to 25.2% at P4.

The highest concentrations of plant accumulated metals were measured at P2 (Table 2), with maximal concentrations of up to 101 and 147 mg kg^{-1} of Cd and 695 and 1,460 mg kg^{-1} of Pb, in the leaves and roots respectively. A relatively large portion (42–61%) of Pb and even larger portion (63–83%) of Cd was accumulated in the shoots. Photosynthetic pigments showed no statistically significant differences among the plots (Table 2).

Root fungal colonisation of *Salix caprea*

Microscopic examination of stained root segments of *Salix caprea* revealed presence of hyphae and distinct microsclerotia of dark septate endophytes (DSE), as well as vesicles and non-septate hyphae of AM fungi. AM colonisation levels (F%) ranged from 0 on P1 to 3.0%±1.6% on P2 representing an average of 1.0%± 0.4% within the total colonisation level (Table 2). The highest level of DSE colonisation was observed on P1 (20.4%±7.0%) whereas on plots P2-4 it did not vary significantly with less than 5% of all root segments colonised. EM colonisation levels with 44.5%±1.8% of colonised root tips on average represented the

Table 2 Plant accumulated metals, photosynthetic pigments and root fungal colonization (%) of S. caprea (Mean ±SE, *n*=5). Different letters show statistically significant differences at *p*< 0.05, ANOVA, Duncan's post hoc test

	P1	P2	P3	P4
Plant metals				
Cd leaf (mg/kg)	36.8±6.8b	63.3±11.5a	23.1±0.6b	30.3±3.6b
Cd root (mg/kg)	44.5±6.8b	85.6±15.7a	36.5±1.3b	42.2±6.4b
Cd BAF	0.40±0.07b	0.64±0.11b	0.50±0.01b	0.92±0.11a
Cd TF	0.83±0.09a	0.74±0.07ab	0.63±0.02b	0.75±0.11ab
Pb leaf (mg/kg)	159±14.5b	485±86.7a	26±6.3c	26.4±2.5c
Pb root (mg/kg)	376±57.5b	1112±156a	42.5±4.1c	48.8±5.7c
Pb BAF	0.003±0.0002b	0.02±0.003a	0.004±0.0008b	0.003±0.0003b
Pb TF	0.48±0.01	0.42±0.04	0.49±0.12	0.61±0.12
Photosynthetic pigments				
Chlorophyll a (mg/g)	0.21±0.03	0.22±0.004	0.15±0.01	0.26±0.04
total chlorophylls (mg/g)	0.32±0.04	0.33±0.06	0.23±0.01	0.38±0.06
total carotenoids (mg/g)	0.09±0.01	0.08±0.01	0.06±0.001	0.1±0.02
Root fungal colonisation				
AM (%)	0±0	3.0±1.6	1±0.5	0.03±0.01
DSE (%)	20.4±7.0a	4.5±1.6b	0.1±0.01b	0.7±0.7b
EM (%)	45.3±1.9a	36.2±0.4b	46.3±0.7a	50.4±4.0a

BAF bioaccumulation factor (BAF=C_{shoot}/C_{soil}), *TF* translocation factor (TF=C_{shoot}/C_{root})

highest portion of colonisation among the three fungal types.

Sporocarp EM community

Sporocarps of seven mycorrhizal species belonging to five different genera were recorded over a 2-year period (Table 3). *Hebeloma* species, e.g. *H. mesophaeum* (Pers) Quélet and *H. collariatum* Bruchet were the most abundant species, producing 113 (41% of the total) and 80 (29% of the total) sporocarps, respectively. Also, *H. mesophaeum* was the only species producing sporocarps on all four plots. *Inocybe* was the second most abundant sporocarp-producing genus (26% of the total), although its representatives were only found on P3. Other recorded species were: *Lactarius subdulcis* (Pers. ex Fr.) S.F. Gray, *Suillus bovinus* (Fr.) O. Kuntze, *Cortinarius decipiens* (Pers.: Fr.) Zaw that formed 5% of total sporocarps. On average 3–4 sporocarp forming species were found on plots 2–4 with the highest number of species on P3, whereas *H. mesophaeum* was the only species found on P1.

