

Dissertation

Evaluation for a plant-assisted bioremediation approach:

Zn (Cd) accumulation properties of indigenous poplar species
and the impact of ectomycorrhizas on phytoextraction characteristics

submitted by

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1 Abstract

1.1 Abstract

Phytoremediation, a plant-based *in situ* approach, is considered an ecologically sensitive strategy to remediate metal affected soils. It may be assisted by ectomycorrhizas known to improve plant vitality on disturbed habitats and to alter metal accumulation.

This research aimed to characterize *Populus* species for use in a Zn(Cd) phytoextraction approach, to evaluate the impact of ectomycorrhizas on *P. tremula* growth and metal accumulation and to develop a synthesis protocol for *P. tremula* ectomycorrhizas under controlled conditions.

Experimental data reveal differential growth and metal accumulation of *P. canescens* and *P. tremula* mainly due to species specificities and their provenance from non-metalliferous/metalliferous habitats.

P. canescens growth was not affected by NH_4NO_3 -extractable soil Zn concentrations up to 60 mg kg^{-1} . Foliar Zn concentrations increased to just above $2000 \text{ mg Zn kg}^{-1}$. Toxicity threshold concentrations and relations between foliar and soil Zn concentrations suggest *P. canescens* suitable for phytoextraction processes on sites that contain up to $30 \text{ mg extractable Zn kg}^{-1}$.

P. tremula exceeded foliar Zn(Cd) concentrations and translocation factors of numerous *Salicaceae* species. In contrast, extracted Zn(Cd) contents were low due to restricted biomass yields. Studies on ectomycorrhizal *P. tremula* revealed retarded growth in mycorrhizal as compared to irradiated control treatments which may be explained by side effects of soil irradiation (Fe mobilization), the absence of microbial competitors and by an imbalance of the symbiosis at early growth stage. However, Zn translocation was inhibited at high soil Zn levels indicating barrier functions of the symbiosis. Thus mycorrhizal inoculation of *P. tremula* may be a promising strategy to enhance phytostabilization of metal-polluted sites.

The formation of ectomycorrhizas with *P. tremula in vitro* was primarily achieved by an improved nutrient composition added to the synthesis medium. It may be applied in non-sterile inoculation techniques that promise more vigorous mycorrhizal plants for use in revegetation and phytoremediation processes.

1.2 Zusammenfassung

Phytoextraktion, ein Verfahren zur Sanierung metallbelasteter Böden, beruht auf dem Einsatz von Pflanzen, welche Metalle in hoher Konzentration aufnehmen und in der oberirdischen Biomasse speichern.

Ziel der Studie war die Prüfung einheimischer *Populus*-Arten für die Phytoextraktion von Zn(Cd), die Beurteilung des Einflusses der Ektomykorrhiza auf Wachstum und Extraktionsverhalten von *P. tremula* und die Entwicklung eines Synthese-Protokolls für *P. tremula* mit Ectomykorrhiza-Pilzen unter kontrollierten Bedingungen.

Die Eignung von *P. canescens* und *P. tremula* für die Phytoextraktion wurde unterschiedlich beurteilt und liegt vor allem in artspezifischen Wachstumsmerkmalen und ihrer Herkunft (nicht belasteter/metallbelasteter Boden) begründet. *P. canescens* zeigte sich unempfindlich gegenüber extrahierbaren ($1\text{M NH}_4\text{NO}_3$) Boden-Zn-Konzentrationen bis 60 mg kg^{-1} und erreichte Blatt-Zn-Konzentrationen über 2000 mg kg^{-1} . Aufgrund errechneter Toxizitäts-Grenzwerte und der vorliegenden Beziehung zwischen Blatt- und Boden-Zn-Konzentration kann *P. canescens* für die Phytoextraktion empfohlen werden.

P. tremula übertraf die Blatt-Zn(Cd)-Konzentrationen sowie die Transferfaktoren vieler *Salicaceae*-Arten, während die Zn(Cd)-Gehalte aufgrund des schwachen Wachstums deutlich zurückblieben. Untersuchungen zur Ektomykorrhiza von *P. tremula* zeigten hingegen deutlich kräftigeres Wachstum in nicht-mykorrhizierten Kontrollen, basierend auf einem Ungleichgewicht der Symbiose in der ersten Wachstumsphase und der γ -Bestrahlung des Kontroll-Inokulums. Während mykorrhizierte Pflanzen deutlich höhere Blatt-Zn-Konzentrationen aufwiesen, wurde der Zn(Cd)-Transfer durch die Symbiose reduziert. Eine Mykorrhizierung von *P. tremula* mit Ektomykorrhiza-Pilzen ist daher vielversprechend für eine Phytostabilisation hochbelasteter Böden.

Die Synthese von *P. tremula* mit Ektomykorrhiza-Pilzen *in vitro* wurde durch eine ausgewogene Nährstoffzugabe erreicht. Diese kann auch unter veränderten Synthesebedingungen *in vivo* zugesetzt werden und verspricht kräftigere Pflanzen mit ausreichender Mykorrhizierung für eine mykorrhiza-gestützte Phytosanierung.

2 Introduction

2.1 Plant assisted bioremediation

Soil pollution by toxic levels of trace metals and metalloids (in the following referred to as metals) are of major environmental concern as they harm human health and restrict multifunctional soil use and fertility (Adriano 2001). High metal concentrations in soil may derive from geogenic sources, metalliferous parent material such as calamine ores (Cd, Pb, and Zn) or ultramafic rocks (Co, Cr, and Ni) (Baker et al. 2000). Widespread areas throughout the globe have been affected by anthropogenic inputs of metals such as atmospheric deposits and waste disposal from metal mining and processing industries (e.g. As, Cd, Cu, Pb, Zn) or the excessive use of sewage sludge and phosphate fertilizers (Cd) in agriculture.

Numerous strategies have been developed to remediate the affected soils or to restrict movement of contaminants into non-polluted areas. Phytoremediation, a plant-assisted *in situ* approach, is considered an ecologically sensitive strategy particularly maintaining biological properties and physical structure of the affected soils (Chaney et al. 1997; Khan et al. 2000; Wenzel 2009).

2.1.1 Definition

The plant assisted bioremediation (phytoremediation) approach has been defined as “the use of green or higher terrestrial plants to treat chemically or radioactively polluted soils” and comprises the main techniques: (1) phytostabilization, (2) phytoextraction, (3) phytovolatilization and (4) phytodegradation (Wenzel 2009).

Processes mainly discussed with respect to metal-affected areas are phytostabilization and phytoextraction. Both techniques require plants highly tolerant to a range of heavy metals and their excess concentration levels. But whereas phytostabilization (immobilization) aims at colonizing metal affected habitats to mechanically stabilize topsoil and to prevent movement of the contaminants into non-polluted areas via leaching or erosion, phytoextraction intends to remove detrimental metals from soil by means of metal accumulating plants that concentrate metal elements in the aerial, harvestable plant parts.

2.1.2 Plant species suitable for phytoextraction

So called metal hyperaccumulator plants have initially inspired scientists to develop non-destructive *in-situ* phytoremediation technologies (Baker et al. 2000; McGrath and Zhao 2003; Salt et al. 1998; Wenzel et al. 1999). Hyperaccumulators are capable of concentrating unusually high levels of metals such as As, Cd, Zn, Pb, and Ni in their shoots (Baker et al. 2000), and are defined as plants accumulating more than 100 times larger than background concentrations in normal plant species, corresponding to at least 100 mg kg⁻¹ dry weight Cd, 1000 mg kg⁻¹ dry-weight Cu, Co, Cr, Ni or Pb, or more than 10000 mg kg⁻¹ Zn and Mn in shoot tissues. Baker et al. (2000) further suggested to set the threshold for translocation factors (i.e., shoot/root concentration ratios) >1, reflecting the high plant-internal metal transfer from roots to shoots. Moreover the metal concentration in shoots of hyperaccumulator species should typically exceed the total metal concentration in the parent soil (bioconcentration factor >1).

Both in tropical and temperate climate zones more than 400 plant species are known to hyperaccumulate at least one metal element. Most hyperaccumulator species are biennials or short-lived perennial herbs, shrubs and less frequently small trees. Among the large number of taxa hosting metal hyperaccumulators, the most common hyperaccumulator species belong to the family of *Brassicaceae* including 48 *Alyssum* and 23 *Thlaspi* species (Baker et al. 2000).

However, slow growth, poor biomass production and shallow root penetration often limit their extraction potential. Thus plant species such as *Salix* spp. and *Populus* spp. are proposed more suitable for phytoextraction due to their high-yielding biomass production, the extensive root system and their intermediate metal accumulation properties (DosSantos-Utmazian 2006; Komarek et al. 2008; Meers et al. 2007; Pulford and Watson 2003; Robinson et al. 2000).

2.1.3 Heavy metal bioavailability

The phytoextraction approach presupposes the contaminants to be available for plant uptake. In the solid phase of soil, metal ions such as Zn and Cd are attracted to the negatively charged oxygen, hydroxyl-, carboxyl or phenolic groups of clay minerals and organic substances. In soil solution, they may be bound to colloids or found in complexes with inorganic anions and humic substances (Greger 2004). Metal fractions immediately bioavailable are free metal ions and dissolved labile metal complexes in soil solution. The solubility of metals in soil solution and their availability for plant uptake is mainly influenced by soil chemical properties such as pH, cation exchange capacity (CAC) and redox-potential. Moreover clay content and organic matter substantially affect bioavailability of metal ions (Greger 2004).