Analysis of relationship of sporocarp data with environmental factor or EM plant community composition, using Mantel test showed no statistically significant correlation.

Description of EM community on roots of *S. caprea*

Morphotyping resulted in seven distinct EM morphotypes (labelled A–G), which yielded 26 different RFLP patterns. Morphotypes B, C and G were only represented by 1–2 RFLP patterns, while other morphotypes showed greater genetic heterogeneity (4–7 RFLP patterns). A comparison of the ITS sequence restriction fragments of sporocarps and EM root tips resulted in two matches. Morphotype B showed similarity with the RFLP pattern of *Hebeloma mesophaeum* (Tricholomataceae), while one of the RFLP patterns of morphotype D was similar to that of *Cortinarius decipiens* (Cortinariaceae).

All fungal RFLP patterns exceeding total colonisation of roots above 1.5%, as well as the representatives of EM morphotypes G and C (less than 1.5% of all root tips) were sequenced. Sequencing yielded 11 fungal sequences (Table 4). Assuming that they share the same trophic strategy as their closest GenBank relatives, two of the ascomycete sequences with similarities to Leotiomycetes could be plant endophytes, whereas the other 9 sequences most likely represent EM fungi. The three most abundant fungal species (Table 4, AY916066-AY916068) colonizing *S. caprea* belonged to the ascomycete family Sordariaceae (Fig. 1a) and grouped within morphotype E. Other ascomycete taxa were represented by sequences from Sarcosomataceae (morphotype F) and ascomyceteous endophytes from Leotiomycetes (morphotypes A and G). Basidiomycete sequences belonged to Thelephoraceae (morphotype A and D) and Cortinariaceae (morphotype D) (Table 4, Fig. 1b). Unfortunately, no sequences from the morphotypes B and C were obtained.

Ascomycetes dominated the EM fungal community at the most degraded and exposed plots respectively (P1 and P3; Fig. 2a), while basidiomycetes formed the bulk of EM fungal species at the least polluted plot (P4; Fig. 2a). In general Sordariaceae colonised on average 40% of EM root tips (21% of all root tips), followed by Thelephoraceae with 22% (10%), Sarcosomataceae 5% (2.5%), genus *Hebeloma*/Tricholomataceae 4% (2%), and Cortinariaceae 4% (2%).

Table 3 Diversity of fungal sporocarps collected on selected plots at the polluted site. Numbers represent the actual number of observed sporocarps

Species	P1	P2	P3	P4
Cortinarius decipiens (Pers.: Fr.) Zaw		3		1
Hebeloma collariatum Bruchet		1	79	
Hebeloma mesopheum (Pers) Quélet	1	7	89	16
Inocybe dulcamara (Alb. & Schw. ex Pers.) Kummer			43	
Inocybe sp.			30	
Lactarius subdulcis (Pers. ex Fr.) S.F. Gray				1
Suillus bovinus (Fr.) O. Kuntze				8
Total	1	11	241	26

Table 4 Genbank accession numbers of fungal ITS sequences from the most abundant RFLP patterns on *Salix caprea* roots with nearest matches with corresponding *E* values and similarity percentages

| Fungal group	Tentative identification	Acc. No.	Nearest match		Morphotype
Ascomycota	Helotiales, Leotiomycetes	AY916069	DQ182427	Uncultured Helotiales (6e-125, 89%)	A
			AJ875369	Uncultured fungus (7e-95, 89%)	
	Unknown ascomycetes from Leotiomycetes	AY916070	DQ491510	Potebniamyces pyri (1e-118 79%)	G
			DQ317350	Thelebolaceae (3e-115, 89%)	
	Sordariaceae #1	AY916066	DQ447981	Uncult, Sordariaceae (3e-150, 95%)	E
			AB089817	Uncult, fungus (1e-118, 89%)	
	Sordariaceae #2	AY916068	DQ447981	Uncult, fungus (0.0, 88%)	E
			AB089817	Uncult, Sordariaceae (2e-167, 98%)	
	Sordariaceae #3	AY916067	AB089817	Uncult, Sordariaceae (3e-172, 93%)	E
			DQ447981	Uncult, fungus (3e-138, 90%)	
	Sarocosomataceae	AY916071	AY351623	Sarcosomataceous mycorrhiza (2e-64, 87%)	F
			AJ968676	EM isolate (Pezizales) (7e-64, 100%)	
Basidiomycota	*Tomentella lilacinogrisea*	AY916075	DQ068472	*Tomentella lilacinogrisea* (0.0, 99%)	A
			AY953422	*Tomentella* sp, (0.0, 98%)	
	Tomentella sp. #1	AY916073	DQ068472	*Tomentella lilacinogrisea* (4e-56, 91%)	A
			U83475	*Tomentella* sp, (4e-56, 91%)	
	Tomentella sp. #2	AY916077	AY635176	*Tomentella* sp, (0.0, 98%)	A
			AY635170	*Tomentella* sp, (0.0, 98%)	
	Thelephoraceae	AY916074	AJ893295	Thelephoraceae (0.0, 96%)	D
			AY656947	Uncult, thelephoraceous EM fungus (0.0, 93%)	
	Cortinarius sp.	AY916076	AY916076	Uncult, EM fungus (Cortinariaceae) (0.0, 100%)	D
			AY351630	Uncult, EM fungus (Cortinariaceae) (0.0, 100%)	

Unidentified RLFPs that represented 47% of all RLFP patterns colonised 17% of all EM root tips (8% of all root tips) (Fig. 2b).

Relations between environmental factors and colonisation

Mantel test showed significant positive correlation between colonisation levels and environmental variables ($R=0.47$, $p<0.0001$). The nonmetrical multidimensional scaling (MDS) returned two-dimensional ordinations for colonisation data (Stress = 6.07, nonmetric fit R^2 = 0.99; Fig. 3). Joint plots of the colonisation data with fitted environmental gradients showed positive correlation of DSE colonisation levels with increasing Pb concentrations and decreasing OM. EM and AM colonisation levels showed no correlation with environmental gradients. The indirect ordination analysis of RFLP data showed no statistically significant correlations with environmental gradients.

Discussion

Salix caprea was shown to accumulate high levels of Pb and Cd. The majority of Pb was immobilised in the roots of *S. caprea*, which is also common for other *Salix* species and other plant genera (Dahmani-Muller et al. 2000; Pulford and Watson 2003), indicating the less efficient internal transport of Pb from roots to shoots (Huang and Cunningham 1996). Cd showed higher mobility and reached leaves/roots ratio of 0.8 at the most polluted plot. Several studies have shown that willows can accumulate high levels of Cd (Landberg and Greger 1996; Sander and Ericsson 1998; Vandecasteele et al. 2002; Vandecasteele et al. 2005), although they are considered as good accumulators rather than hyperaccumulators of Cd (Reeves and Baker 2000). Still, their high biomass production could make willows useful in the removal of Cd from the soil. In our case of high soil metal concentrations, metal removal makes little sense; nevertheless the