Apart from chemical soil characteristics, plants and microbial consortia substantially affect the bioavailability of the contaminants (Wenzel 2009) particularly by H^+ release or the extrusion of organic acids (Ahonen-Jonnarth et al. 2000) or phytochelatins (Prasad 2004). Metal availability may also be influenced by the microbial surface area such as with increasing growth of extra-radicle mycelium. Fungal structures offer numerous binding sites and affect chemical and physical soil properties beyond the rhizosphere (Gadd 1993). Moreover plants may differentially affect the metal equilibrium in soil due to their species/isolate specific metal uptake capacity, realized by physiological adaptation (Lasat et al. 1996) or modifications in root structure and morphology (Haines 2002; Vaculik et al. submitted; Whiting et al. 2000).

2.1.4 Measuring the bioavailable metal fraction in soil

Heavy metal pollution of soils is primarily characterized by total metal concentrations based on the analysis of soil digests. However the level of metal contamination of a site may preferentially be described by a measure of the available soil metal concentration (Pulford and Dickinson 2005). This is because metal accumulation by plants is frequently more closely correlated with extractable than with total soil metal concentrations (Madejón et al. 2004; Nolan et al. 2005). Of the various extractants used for the quantification of extractable soil metal pools, 1 M NH_4NO_3 solution has been widely used and was adopted as German norm (DIN-19730 1997; Prueß 1992). In terms of extraction efficiency, this method compares quite well with extractions using 0.1 M $NaNO_3$ or 0.01 M $CaCl_2$ at the same solution:soil ratio. Relationships between 1 M NH_4NO_3 -extractable metals and uptake in various vegetables and other plants have been established with some success (Gryschko et al. 2005).

2.2 *Salicaceae*

The *Salicaceae* family hosts an expanding number of genera since molecular identification of the genetic information has simplified relational affiliation. *Salix* and *Populus* are two of the initial genera assigned to this family. They host pioneer plants that tolerate a wide range of climatic and soil conditions (Hörandl et al. 2002; Worrell 1995). In silviculture they are mainly grown in short – rotation coppice systems. Their potential for use in energy-cropping processes has particularly brought them into the focus of power industry and regional policy. Moreover *Salix* and *Populus* species have attracted attention in the scientific community since they have been proposed suitable for the plant-assisted bioremediation approach. In addition the *Salicaceae* family hosts the first

model tree in plant biology (*Populus trichocarpa*) with its genome fully sequenced (Tuskan et al. 2006).

2.2.1 Metal (Zn, Cd) accumulation properties of *Salix* and *Populus* species

Heavy metal contaminated habitats in Central Europe host a variety of *Salix* species such as *S. caprea*, *S. purpurea*, and *S. fragilis* and the *Populus* species *P. tremula* and *P. nigra* that impressively demonstrate tolerance to excess levels of Zn, Cd and Pb (Unterbrunner et al. 2007). Metal accumulation properties do not attain defined values of hyperaccumulating plant species. However, individual adult plants have shown to accumulate Zn and Cd up to foliar concentrations of 4600 mg kg⁻¹ and 50 mg kg⁻¹ dry-weight respectively. Poplar trees grown near Aubry (France) have even shown to accumulate 200 mg Cd kg⁻¹ in leaf tissues (Robinson et al. 2000).

Foliar accumulation properties with Zn concentration values of 2000 and 2500 mg kg⁻¹ have been proved under experimental conditions, in both field trials and pot experiments (Castiglione et al. 2009; DosSantos-Utmazian et al. 2007). In pot cultures the amount of Cd concentrated in leaves was clearly enhanced showing 300 - 400 mg kg⁻¹ dry-weight (DosSantos-Utmazian et al. 2007).

Comparisons of several *Salix/Populus* species reveal considerable differences in the capacity to accumulate metals in plant organs and suggest a broad genetic variety within the *Salicaceae* family (Castiglione et al. 2009; DosSantos-Utmazian and Wenzel 2007; DosSantos-Utmazian et al. 2006; Puschenreiter et al. 2010). Recent studies also reveal that plant provenance from either metalliferous or non-metalliferous habitats may significantly affect the metal accumulation behavior (Puschenreiter et al. 2010).

2.2.2 Plant accumulation behavior as affected by soil metal concentration

Species and clones of the *Salicaceae* family have been shown to differ in their capacity to accumulate metals in their shoots. Apart from the genetic variability, and soil/climatic factors influencing growth, the amount of metal accumulated by a clone is most notably affected by the substrate metal concentration. It increases with increasing substrate metal concentrations, at least up to toxic concentrations which negatively affect plant growth. In contrast, the ratio between foliar and substrate metal concentrations generally decreases with increasing substrate concentrations (Unterbrunner et al. 2007). This ratio (termed accumulation factor = AF, or bioconcentration factor = BC) has been used by some authors to evaluate the extraction capacity of a plant (McGrath and Zhao 2003).

2.2.3. Plant responses to toxic levels of trace elements

Toxic levels of trace elements diversely affect plant development and viability.

Metals may form complexes with molecules in plant cells or replace essential elements thereby inhibiting/changing functional properties of molecules such as enzymes and proteins. High levels of metal supply may additionally cause oxidative stress in plant tissues induced by an imbalance of reactive oxygen species and anti-oxidative compounds (Shaw et al. 2004). On the cellular level toxic metal concentrations may particularly affect enzymes, proteins and genes related to metal transport and homeostasis, cell wall and cell metabolism, proper protein synthesis and defense responses (Cuypers et al. 2009).

On the physiological level plants may express (1) changes in mineral nutrition, (2) show interferences of metals with stomata conductivity and transpiration rates, and/or (3) express responses in photosynthesis, due to modifications of the photosystems PS I and PS II or altered CO₂ fixation (Cuypers et al. 2009; Shaw et al. 2004).

Visible symptoms that may be observed frequently are stunting, chlorosis and necrosis. High levels of Zn mainly affect root elongation and cause chlorosis particularly in young leaves. Cd toxicity is characterized by brown colored and stunted roots, reduced growth, chlorosis and necrosis of leaf tissues, reddish veins and petioles and purple coloration (Marschner 1995; Shaw et al. 2004)

2.2.4 Propagation methods

Salix and *Populus* species form remarkably small seeds with a short lifespan that may germinate within a few days, immediately after maturation and release from catkins (Bärtels 1989). Thus regeneration from seeds is scarce in the field and not common practice in tree nurseries. In the field *Salix* and *Populus* species mainly regenerate from root suckers. Tree nurseries preferentially propagate individual clones of *Salicaceae* species by means of woody- or green-cuttings. These cuttings may take new roots within few weeks and guarantee the genetic information of their mother plants.

Most *Populus* species such as *P. canadensis*, *P. alba*, *P. canescens* and *P. nigra* that have been tested for their metal tolerance and accumulation potential, have successfully been propagated by cuttings (DosSantos-Utmazian 2006; Sell et al. 2005). However, Bärtels (1989) refers to the low rooting capacity of the European *Populus* species *P. tremula*. Thus the European aspen may more likely be generated from seeds at the expense of low germination rates and slow development in the juvenile stage of growth.

2.3 Mycorrhiza

The mycorrhiza symbiosis is a mutualistic association between plant roots and specific soil fungi. It is particularly characterized by a bi-directional movement of nutrients whereby plants provide organic carbon in exchange for essential nutrients.

Mycorrhizal structures such as hyphae, vesicles and arbuscles have already been verified in fossil relics of the first land colonizing plants. Although these plants had no true root systems it has been suggested that fungal structures provided the uptake of essential nutrients and transport to the symbiotic host. Today around 80% of all land plants form symbioses with mycorrhizal fungi (Smith and Read 1997).

2.3.1 Classification of mycorrhizas

Functional mycorrhizal associations show a large variety of symbiotic structures such as hyphal cell ingrowth, hyphal coils and arbuscles, hyphal sheaths (mantle) and the Hartig net. These structures differ with their symbiotic partners and reflect the affiliation to plant and fungal genera, families and species. Based on these structures up to seven mycorrhiza association types have been recognized: (1) arbuscular-, (2) ecto-, (3) ectendo-, (4) arbutoid-, (5) monotropoid-, (6) ericoid-, (7) orchide-mycorrhiza (Brundrett et al. 1996; Smith and Read 1997).

The arbuscular mycorrhiza (AM) represents the most frequent type of mycorrhiza. It is characterized by extra-radicle hyphae, auxiliary bodies and spores produced in the mycorrhizosphere, hyphal growth within roots and the formation of arbuscles within root cortex cells. AM fungi may additionally form vesicles within the cortical cell layers (Brundrett et al. 1996).

The AM is formed by fungi of the phylum *Glomeromycota* in symbiosis with an enormous variety of vascular plants including most herbaceous plants, with primary importance for agrarian plant species.

Although AM may also be established by trees including species of the *Salicaceae* family its importance is mainly restricted to mineral soils (Smith and Read 1997) and the juvenile stage of plant growth. AM associations with individual *Populus* species are supposed to be replaced by ectomycorrhizas with time (Lingua G 2008, personal communication).

2.3.2 Ectomycorrhiza

Ectomycorrhizas (EcM or EM) are mainly formed by fungal species of the phylum *Basidiomycota*, and to less extent by fungi of the phyla *Ascomycota* and *Zygomycota* in symbiosis with angiosperm and gymnosperm host plants. Although ectomycorrhizas are established between numerous plant and fungal species, functional associations are highly plant and fungus specific even on the species/isolate level and their individual combinations. The mycorrhiza formation particularly depends on mechanisms of recognition and compatibility and the inoculum potential of the symbiotic fungi (Smith and Read 1997).

The ectomycorrhizal symbiosis is characterized by (1) extra-radical hyphae that proliferate within and beyond the rhizosphere, (2) the hyphal sheath (mantle) and (3) the Hartig net, hyphal root ingrowths, which form an inter-cellular hyphal net between cells of the rhizodermis and possibly penetrate between cells of the root cortex (Figures 1 and 2). The Hartig net substantially increases the contact area between the symbiotic partners and constitutes the main site of nutrient exchange (Brundrett et al. 1996).