Springer
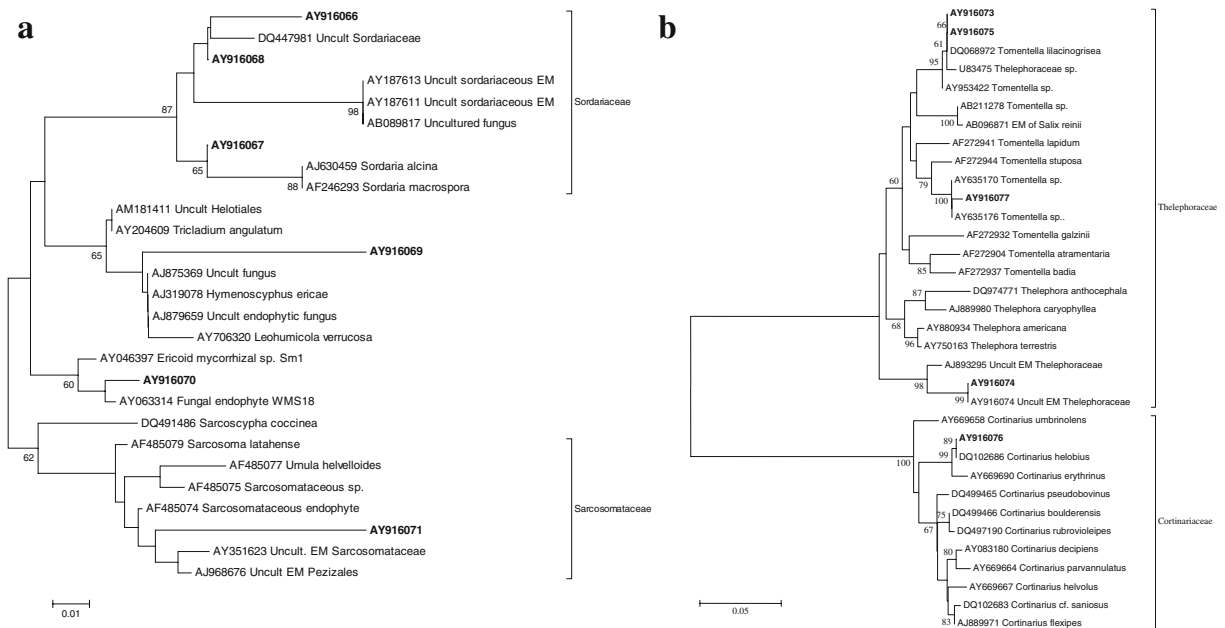
Fig. 1 Phylogenetic placement of **a** ascomycete and **b** basidiomycete fungi associated with roots of *Salix caprea* in a neighbour-joining tree. Analysis was performed by with 1,000 bootstrap replicates (values represent bootstrap results above 60%)

concentrations indicate the tolerance of *Salix* to the existing levels of pollution.

Despite the high levels of plant accumulated metals and several reports on the impact of heavy metals on

photosynthetic pigments (Van Assche and Clijsters 1990) we observed no differences in chlorophyll or carotenoid content among the willows growing at different levels of pollution, further confirming its
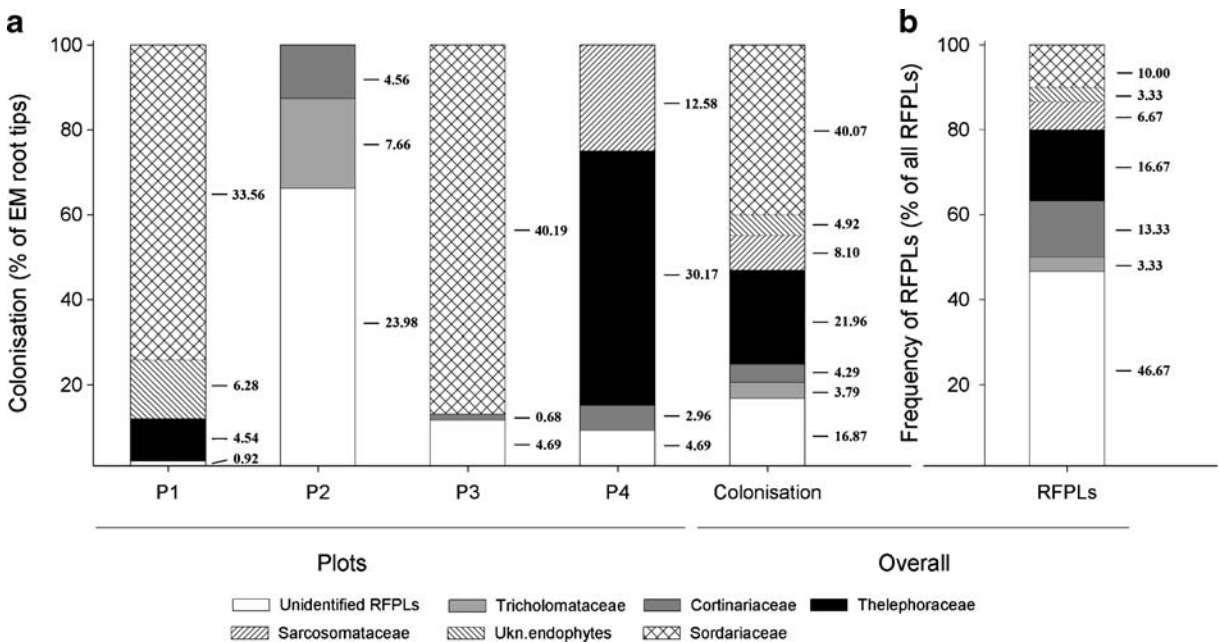
Fig. 2 Abundance of the main fungal groups on roots of *S. caprea*, expressed as **a** percentage of sampled ectomycorrhizal root tips: at the individual plot and as overall average, **b** as

percentage of total number of RFLP types observed. Data represents combined results for five *Salix caprea* shrubs
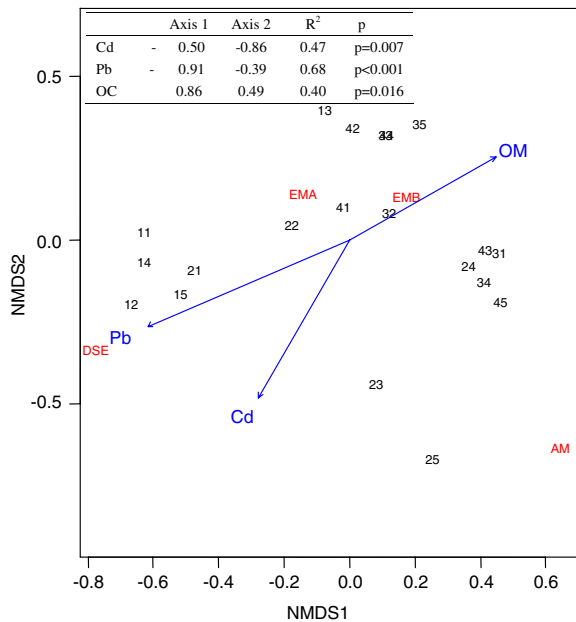
Fig. 3 Plot of nonmetric multidimensional scalling (MDS) ordinations for colonisation levels with fitted environmental gradients for soil Cd, Pb, organic matter and pH. Legend: Pb, Cd...total soil concentrations of metal, OM...organic matter. Legend: DSE...dark septate endophytes, AM...arbuscular mycorrhizal fungi, EMA...ascomycete EM fungi and EMB... basidiomycete EM fungi. Individual samples are represented by plot and plant number (ex. 13 = 3rd plant from plot 1). Only physicochemical factors with p values < 0.5 are plotted (correlations of the physicochemical factors with Axis 1 and Axis 2, their R^2 and p values are presented in the table)

physiological suitability for phytostabilisation of the site.

Distinct AM, DSE, and EM structures were found in association with roots of *S. caprea*. Examination of *S. caprea* root showed that on average 44% of root tips were colonised by EM fungi. DSE colonised 6% of examined root segments, while AM fungi were observed only on 1% of the examined root segments. Similar colonisation levels were reported also for other *Salix* species (van der Heijden and Vosatka 1999; Trowbridge and Jumpponen 2004: Cazares et al. 2005; Obase et al. 2007). AM colonisation may be favoured in natural environments where EM propagules are sparse or absent or at early establishment (Smith et al. 1998). Indeed, highest AM colonisation levels coincided with lowest EM colonisation levels. In our study, DSE colonisation increased with higher soil Pb content and lower organic matter content. Presence of melanin in the cell wall of DSE fungi could contribute to their heavy metal tolerance (Gadd 1993) and consequently