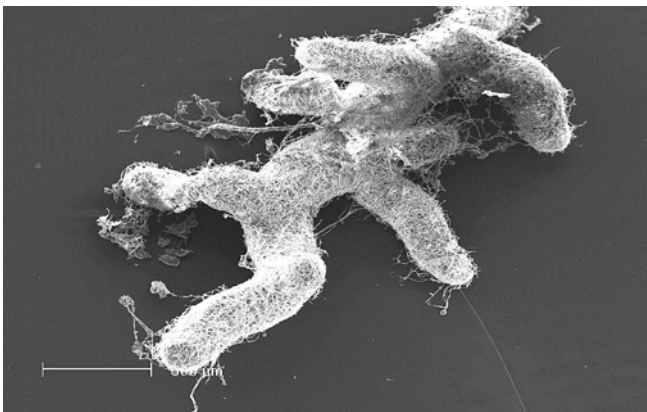


Figure 1 Root tips of *Populus tremula* in symbiosis with ectomycorrhizal fungi viewed with an electron microscope (bar: 500μm).

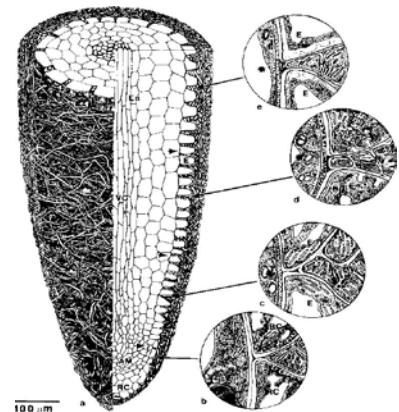


Figure 2 Anatomy of the ectomycorrhizal symbiosis (Smith and Read 1997).

The EM symbiosis may further be characterized by extra-radical hyphal strands and rhizomorphs produced in soil that may substantially differ in their structure and morphology due to the symbiotic partners, and by cystidia, spherical or clubbed structures formed on the surface of the hyphal sheath. Whether fungi penetrate the first layer of epidermal cells (rhizodermis) or additional layers of the root cortex may indicate plant affiliation to angiosperm or gymnosperm plant species. The

symbiotic partners may also differ in the structure of the Hartig net, either completely or partially enveloping plant cells (Brundrett et al. 1996).

Characteristics of the mycorrhizal root tips such as color, shape and ramification, as well as the surface structure of the hyphal sheath (Figure 3) may indicate specific host-symbiont associations and are utilized for the morphotyping approach, a non-molecular and classic method to identify the symbiotic partners (Agerer 1990; 2001).



Figure 3 *Populus tremula* ectomycorrhizae showing differences in color, shape, ramification and surface texture.

2.3.3 Function of ectomycorrhizas

The mycorrhiza symbiosis is regarded as a mutualistic association between dissimilar organisms providing benefits for both partners.

Ectomycorrhizal fungi are thought to have at least limited ability to use lignin or cellulose as carbon source. However, main requirements for organic carbon are met by photosynthetic products delivered by their hosts. The fungal symbiont readily utilizes the monosaccharide glucose followed by fructose whereas sucrose disaccharides have to be hydrolysed in plant cell walls involved in the symbiotic structures (Smith and Read 1997).

The mycorrhiza association constitutes a considerable sink for carbon allocation. This has been shown inter alia for *Salix viminalis* that significantly increases carbon allocation belowground with ectomycorrhiza symbiosis (Jones et al. 1991). However, the demand for organic carbon may show seasonal shifts and differences with the stage of mycorrhizal formation and development.

In exchange for organic carbon, mycorrhizal fungi deliver nutrients to their hosts. Extra-radical hyphae are supposed to exploit nutrients via increase of the surface area for nutrient uptake most notably beyond the rhizosphere and nutrient depletion zones. The element most relevant for the symbiotic exchange is the macronutrient phosphorus, a most prominent constituent of

macromolecular structures such as nucleic acids in DNA molecules and phospholipids in bio-membranes (Marschner 1995).

Numerous studies have shown that mycorrhiza colonization may particularly enhance P concentrations in leaf tissues. Moreover mycorrhizas are supposed to enhance shoot and root growth (on the dry-weight basis) and decrease the root-shoot weight-ratios, enhance the photosynthetic rate, ameliorate water deficiency (Smith and Read 1997) and alter biotic stress responses (Carlsen et al. 2008; Liu et al. 2007).

However, mycorrhizal associations do not invariably improve plant growth and development. Ectomycorrhizas have repeatedly shown decreased plant biomass production particularly in the early stage of growth. Moreover plant and fungus species/isolates and their combinations may also affect the mutualistic balance (Smith and Read 1997). Johnson et al. (1997) stress individual factors such as (1) genetic characteristics of the involved organisms, (2) their developmental stage and (3) environmental stresses relevant for a balanced mutualistic symbiosis. Thus the mycorrhizal symbiosis appears to move along a mutualism-parasitism continuum related to a cost:benefit balance of the plant-fungus association.

2.3.4 Ectomycorrhiza role in phytoremediation

Soil micro-organisms are generally known to affect rhizosphere processes fundamental for the plant assisted remediation processes (Wenzel 2009). Ectomycorrhizal fungi colonizing tree roots are particularly proposed to affect metal bioavailability (Leyval et al. 1993). Ectomycorrhiza has been demonstrated to enhance plant growth and vitality on metal disturbed habitats and to ameliorate detrimental impacts of excess metal supply, affecting plant metal uptake and the accumulation in plant tissues (Jentschke and Godbold 2000; Leyval et al. 1997). Jentschke and Godbold (2000) compiled existing information on some general strategies such as alterations of the adsorptive surface area, modified mobility in the root apoplast due to fungal ingrowth, changes in the hormonal metabolism or the release of chelating agents. Khan et al. (2000) particularly emphasized barrier properties of ectomycorrhizal structures together with metabolic processes crucial for mycorrhiza-driven amelioration of metal toxicity. Thus mycorrhizas may constitute a valuable and integral part of plant assisted bioremediation processes.

However the impact of mycorrhizal formation on plant growth and metal uptake cannot be generalized as shown by differential responses of *Pinus*, *Abies*, *Salix* or *Populus* species (Baum et al. 2006; DosSantos-Utmazian et al. 2007; Godbold et al. 1998; Sell et al. 2005). Moreover plant responses have been demonstrated to vary with the fungal species/isolates. This may reflect fungal

properties such as metal tolerance and/or specificities in functional compatibility with their symbiotic host (Baum et al. 2006; Colpaert and VanAssche 1992; Lingua et al. 2008; Sell et al. 2005). In addition metal- affected responses of the symbiotic association may be influenced by the bioavailable metal concentration (Adriaensen et al. 2003; Bücking and Heyser 1994; Colpaert and VanAssche 1992).

3 Objectives

The aims of the presented study were to

1. characterize the potential of the indigenous European poplar species *Populus tremula* (European aspen) and *Populus canescens* (*P. tremula* x *P. alba*, Gray poplar) for phytoextraction of Zn;
2. determine the Zn phytotoxicity thresholds;
3. establish the relationship between accumulation factors (ratio between foliar and soil metal concentrations) and extractable soil Zn concentrations;
4. determine the effect of a fungal community from a metalliferous soil and naturally forming mycorrhizas with *P. tremula* on plant growth and metal accumulation under variable Zn bioavailability;
5. develop an EM inoculation protocol specifically suitable for *P. tremula*;
6. confirm the suitability of an improved medium composition for EM formation with *Laccaria*, *Hebeloma*, and *Paxillus* isolates.

To this end, the European poplar species *P. canescens* and *P. tremula* were examined in a series of dose response studies. Effects of increasing substrate Zn concentrations on growth and Zn (Cd) uptake were quantitatively described.

Green-cuttings of the European poplar species *P. canescens* were grown in two pot experiments using soil with low native Zn content. Soil portions were spiked with Zn salt solutions resulting in 15 and 20 levels of soil Zn addition, ranging from 0 to 100 mg Zn added kg⁻¹ soil and from 0 to 2500 mg Zn kg⁻¹ respectively. Final data were also used to calculate the phytotoxicity threshold, as well as to establish the relationship between accumulation factors and extractable soil Zn concentration.

Seedlings of *P. tremula* were grown in a soil substrate containing both non-contaminated agricultural soil and metalliferous soil from an aspen stand containing the inherent mycorrhizal community of *P. tremula*. Substrate portions were additionally spiked with increasing amounts of Zn₂SO₄ (0, 10, 20, 40, 80 mg Zn addition kg⁻¹ soil substrate). For non-mycorrhizal controls substrate was γ -irradiated to eliminate living microbial propagules. Experimental results characterized the Zn and Cd accumulation potential of *P. tremula* indigenous to a metal contaminated habitat and

revealed effects of an ectomycorrhizal community naturally forming mycorrhizas with *P. tremula*, on plant growth and metal accumulation under variable Zn additions.

In a final approach pre-grown *P. tremula* seedlings were transferred to various nutrient media and inoculated with *Paxillus involutus* isolates using modified sandwich techniques. Mycorrhiza formation was evaluated macroscopically and further confirmed by microscopic examination of semi-thin sections for anatomical features of the mantle and the Hartig net. In a follow-up experiment, the adapted media formulation was tested for successful ectomycorrhiza formation by various *Laccaria* and *Hebeloma* isolates in association with *P. tremula*.