enhance their competitiveness and establishment in polluted environments, although the understanding of the ecological role of fungal melanin in DSE fungi is still lacking. Even though the nutritional role of DSE colonisation on roots is often unclear (Smith and Read 2008), enhanced host mineral nutrition was shown for *Phialocephala fortinii* on *Salix glauca* (Fernando and Currah 1996) indicating their presumable functional role at highly polluted sites. However, as no correlation between colonisation levels of *S. caprea* with different fungal groups and plant accumulated metal concentrations, bioaccumulation factors or translocation factors could be observed, the benefit of fungal colonisation in this extreme environment may be a result of functional importance of individual fungal species or an effect of improved nutrition and tolerance to stress factors other than heavy metal pollution. In addition, a more precise examination of the significance of fungal isolates on the growth of *S. caprea*, and their persistence on polluted sites over a longer period of time is needed for evaluation of the importance of different fungal colonisers on *S. caprea* fitness and survival at a polluted site.

In order to reveal the most frequent ectomycorrhizal colonisers, the EM root tips were sorted to morphotypes that were characterized with RFLP typing. Sequencing of the most frequent RFLP patterns from ectomycorrhizal root tips revealed asco- and basidiomycete taxa previously reported to form ectomycorrhizal associations with *Salix* species (Jumpponen et al. 2002; Trowbridge and Jumpponen 2004; Obase et al. 2007; Mühlmann and Peintner 2008). In all, 15 EM taxa were revealed on the roots of *S. caprea* studied at the polluted site, by combining the EM phenotyping and sporocarp identification. The members of Sordariaceae were the most frequent colonisers with an average colonisation of 21% of all root tips, followed by Thelephoraceae with 10%. A study from a primary succession site at the forefront of a receding glacier also reported Sordariaceae as dominant EM fungi of willows (Trowbridge and Jumpponen 2004). The presence of "sordariales" EM could be extremely important data as we have little knowledge on the EM of those ascomyceteous lineages. No statistically significant correlations of environmental gradients with RFLP data ordination could be found, which could be a result of the absence of DSE fungi among the sequenced fungal groups, as only EM root tips were used. Still, ascomycetes

dominated the EM fungal community at the most degraded and exposed plots, whereas basidiomycetes were more common at the least polluted plot, with highest organic matter content. Tomentelloid fungi formed the bulk of observed EM basidiomycetes. They form thin, resupinate sporocarps on the underside of dead plant and soil debris (Kõljalg 1996) and have been, due to this apparent association with organic matter, previously considered as saprotrophic fungi (Larsen 1968, 1974).

An additional group consisting of representatives from Helotiales, which includes root biotrophs and mycorrhizal species (Vralstad 2004) was observed. These ascomycetes could be intercellular endophytes or rhizoplane colonisers and their role for *S. caprea* deserves further attention. Besides DSE, ascomycete EM fungal taxa seem to be most numerous at the plots with high metal and low levels of organic matter and more sparse vegetation cover (e.g. early successional stages).

Conclusions

- Despite the high levels of plant accumulated metals we observed no differences in chlorophyll or carotenoid content among the willows growing at different levels of pollution, suggesting high tolerance of *S. caprea* to increased heavy metal pollution, thus making it suitable for phytoremediation efforts.
- Root colonisation of willows with three clearly distinct functional fungal groups may add to the adaptability of willows on the polluted sites.
- Members of Sordariaceae were the most frequent colonisers with an average colonisation of 21% of all root tips, followed by Thelephoraceae with 10%. Still, only DSE colonisation increased with higher levels of pollution and low levels of organic matter suggesting their potential functional role for the *S. caprea* growing at metal enriched soils and stressing the need for their further characterisation in screens of fungal symbionts with the potential application of phytoremedial studies.

References

Adriaensen K, Vralstad T, Noben JP, Vangronsveld J, Colpaert JV (2005) Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonizing Cu mine spoils. Appl Environ Microbiol 71:7279–7284

Adriaensen K, Vangronsveld J, Colpaert JV (2006) Zinc-tolerant *Suillus bovinus* improves growth of Zn-exposed *Pinus sylvestris* seedlings. Mycorrhiza 16:553–558

Agerer R (1996–2006) Colour atlas of ectomycorrhizae Germany, Einhorn Verlag, Schwaebisch Gmuend

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 254:173–181

Cazares E, Trappe JM, Jumpponen A (2005) Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. Mycorrhiza 15:405–416

Colpaert JV, Muller LAH, Lambaerts M, Adriaensen K, Vangronsveld J (2004) Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. New Phytol 162:549–559

Dahmani-Muller H, van Oort F, Gelie B, Balabane M (2000) Strategies of heavy metal uptake by three plant species growing near a metal smelter. Environ Pol 109:231–238

Dickinson NM (2000) Strategies for sustainable woodland on contaminated soils. Chemosphere 41:259–263

Fernando AA, Currah RS (1996) A comparative study of the effects of the root endophytes *Leptodontiudium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. Can J Bot 74:1071–1078

Gadd GM (1993) Tansley review No. 47. Interaction of fungi with toxic metals. New Phytol 124:25–60

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Molec Ecol 2:113–118

Hammer D, Kayser A, Keller C (2003) Phytoextraction of Cd and Zn with *Salix viminalis* in field trials. Soil Use Manage 19:187–192

Hartley J, Cairney JWG, Meharg AA (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant Soil 189:303–319

Haselwandter K, Read DJ (1982) The significance of a root-fungus association in two Carex species of high-alpine plant communities. Oecologia 52:352–354

Huang JW, Cunningham SD (1996) Lead phytoextraction: species variation in lead uptake and translocation. New Phytol 134:75–84

Jumpponen A (2001) Dark septate endophytes—are they mycorrhizal? Mycorrhiza 11:207–211

Jumpponen A, Trappe JM, Cázares E (2002) Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman Glacier (Washington, USA) in relation to time since deglaciation. Mycorrhiza 12:43–49

Kayama M, Choi D, Tobita H, Utsugi H, Kitao M, Maruyama Y, Nomura M, Koike T (2006) Comparison of growth characteristics and tolerance to serpentine soil of three ectomycorrhizal spruce seedlings in northern Japan. Trees Struct Funct 20:430–440

Kõljalg U (1996) On the systematics and phylogeny of Tomentella (Thelephorales, Basidiomycota) and related genera. - In: Abstracts of the First International Conference on Mycorrhizae. Berkeley, University of California, p 72

Kovacs GM, Szigetvari C (2002) Mycorrhizae and other root-associated fungal structures of plants of a sandy grassland on the Great Hungarian Plain. Phyton 42:199–210

Krupa P, Kozdroj J (2007) Ectomycorrhizal fungi and associated bacteria provide protection against heavy metals in inoculated pine (Pinus sylvestris L.) seedlings. Water Air Soil Pollut 182:83–90

Kruskal JB (1964a) Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika 29:1–27

Kruskal JB (1964b) Nonmetric multidimensional scaling: a numerical method. Psychometrika 29:115–129

Krznaric E, Verbruggen N, Wevers JHL, Carleer R, Vangronsveld J, Colpaert JV (2009) Cd-tolerant Suillus luteus: a fungal insurance for pines exposed to Cd. Environ Pol 157:1581–1588

Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinformatics 9:299–306

Landberg T, Greger M (1996) Differences in uptake and tolerance to heavy metals in Salix from unpolluted and polluted areas. Appl Geochem 11:175–180

Larsen MJ (1968) Tomentelloid fungi of North America. Technical Publication 93. State University College of Forestry at Syracuse University, New York

Larsen MJ (1974) A contribution to the taxonomy of the genus Tomentella. Mycol Mem 4:1–145

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7:139–153

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Markkola AM, Ahonen JU, Roitto M, Strommer R, Hyvarinen M (2002) Shift in ectomycorrhizal community composition in Scots pine (Pinus sylvestris L.) seedling roots as a response to nickel deposition and removal of lichen cover. Environ Pollut 120:797–803

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102

Mühlmann O, Peintner U (2008) Mycobionts of Salix herbacea on a glacier forefront in the Austrian Alps. Mycorrhiza 18:171–180