4 Publications

- 4.1 Zinc accumulation potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone in a dose-response study.**

Langer I, Krpata D, Fitz W J, Wenzel W W and Schweiger P F 2009

Environmental Pollution 157, 2871-2877.



Zinc accumulation potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone in a dose–response study

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Quantitative information about the concentration-dependent Zn accumulation of *Populus canescens* contributes to assess its suitability for phytoremediation.

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ABSTRACT

The effect of increasing soil Zn concentrations on growth and Zn tissue concentrations of a metal-accumulating aspen clone was examined in a dose–response study. Plants were grown in a soil with a low native Zn content which was spiked with Zn salt solutions and subsequently aged. Plant growth was not affected by NH_4NO_3 -extractable soil Zn concentrations up to $60 \mu\text{g Zn g}^{-1}$ soil, but it was completely inhibited at extractable concentrations above $90 \mu\text{g Zn g}^{-1}$ soil. From these data an effective concentration of $68.5 \mu\text{g extractable Zn g}^{-1}$ soil was calculated at which plant growth was reduced by 50%. The obtained information on toxicity threshold concentrations, and the relation between plant Zn accumulation and extractable soil Zn concentrations may be used to assess the suitability of the investigated *Populus canescens* clone for various phytoremediation strategies. The potential risk of metal transfer into food webs associated with *P. canescens* stands on Zn-polluted sites may also be estimated.

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1. Introduction

Contamination of soils with various metals poses considerable risks to the environment including human health. Various strategies have been developed to minimize the negative impact of soil metal contamination on the environment. One of these strategies is phytoremediation, a generally *in situ*, low-input and ecologically sensitive technique (Chaney et al., 2007). In phytoremediation of metal-contaminated soils plants are used either to remove metals from the soil (phytoextraction) or to prevent their further spread via erosion or leaching (phytostabilization). The ability of the chosen plants to tolerate the type and level of contamination is a prerequisite for successful phytoremediation. An additional plant attribute required for phytoextraction is the capacity of the plants to take up large amounts of metals into their harvestable above-ground biomass. This can either be achieved by hyperaccumulators (McGrath et al., 2006) or high-yielding plants that accumulate metals to moderate amounts (Greger and Landberg, 1999). Fast-growing trees of the family Salicaceae (willows (*Salix* spp.) and poplars (*Populus* spp.)) fulfil the latter criterion and have therefore

been considered suitable for phytoextraction (Pulford and Dickinson, 2005). Their large root biomass also contributes to physically stabilize the substrate.

Species and clones of willows and poplars differ in their capacity to accumulate metals in their shoot tissues (Klang-Westin and Eriksson, 2003; Laureysens et al., 2004). Apart from soil and climatic factors influencing growth, the amount of metal accumulated by a specific clone is most notably affected by the substrate metal concentration. It increases with increasing substrate metal concentrations, at least up to toxic concentrations which negatively affect plant growth. In contrast, the ratio between foliar and substrate metal concentrations generally decreases with increasing substrate concentrations (Unterbrunner et al., 2007). This ratio (sometimes termed accumulation factor = AF or bioconcentration factor) has been used by some authors to evaluate the extraction capacity of a plant (McGrath and Zhao, 2003). In spite of this direct dependence of AFs on soil metal concentrations, only scant quantitative information is available on the relationship between substrate metal concentrations and AF of individual tree clones considered suitable for phytoremediation (Shanahan et al., 2007). Similarly, only little information is available on metal toxicity thresholds for appropriate trees.

Native zinc (Zn) concentrations in soils are generally low. At these concentrations Zn is an essential element for plant

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metabolism and growth (Marschner, 1995). However, human activities such as mining operations have resulted in numerous sites contaminated with Zn to concentrations potentially harmful to the environment. Contaminations of these sites are variable with respect to Zn concentration, Zn availability and other metal pollutants present. These are key factors on which the appropriate techniques for rehabilitation, restoration and remediation of the contaminated land are decided on (Ernst, 2005; Van Nevel et al., 2007). For the assessment of the feasibility of phytoextraction for a specific site, prospective plant growth and metal uptake needs to be put in relation to the soil metal concentration at the site (Maxted et al., 2007; Van Nevel et al., 2007). The scant information on growth and metal uptake by trees suitable for phytoremediation across a wide range of soil metal availabilities however contributes to our currently limited ability to predict the feasibility of phytoremediation technologies.

An additional aspect that needs to be considered in phytoremediation are the potential risks it may pose to the environment. Several risks have been identified (Angle and Linacre, 2005) of which the following three were considered the most important for phytoextraction (Van Nevel et al., 2007): (i) metal dispersal into adjacent environments, (ii) metal accumulation in topsoil and (iii) harmful effects of metals on herbivores. The severity of these risks is mainly determined by foliar metal concentrations. In a comparative field study, redistribution of Zn and Cd from deeper soil layers to the topsoil was thus only observed under a metal-accumulating poplar clone (Mertens et al., 2007). In combination with an observed acidifying effect of the poplar litter, this clone was concluded undesirable for planting on contaminated sites due to a potential risk of metal transport into the food web (Mertens et al., 2007). Information on expected metal concentrations in the foliage is therefore necessary for a proper assessment of the potential risk of phytoextraction.

The level of metal contamination of a site is preferentially described by a measure of the available soil metal concentration (Pulford and Dickinson, 2005). This is because metal accumulation by plants is frequently more closely correlated with extractable than with total soil metal concentrations (Nolan et al., 2005; Madejón et al., 2004). Of the various extractants used for the quantification of extractable soil metal pools (Young et al., 2000), 1 M NH_4NO_3 solution has been widely used and was adopted as German norm (Prüß, 1992; DIN 19730, 1997). In terms of extraction efficiency, this method compares quite well with extractions using 0.1 M NaNO_3 or 0.01 M CaCl_2 at the same solution:soil ratio (Wenzel and Blum, 1997; Pueyo et al., 2004). Relationships between 1 M NH_4NO_3 -extractable metals and uptake in various vegetables and other plants have been established with some success (Gryschko et al., 2005).

The aim of the present study was (i) to establish the relationship between AF and extractable soil Zn concentration and (ii) to determine the Zn phytotoxicity threshold for a poplar clone potentially suitable for phytoremediation. To this end, the effect of increasing substrate Zn concentrations on growth and Zn uptake of a clone of European aspen known to accumulate considerable amounts of Zn was quantitatively described. Plants were grown in spiked and subsequently aged soil. Data were also used to calculate phytoextraction efficiency.

2. Material and methods

2.1. Plant material

The plant used in the current study was the *Populus canescens* Sm. (*Populus alba* × *Populus tremula*) clone BOKU 01 AT-001, which was found to accumulate large amounts of zinc and cadmium in the above-ground biomass (DosSantos-Utmazian and Wenzel, 2007; there referred to as a *P. tremula*). Green-cuttings of 12 cm length taken from annual foliate shoots of greenhouse-grown trees were rooted in a mixture of sand and perlite continuously soaked with a dilute (0.2%) commercial nutrient solution (Wuxal topN; 0.05 g Zn kg^{-1}). After 7 weeks of growth under

controlled conditions (24 °C, 12 h supplemented light, ≥90% air humidity), rooted plantlets of relatively uniform size were transplanted into the experimental pots.

2.2. Soil

The experimental soil was a relictic B horizon rich in iron (red loam) buried 2 m below the surface of a forest Cambisol collected near Lockenhaus in the province of Burgenland/Austria. It was chosen based on its low total ($40.0 \mu\text{g Zn g}^{-1}$; aqua regia digestion) and extractable ($0.04 \mu\text{g NH}_4\text{NO}_3$ -extractable Zn g^{-1}) soil Zn concentrations and very low organic matter content (0.51% C_{org} ; 0.08% N_{tot}). The very acidic soil (pH (H_2O) 4.10; pH (0.01 M CaCl_2) 3.65) was sieved to <4 mm, homogenized and subsequently gamma-irradiated using a ^{60}Co source to eliminate any viable mycorrhizal propagules. For the spiking, the following procedure adapted from a method employed by Barrow (1998) was used: Fifty-gram soil portions, as many as the total number of pots used in the experiments, were weighed into plastic vials and amended with increasing amounts of Zn as aqueous solutions of ZnSO_4 . Zinc amendments to these portions were dimensioned to result in Zn addition rates to the final pot substrate given in the 'Experimental setup' section (see below). The still wet, spiked soil portions were placed in an incubator set at 60 °C and dried to constant weight. Thereafter, portions were thoroughly mixed and remoistened with reverse osmosis (RO) water to 60% of the soils water holding capacity. The vials were subsequently sealed and the moist soil portions again incubated at 60 °C. After a 2 weeks incubation period the still moist soil portions were again dried at 60 °C. Soil portions were then homogenized and mixed with the additional amount of soil needed for each pot (1.425 kg soil). This dry soil substrate was aged for a further 5 days at room temperature.

The soil substrate was subsequently amended with a set of macro- and micro-nutrients to avoid nutrient deficiencies. Nutrients were added as concentrated aqueous solutions of their various salts to give the following final concentrations (in mg kg^{-1}): K_2SO_4 , 75; $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 75; KH_2PO_4 , 1100; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 45; NH_4NO_3 , 76; $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 2.1; $\text{MnSO}_4 \times \text{H}_2\text{O}$, 10.5; $\text{CoSO}_4 \times 7\text{H}_2\text{O}$, 0.39; $\text{NaMoO}_4 \times 2\text{H}_2\text{O}$, 0.18. After the solutions had dried, 1.65 g $\text{CaCO}_3 \text{ kg}^{-1}$ soil was added to adjust the soil to pH (0.01 M CaCl_2) 5.5. Nutrients and CaCO_3 were mixed throughout the experimental soil. Finally, the pot substrate was obtained by mixing 25 g perlite into the soil (equals a 1:3 (v:v) ratio perlite:soil) to ensure adequate aeration and water percolation.