Mullen RB, Schmidt SK, Jaeger CH (1998) Nitrogen uptake during snow melt by the snow buttercup, Ranunculus adoneus. Arctic Alpine Res 30:121–125

Nelson DW, Sommers LE (1982) Total carbon, organic carbon, and organic matter. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil Analysis. Part 2. Agron Monogr 9. ASA and SSSA, Madison, pp 539–580

Obase K, Tamai Y, Yajima T, Miyamoto T (2007) Mycorrhizal associations in woody plant species at the Mt. Usu vocano, Japan. Mycorrhiza 17:209–241

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Pinto E (2003) Heavy metal-induced oxidative stress in algae. J Phycol 39:1008–1018

Pulford ID, Watson C (2003) Phytoremediation of heavy metal-contaminated land by trees—a review. Environ Int 29:529–540

Punshon T, Dickinson NM (1997) Acclimation of Salix to metal stress. New Phytol 137:303–314

Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems—a journey towards relevance? New Phytol 157:475–492

Reeves RD, Baker AJM (2000) Metal—accumulating plants. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals: using plants to clean up the environment. Willey, New York, pp 193–229

Regvar M, Vogel-Mikuš K, Kugonič N, Turk B, Batič F (2006) Vegetational and mycorrhizal successions at a metal polluted site-indications for the direction of photostabilisation? Environ Pollut 144:976–984

Ruotsalainen AL (2003) Mycorrhizal colonization and plant performance in arcto-alpine conditions. Ph.D thesis, Department of Biology, University of Oulu, Oulu, Finland, 43pp

Sander M-L, Ericsson T (1998) Vertical distribution of plant nutrients and heavy metals in Salix viminalis stems and their implications for sampling. Biomass Bioenerg 14:57–66

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Smith JE, Johnson KA, Cazares E (1998) Vesicular mycorrhizal colonization of seedlings of Pinaceae and Betulaceae after spore inoculation with Glomus intraradices. Mycorrhiza 7:279–285

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1986) Mesure de taux de mycorhization VA dun systeme radiculaire. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Recherche de methodes destimation ayant une signification fonctionnelle. Mycorrhizae: physiology and genetic. INRA, Paris, pp 216–222

Trowbridge J, Jumpponen A (2004) Fungal colonization of shrub willow roots at the forefront of a receding glacier. Mycorrhiza 14:283–293

Unterbrunner R, Puschenreiter M, Simmer P, Wieshammer G, Tlustoš P, Zupan M, Wenzel WW (2007) Heavy metal

accumulation in trees growing on contaminated sites in Central Europe. Environ Pol 148:107–114

Van Assche JA, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:195–206

van der Heijden EW (2001) Differential benefits of arbuscular mycorrhizal and ectomycorrhizal infection in *Salix repens*. Mycorrhiza 10:185–193

van der Heijden EW, Vosatka M (1999) Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. II. Mycorrhizal dynamics in interactions of ectomycorrhizal and arbuscular mycorrhizal fungi. Can J Bot 77:1833–1841

Vandecasteele B, De Vos B, Tack FMG (2002) Cadmium and zinc uptake by volunteer willow species and elder rooting in polluted dredged sediment disposal sites. Sci Total Environ 299:191–205

Vandecasteele B, Meers E, Vervaeke P, De Vos B, Quataert P, Tack FMG (2005) Growth and trace metal accumulation of two *Salix* clones on sediment-derived soils with increasing contamination levels. Chemosphere 58:995–1002

Vogel-Mikuš K, Pongrac P, Kump P, Nečemer M, Regvar M (2006) Colonisation of a Zn, Cd and Pb hyperaccumulator Thlaspi praecox Wulfen with indigenous arbuscular mycorrhizal fungal mixture induces changes in heavy metal and nutrient uptake. Environ Pollut 139:362–371

Vralstad T (2004) Are ericoid and ectomycorrhizal fungi part of a common guild? New Phytol 164:7–10

White TJ, Bruns T, Lee S, Taylor J (1990) Amplication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR - protocols and applications—a laboratory manual. Academic, London, pp 315–322