2.3. Experimental setup

Two almost identical pot experiments were conducted to quantitatively describe the growth response of *P. canescens* to increasing Zn additions. The first experiment aimed at determining the substrate Zn tolerance and toxicity threshold of *P. canescens*. Plants were grown at 15 Zn levels ranging from the native Zn status of the soil up to Zn additions expected to be toxic (0, 5, 10, 20, 40, 70, 100, 150, 250, 500, 750, 1000, 1500, 2000 and 2500 mg Zn added kg^{-1} dry pot substrate). Based on visual assessment of plant performance after one week in the first experiment, the second experiment with twenty rates of added Zn (0, 0.005, 0.01, 0.02, 0.04, 0.08, 0.15, 0.3, 0.6, 1, 1.5, 2, 2.5, 3, 4, 6, 10, 20, 40, 100 mg Zn added kg^{-1} dry pot substrate) was set up one month later to examine the Zn uptake and accumulation capacity of *P. canescens*. These rates were chosen to also include levels of Zn limiting to plant growth. In both experiments each Zn treatment was replicated three times. From the start of the experiments each pot was individually identifiable by Zn treatment and replicate number.

Two-litre pots coated with plastic bags were filled with 1500 g of pot substrate (=soil + perlite) on a dry weight basis. Rooted cuttings of *P. canescens* were planted one per pot, and the soil surface was covered with plastic beads (polyethylene) to avoid excessive evaporation. Plants were grown for twelve weeks in a greenhouse in the period between late July and mid November. Substrate moisture was adjusted to 60% water holding capacity by weight every third day. Reverse osmosis irrigation water was supplemented with NH_4NO_3 (114 mg per pot) every third week. Greenhouse temperatures were regulated to between 16 °C and a maximum of 30 °C by means of spray mist and ventilation. From September onwards daylight lamps (20 000 Lx) were used to supplement ambient light to obtain a 16 h day length.

2.4. Harvest and analyses

After 12 weeks of growth plants were harvested by cutting the shoots at the soil surface. The shoots were further separated into the stem and leaves. Entire root systems were harvested by shaking off all soil and torn-off roots were picked from the pot substrate. Subsamples of the pot substrate were taken for analysis of extractable substrate Zn concentrations. All plant parts (leaves, stems and roots) were carefully washed with tap water to remove any adhering soil particles and further rinsed twice with RO water. Roots were finally exposed to a 5 mM CaCl_2 solution in an ultrasonic bath for 3 min to remove any sorbed Zn ions. Subsamples of the roots were stored in 50% ethanol for later microscopic evaluation of their mycorrhizal status by a standard clearing and staining technique (Gazey et al., 1992). The remaining root sample and shoot tissues were dried to constant weight at 60 °C and their dry weight was recorded. For plant tissue Zn analyses 0.2 g aliquots of ground tissues were digested in a 4/1 (v/v) mixture of $\text{HNO}_3/\text{HClO}_4$ using an open digestion system (Velp Scientifica DK Heating Digester). Ammonium nitrate-extractable soil Zn concentrations were quantified on samples of air dried pot

substrate collected before the start of the experiment as well as following plant growth (DIN 19730, 1997). This extractable soil Zn fraction is the main source of phyto-available soil Zn. All data on extractable soil Zn concentrations presented in the figures are from pot substrate samples collected at harvest following plant growth. Zinc concentrations in plant tissue digests were measured by ICP-MS (Elan 9000 DRCE, Perkin Elmer) and in soil extracts by atomic absorption spectroscopy (Perkin Elmer 2100). All leaf samples were analysed for Zn, while stem and root Zn concentrations were only measured in plants from replicate number 1 to reduce analysis costs. Metal analyses were validated with an identically processed internal soil standard (Eurosoil 7; Weissteiner et al., 1999) and certified plant reference material (oriental tobacco leaves, CTA-OTL-1, Institute of Nuclear Chemistry and Technology, Warszawa, Poland; purchased from LGC Promochem, Germany).

2.5. Toxicity measures and statistical analysis

All regression analyses were performed in SigmaPlot (Systat Software Inc.). For the calculation of the substrate concentration that causes a 50% decrease in above-ground plant biomass (=EC50; EC = effective concentration) a dose–response relation was fitted to the log-transformed shoot (=stem + leaves) biomass data assuming that 1% of unaffected growth represented lethal Zn concentrations. From this relation, the EC50 was calculated. A critical leaf Zn concentration, above which leaf dry mass was reduced, was calculated by the Cate–Nelson procedure (Cate and Nelson, 1971). This critical concentration was compared to the phytotoxicity threshold PT50, which in this study defines the leaf Zn concentration that corresponds to a 50% reduction in leaf dry mass (adopted from Shanahan et al., 2007). In this study, the PT50 was calculated on the basis of a logistic regression fitted to the leaf dry mass and leaf Zn concentration data. Zinc root–shoot transfer data from experiment 2 were described by two relations using a simple iterative procedure. Starting from the highest extractable Zn concentration, the data were divided into two groups sharing one data point. The groups were then separately described and an overall R^2 was calculated. Moving successively to the next lower extractable Zn concentration, this procedure was repeated. The lines in Fig. 7 show the two relations where the overall R^2 was maximum. Metal extraction ratios were calculated analogous to Mertens et al. (2005; MER = shoot Zn content/soil Zn content*100).

3. Results

Cuttings of *P. canescens* planted in soil with Zn additions $>250 \mu\text{g g}^{-1}$ died within a few days after transplanting. Extractable Zn concentrations in the soil of surviving plants ranged from approximately 0.05 to just below $100 \mu\text{g Zn g}^{-1}$ soil. Extractable soil Zn concentrations were closely correlated with total soil Zn concentrations ($R^2 = 0.985$; Fig. 1) that ranged from 38.6 to $290 \mu\text{g Zn g}^{-1}$ soil. These concentrations were calculated on the basis of the native Zn content of the soil as determined by aqua regia digestion, the various Zn additions and the weight proportion of the soil in the growth substrate.

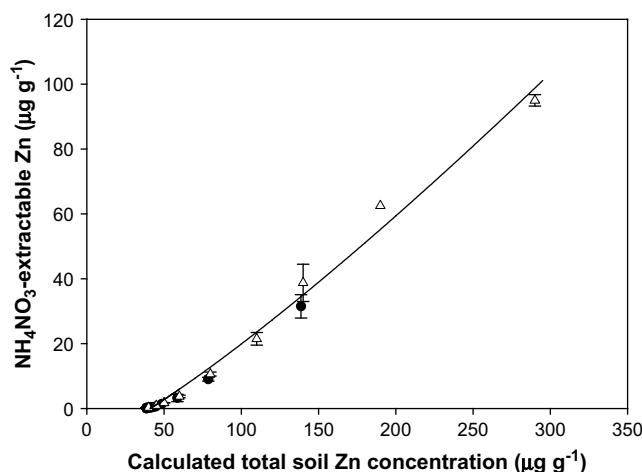


Fig. 1. Relationship between NH_4NO_3 -extractable and total Zn concentrations in the pot substrate. These were calculated on the basis of the native Zn concentration of the soil as determined by aqua regia digestion, the various Zn additions and the weight proportion of the soil in the pot substrate. Symbols: experiment 1 = open triangles, experiment 2 = filled circles. Error bars give standard deviation of measurements from three replicate pots. Fitted equation: $y = -10.32 + 0.117 * x^{1.21}$. $R^2 = 0.985$.

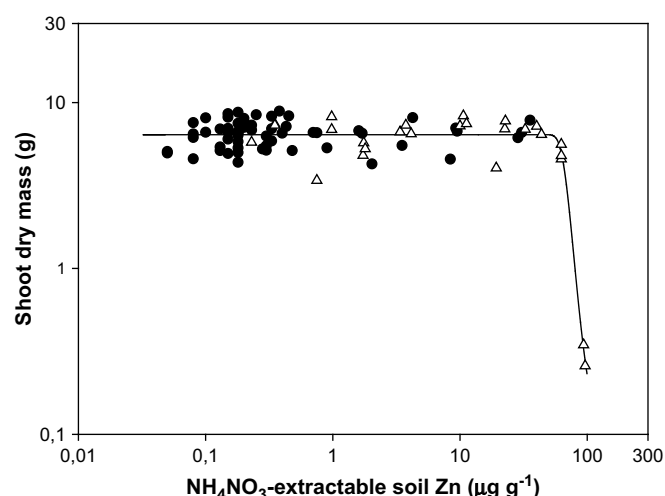


Fig. 2. Shoot (=leaves + stem) dry mass production by *P. canescens* as affected by extractable soil Zn concentrations. Symbols as in Fig. 1. Fitted equation: $\log_{10}(y) = 0.807 - 2 * \exp(-\exp(-10.81 * (\log_{10}(x) - 1.895)))$. $R^2 = 0.854$.

Dry mass of *P. canescens* shoots was largely unaffected by extractable Zn concentrations below $65 \mu\text{g Zn g}^{-1}$ soil (Fig. 2). At extractable soil concentrations between 93 and $96 \mu\text{g Zn g}^{-1}$ soil, shoot dry mass was decreased to 4–5% of the dry mass produced by unaffected plants. From the fitted sigmoid relation, the EC50 was calculated to $68.54 \mu\text{g NH}_4\text{NO}_3$ -extractable Zn g^{-1} soil. Plants remained non-mycorrhizal throughout the experimental period.

Foliar Zn concentrations in plants grown without any Zn added to the soil were in the range of $50\text{--}60 \mu\text{g Zn g}^{-1}$ dry mass. Concentrations increased with increasing extractable soil Zn concentrations (Fig. 3). The highest concentrations were thus measured in plants grown at $60\text{--}96 \mu\text{g NH}_4\text{NO}_3$ -extractable Zn g^{-1} soil and reached around $2000 \mu\text{g Zn g}^{-1}$ leaf dry mass. The critical leaf Zn concentration estimated by the Cate–Nelson procedure above which leaf dry mass was negatively affected was $1360 \mu\text{g Zn g}^{-1}$ leaf dry mass (Fig. 4). The toxicity threshold for foliar Zn concentration (PT50) was calculated to be $2080 \mu\text{g Zn g}^{-1}$ (Fig. 4). Similarly to leaves, Zn concentrations in stems and roots also increased exponentially but were mostly below leaf Zn concentrations (Fig. 5).

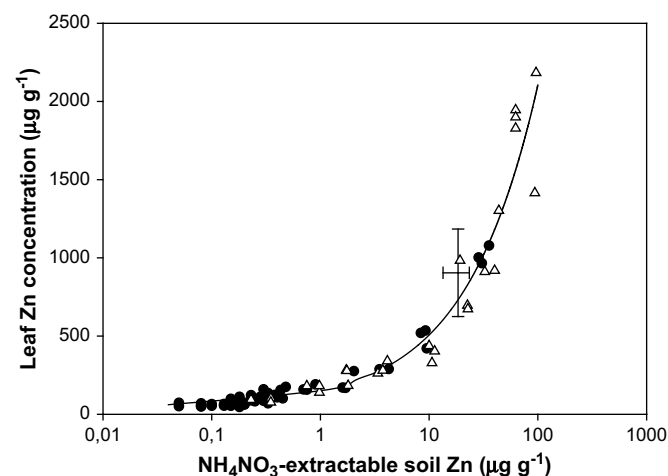


Fig. 3. Leaf Zn concentrations as affected by extractable soil Zn concentrations. The cross-hair shows leaf Zn concentrations measured in *P. canescens* explants which were grown for four months at a metal-contaminated field site. Symbols as in Fig. 1. Fitted equation: $y = 48.69 + 102.063 * \exp(1.502 * \log_{10}(x))$. $R^2 = 0.946$.

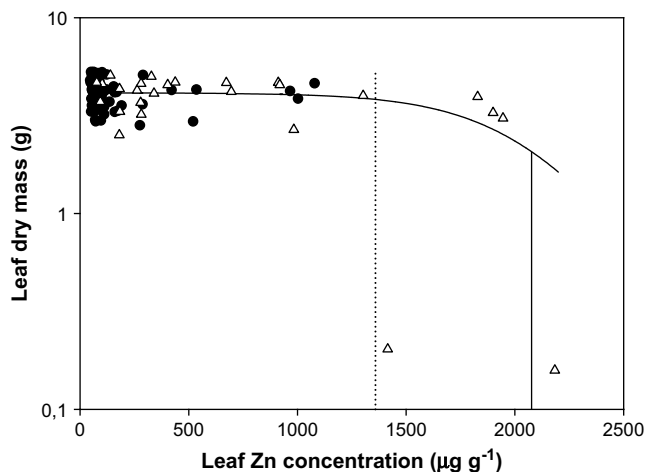


Fig. 4. Relationship between leaf dry mass and leaf Zn concentrations. Symbols as in Fig. 1. Fitted equation: $y = 4.131/(1 + \exp(-7.29 + 0.0035 \cdot x))$. $R^2 = 0.23$. Dotted line: critical concentration (Cate–Nelson procedure), solid line: toxicity threshold PT50.

\log_{10} -transformed accumulation factors (AF = leaf concentration:soil concentration) were linearly correlated with \log_{10} -transformed NH_4NO_3 -extractable soil Zn concentrations ($R^2 = 0.96$; Fig. 6). Zn transfer factors (TF = leaf concentration:root concentration) in the second experiment increased from below 1 at the native Zn status of the soil up to 2.1 at an extractable soil Zn concentration of approximately $3 \mu\text{g Zn g}^{-1}$ soil (Fig. 7). Zn TFs were more variable in the first experiment where they reached a value of 2.1 at approximately $65 \mu\text{g Zn g}^{-1}$ soil. Values for TF did not increase any further and were finally markedly decreased when plant growth was reduced by toxic soil Zn concentrations.

The amount of Zn taken up into *P. canescens* leaves as a proportion of the total amount of extractable Zn in the pot is shown in Fig. 8. Leaf Zn content was identical to the calculated total pot amount of NH_4NO_3 -extractable Zn at an extractable soil Zn concentration of $0.23 \mu\text{g Zn g}^{-1}$ soil. The concentration increased to $0.37 \mu\text{g Zn g}^{-1}$ soil, when Zn removal was calculated on the basis of total shoot Zn content (leaves + stem; data not shown). Below these concentrations, some plants took up considerably more Zn than the calculated total amount of extractable Zn in the pot. Nonetheless, extractable soil Zn concentrations were not decreased by plant Zn uptake (data not shown). The total amount of Zn taken

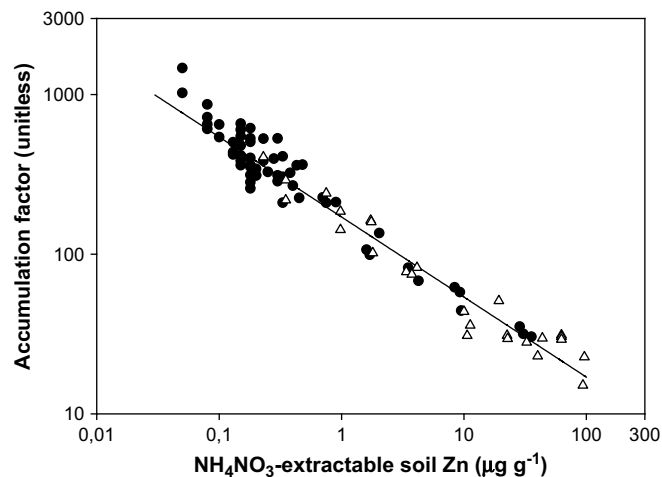


Fig. 6. Log-log plot of accumulation factors (leaf concentration:soil concentration) versus extractable soil Zn concentrations. Symbols as in Fig. 1. Fitted equation: $\log_{10}(y) = 2.233 - 0.5016 \cdot \log_{10}(x)$. $R^2 = 0.963$.

up into *P. canescens* shoots was between 0.5% up to a maximum of 2.5% of total pot Zn (Fig. 9). It was 0.2–0.3% less when only considering Zn uptake into leaves.

4. Discussion

Two dose–response experiments enabled us to accurately determine the toxic soil Zn threshold concentration for a clone of *P. canescens*. Based on its growth and metal uptake capacities this clone has been considered suitable for use in phytoremediation (DosSantos-Utmazian and Wenzel, 2007). Although information on toxicity threshold concentrations of suitable plant species is crucial for the decision whether phytoremediation is a feasible management option for a specific contaminated site, such information is so far mostly lacking (Shanahan et al., 2007). Additionally, the scant information available has mostly been obtained in nutrient solution studies (Di Baccio et al., 2003; Reichman et al., 2001; Shanahan et al., 2007), with the inherent difficulty of extrapolating to real soil (DosSantos-Utmazian and Wenzel, 2007). The experimental setup

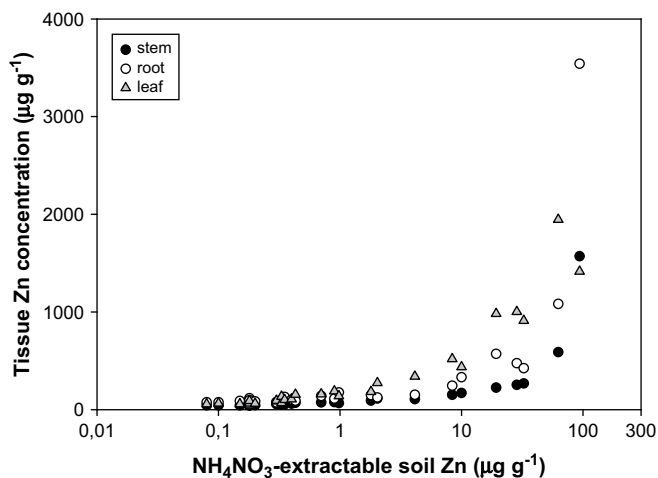


Fig. 5. Zn concentrations in the various plant organs as affected by extractable soil Zn concentrations. Data are from only one replicate.

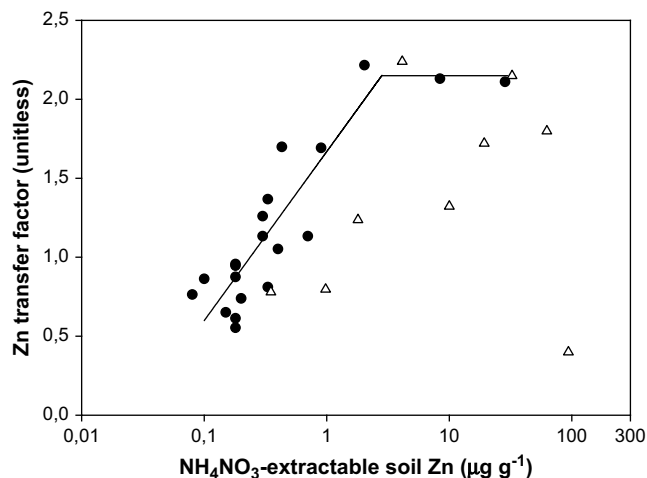


Fig. 7. The effect of labile soil Zn concentrations on Zn root-to-shoot transfer factors (leaf concentration:root concentration). Symbols as in Fig. 1. Fitted equations: $y = 1.67 + 1.07 \cdot \log_{10}(x)$ for $x < 2.83$; $y = 2.15$ for $x \geq 2.83$. Data are from only one replicate and regression lines are fitted to data from experiment 2 only.

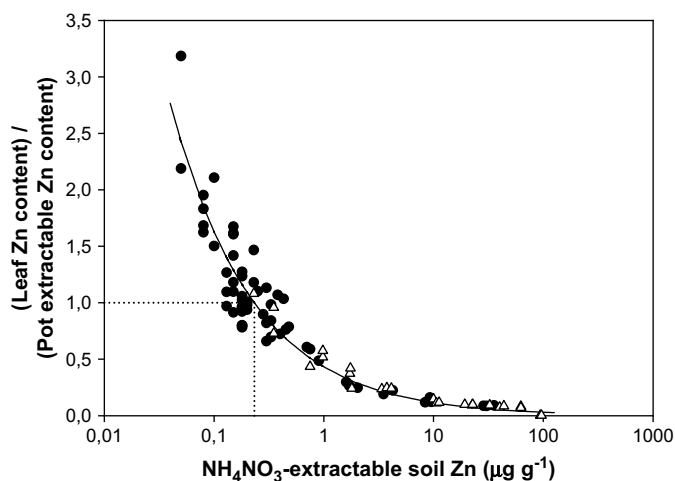


Fig. 8. The effect of the extractable soil Zn concentration on the ratio between leaf Zn content and the total amount of extractable soil Zn in the entire pot soil volume. Symbols as in Fig. 1. Fitted equation: $y = 0.432 \cdot \exp(-1.326 \cdot \log_{10}(x))$. $R^2 = 0.899$.

used in this study represents a more realistic situation, which however is still very different to on-site situations.

In this study, extractable soil Zn concentrations up to $65 \mu\text{g Zn g}^{-1}$ soil had no negative effect on the growth of *P. canescens*. An increase in the extractable concentration to around $95 \mu\text{g Zn g}^{-1}$ soil markedly decreased growth to around 5% of the dry mass produced by unaffected plants. Plants did not survive when planted at higher concentrations. From these data, an effective concentration of $68.5 \mu\text{g NH}_4\text{NO}_3$ -extractable Zn g^{-1} soil was determined at which *P. canescens* shoot growth was reduced by 50%. This concentration is within the upper range of extractable soil Zn concentrations reported for extremely contaminated sites affected by mining and smelting operations (Unterbrunner et al., 2007; range = 0.06 – $434 \mu\text{g 1 M NH}_4\text{NO}_3$ -extractable Zn g^{-1} soil) but considerably higher than expected for most contaminated land (e.g. Laureysens et al., 2004; French et al., 2005; around $5 \mu\text{g 0.01 M CaCl}_2$ -extractable Zn g^{-1} soil). Soils beneath field stands of *P. tremula* have been found to contain up to $58 \mu\text{g NH}_4\text{NO}_3$ -extractable Zn g^{-1} soil (Krpata et al., 2008), which is the highest extractable Zn concentration of a soil we are aware of beneath *P. tremula* or *P. canescens* stands. Specimens of some willow species, however, have been observed to grow on sites with considerably higher extractable Zn concentrations (maximum $434 \mu\text{g extractable Zn g}^{-1}$ soil; Unterbrunner et al., 2007).

The various soil Zn concentrations were obtained by spiking the soil with Zn salt solutions followed by a two week incubation period at 60°C in a moist state. It is well known that the availability of metals immediately after spiking as salt solutions is very high (Smolders et al., 2004), but the employed ageing procedure is assumed to have decreased soil Zn availability (see Barrow, 1993, 1998). However, the proportions of extractable relative to total Zn concentrations measured in this study (Fig. 1) are clearly above the proportion of extractable to total Zn generally found in long-term contaminated field soils ($<10\%$, often $<1\%$, compare: Hammer and Keller, 2002; Pueyo et al., 2004; Unterbrunner et al., 2007). Exceptions to this general pattern are soils with extremely low pH and/or high sand content (Pueyo et al., 2004). Nonetheless, risk assessment studies have shown that results obtained on Zn-spiked and aged soil were comparable to those using contaminated field soil (Efroymson et al., 2004), especially if a measure of the available Zn fraction is used (Lock and Janssen, 2003).

Foliar Zn concentrations increased with increasing extractable soil Zn to just above $2000 \mu\text{g Zn g}^{-1}$ dry mass. Zinc concentrations in

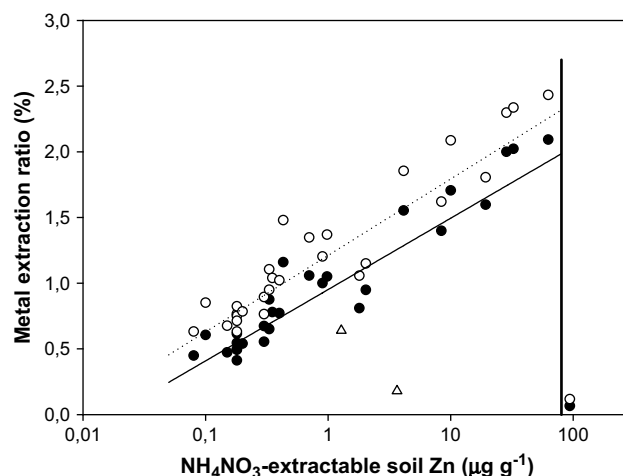


Fig. 9. The effect of the labile soil Zn concentration on the metal extraction ratio. Metal extraction ratios are the amounts of Zn taken up into the leaves (filled circles, solid line) or the total above-ground biomass (open circles, dotted line) expressed as the percentage of the total amount of Zn in the pot substrate at the start of the experiment. Lines were only fitted to data from plants showing no toxicity symptoms (data points left of the vertical divider). Fitted equation (for total above-ground biomass): $y = 1.21 + 0.582 \cdot \log_{10}(x)$. $R^2 = 0.83$. Open triangles are results from DosSantos-Utmazian and Wenzel, unpublished.

leaves of cuttings of the same clone that had been transplanted to a heavily contaminated field site ($1957 \mu\text{g total Zn g}^{-1}$ soil; Krpata, 2008) and grown at the site for four months were within the range of the regression line (Fig. 3). In another pot study, however, foliar Zn concentrations of the same clone grown on two different soils were clearly above the regression line obtained in this study (405 and $920 \mu\text{g Zn g}^{-1}$ leaf dry mass at 1.28 and $3.65 \mu\text{g extractable Zn g}^{-1}$ soil; DosSantos-Utmazian and Wenzel, unpublished), which may be due to differences in experimental procedures. Foliar Zn concentrations in leaves of adult *P. tremula* trees (Krpata et al., 2008; Unterbrunner et al., 2007) tend to lie on or slightly above the obtained regression line. Apart from differences in genotype such small deviations may also be caused by differential leaf age (Vandecasteele et al., 2003). Leaf dry mass production of *P. canescens* was reduced at foliar Zn concentrations above $1300 \mu\text{g Zn g}^{-1}$. This concentration was measured in a plant grown at $95 \mu\text{g g}^{-1}$ extractable soil Zn. Physiological processes in leaves of various *Populus* species are already negatively affected by lower Zn concentrations (*P. canescens*: Bittsanszky et al., 2005; other *Populus* species: Castiglione et al., 2007; Di Baccio et al., 2003). Based on this information and the obtained growth and leaf Zn concentration data, we conclude that the examined *P. canescens* clone may be suitable for planting on sites that contain up to $30 \mu\text{g extractable Zn g}^{-1}$ soil, where leaf Zn concentrations in this study reached $1000 \mu\text{g Zn g}^{-1}$. The suggested upper margin of $30 \mu\text{g extractable Zn g}^{-1}$ soil is higher than measured in the vast majority of contaminated field sites.

Accumulation factors decreased linearly with increasing extractable soil Zn concentrations when plotted on log-log scale. The regression line was slightly less inclined than previously observed for field-grown *P. tremula* (Krpata et al., 2008). Such relations indicate the decreasing relative extraction efficiency with increasing soil Zn concentrations (Efroymson et al., 2001). In combination with growth data this relation may be used to assess the potential of *P. canescens* for use in bioavailable contaminant stripping (Hamon and McLaughlin, 1999). For an assessment of the feasibility of this technique, data on Zn uptake need to be combined with data on the time-course of net reductions in the bioavailable metal pool for each site separately (Van Nevel et al., 2007). In this study, extractable soil

Zn concentrations were not decreased by 12 weeks of plant growth and Zn uptake. This is most likely due to the use of spiked soil which resulted in a very high proportion of soil Zn in the extractable pool. Predictions about the possible time-course of reductions in the extractable soil Zn pool in other soils or even in field situations can therefore not be based on the results of this study.

The extraction capacity of a plant is typically expressed on the basis of total soil metal concentrations (McGrath and Zhao, 2003; Mertens et al., 2005). In this study, metal extraction ratios (MER = shoot Zn content/soil Zn content*100) ranged from 0.5 to just above 2%. These values are considerably higher than data from another pot study with the same *P. canescens* clone (Fig. 9; DosSantos-Utmazian and Wenzel, unpublished). This difference is most likely a consequence of the use of spiked soil in this study, which also will have caused the observed increase in MER with increasing extractable soil Zn concentrations. Thus, comparisons between Zn extraction measured in this study and Zn extractions published by other groups are difficult and can only be done very cautiously, especially when MER were calculated for field-grown trees (e.g. Maxted et al., 2007; Mertens et al., 2005). Similarly, the results of this study cannot be used to predict the feasibility of phytoextraction with any confidence.

Phytoremediation in general is considered an ecologically sensitive technique (Chaney et al., 2007). However, especially phytoextraction may carry the risk of metal dispersal into the environment (Van Nevel et al., 2007). High metal concentrations in the litter may for example contribute to the buildup of metal-enriched organic matter horizons on polluted sites (Gillet and Ponge, 2002; Mertens et al., 2007). Accumulation of humus material has been observed in aspen stands with Zn concentrations in mature leaves above $450 \mu\text{g g}^{-1}$ (Krpata et al., 2008). No humus and thus metal accumulation had occurred on a site with foliar Zn concentrations below $350 \mu\text{g g}^{-1}$. These leaves had however also lower Cd concentrations ($4.8 \pm 2 \mu\text{g Cd g}^{-1}$) than leaves from a site where humus material had accumulated ($12.6 \pm 5.2 \mu\text{g Cd g}^{-1}$). Leaf litter Zn concentrations of $1300 \mu\text{g g}^{-1}$ resulted in a redistribution of Zn from deeper soil layers to the topsoil (Mertens et al., 2007). This corresponds to $870 \mu\text{g Zn g}^{-1}$ in mature leaves when assuming that litter is relatively enriched in metals compared to mature leaves by a factor of 1.5 (approximate mean ratio between mature and senescent leaves in Laureysens et al., 2004). In this study, such foliar Zn concentrations were reached by aspen grown at $25 \mu\text{g extractable Zn g}^{-1}$ soil. To avoid the potential risk of metal accumulation in topsoil, planting of the examined aspen clone should therefore be avoided on sites with similar extractable Zn concentrations. Alternatively, when used in phytoextraction, techniques for harvesting also the foliage need to be implemented (Pulford and Dickinson, 2005).

In conclusion, the experimental setup used in this study enabled us to establish quantitative relations between extractable soil Zn concentrations and some plant parameters that determine phytoextraction feasibility. The data presented here may be used for estimating the suitability of the investigated *P. canescens* clone for various phytoextraction strategies and for any given site by measurement of the extractable Zn concentration in soil. Similarly, the relations between extractable Zn in soil and foliar metal concentrations may be useful in assessing the risk of metal transfer into food webs associated with *P. canescens* stands on contaminated sites. This is particularly important as metal-accumulating poplars are widespread tree species frequently established on metal-contaminated sites.

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4.2 Ectomycorrhizal impact on Zn accumulation of *P. tremula* L. grown in metalliferous soil with increasing levels of Zn concentration.

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Ectomycorrhizal impact on Zn accumulation of *Populus tremula* L. grown in metalliferous soil with increasing levels of Zn concentration

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Keywords

ectomycorrhiza; zinc (Zn); cadmium (Cd); lead (Pb); dose response; *Salicaceae*; phytoremediation.

Abstract

Aims: Our study aimed at characterizing the Zn phytoextraction potential of a metal tolerant *Populus tremula* accession in symbiosis with a community of ectomycorrhizal fungi from metal-contaminated soil that is naturally forming mycorrhizae with the experimental plant. Effects of the fungal community on *P. tremula* development, metal translocation and accumulation properties were tested under variable Zn bioavailability.

Methods: In a pot experiment, *P. tremula* seedlings were grown for 88 days in a substrate composed of metalliferous soil from an aspen stand and non-contaminated agricultural soil spiked with ZnSO₄ to yield total Zn additions from 0 to 80 mg kg⁻¹ substrate. The substrate contained the inherent mycorrhizal community of *P. tremula* (Nat-Myc) or was γ -irradiated to eliminate living microbial propagules (Irr-NM treatment).

Results: γ -Irradiation efficiently inhibited the formation of functional ectomycorrhizae in the control treatments. It increased dissolved organic carbon (DOC) in the substrate and affected the extractability of Zn and Cd by 1M NH₄NO₃. We found three times larger biomass and more than four times increased root lengths in the Irr-NM compared to the Nat-Myc treatments which may be explained by the doubled DOC concentrations and related Fe mobilization due to formation of labile complexes in the irradiation treatment and the absence of microbial competitors for (nutrient) resources. Our results indicate an imbalance of the normally mutualistic symbiosis between mycorrhizal fungi and the host at early growth stage, possibly further enhanced by the high susceptibility of the *P. tremula* seedlings obtained from a contaminated site. Foliar Zn concentrations were generally larger in the Nat-Myc treatments and exceeded those reported for numerous *Salix* and *Populus* species. While the Zn concentrations increased with increasing Zn additions, Zn translocation to shoots was inhibited at high Zn levels in the Nat-Myc treatments, indicating a barrier function of the mycorrhizal community.

Conclusions: The observed barrier properties in the mycorrhizal treatments suggest that mycorrhizal inoculation of *P. tremula* may be a promising strategy to enhance revegetation and phytostabilization of metal-polluted sites. However, early-stage growth of *P. tremula* may be limited by imbalances between the fungal and plant partner in such nutrient-deficient, toxic environments.

Introduction

Soil pollution by toxic metals and metalloids is of major environmental concern as they harm human health and restrict multifunctional soil use and fertility (Adriano 2001). Numerous strategies have been developed to remediate the affected soils or to prevent movement of contaminants into non-polluted areas. Phytoremediation, a plant-assisted *in situ* approach, is considered an ecologically sensitive strategy particularly maintaining biological properties and physical structure of the affected soils (Chaney et al. 1997; Khan et al. 2000; Wenzel 2009). Phytostabilization and phytoextraction processes are mainly discussed with respect to metal affected areas. Both require plants

highly tolerant to a range of toxic metal elements. Phytostabilization aims at vegetating metal-affected habitats, thereby mechanically stabilizing topsoil and preventing the contaminants from spreading via leaching or erosion. Phytoextraction is deemed to remove metal pollutants from soil by means of plants that accumulate the metals in their harvestable parts. Herbal hyperaccumulating species such as *Thlaspi* and *Alyssum* indigenous to metal-contaminated habitats fulfill most basic requirements for use in phytoextraction processes (Keller et al. 2003; Lasat et al. 2001). However, slow growth, poor biomass production and shallow root penetration limit their extraction potential. Tree species such as *Salix* spp. and *Populus* spp. have

been proposed more suitable for phytoextraction due to their larger biomass production, extensive root system and their intermediate metal accumulation properties (DosSantos-Utmazian 2006; Komarek et al. 2008; Meers et al. 2007; Pulford and Watson 2003; Robinson et al. 2000). Some factors, however, may limit the phytoextraction approach such as negligible metal bioavailability going along with high soil pH values, or excess metal supply finally impairing plant growth and vitality.

Rhizosphere processes underlying plant-assisted remediation approaches are affected by soil micro-organisms and may increase or decrease the availability of metals and metalloids (Wenzel 2009). It has been proposed that metal bioavailability in particular is affected by ectomycorrhizal fungi colonizing tree roots (Leyval et al. 1993). Ectomycorrhiza (EcM) has been demonstrated to enhance plant growth and vitality on metal-disturbed habitats and ameliorate metal toxicity, affecting plant metal uptake and the accumulation in plant tissues (Jentschke and Godbold 2000; Leyval et al. 1997). Jentschke and Godbold (2000) compiled existing information on some general strategies such as alterations of the adsorptive surface area, modified mobility in the root apoplast due to fungal ingrowth, changes in the hormonal metabolism or the release of chelating agents. Khan et al. (2000) emphasized barrier properties of ectomycorrhizal structures together with metabolic processes to be crucial for mycorrhiza driven amelioration of metal toxicity. Thus mycorrhizae may constitute a valuable and integral part of phytoremediation.

The impact of mycorrhizal formation on plant growth and metal uptake cannot be generalized as shown by differential responses of *Pinus*, *Abies*, *Salix* or *Populus* species (Baum et al. 2006; DosSantos-Utmazian et al. 2007; Godbold et al. 1998; Sell et al. 2005). Moreover, plant responses have been demonstrated to vary with the fungal species/isolates. This may reflect fungal properties such as metal tolerance and/or specificities in functional compatibility with their symbiotic host (Baum et al. 2006; Colpaert and VanAssche 1992; Colpaert and Assche 1993; Lingua et al. 2008; Sell et al. 2005). In addition, the response of mycorrhizal plants to metal toxicity may be influenced by the metal fraction available to the symbiotic host (Adriaensen et al. 2003; Bücking and Heyser 1994; Colpaert and VanAssche 1992).

Several studies have demonstrated the remediation potential of individual species of the *Salicaceae* family associated with EcM fungi. (Baum et al. 2006; DosSantos-Utmazian et al. 2007; Sell et al. 2005). Most plants were inoculated with single fungal species / isolates but these were typically obtained from non-polluted environments, thus not co-occurring with their hosts on metal-contaminated sites.

The European aspen *P. tremula* L. is indigenous throughout Europe and extends to northeastern Asia and into northern Africa, characteristically shows fast growth, tolerance to various climatic conditions and high adaptability to a wide range of soils (Worrell 1995). *Populus tremula* grows on metalliferous habitats affected by calamine ores and mining processes and was found to accumulate up to 3600 mg kg⁻¹ Zn and 45 mg kg⁻¹ Cd in leaf tissues (Krpata et al. 2009; Unterbrunner et al. 2007). Comprehensive field studies on a metal-affected *P. tremula* stand revealed a diverse fungal community forming EcM with this plant species (Krpata et al. 2008).

The aims of the present study were (1) to characterize the Zn accumulation potential of *P. tremula* indigenous to a heavy metal-affected habitat and (2) to determine the effect of a fungal

community that was obtained from a metalliferous soil and naturally forming mycorrhizae with *P. tremula*, on plant growth and metal accumulation under variable Zn additions. As Cd is typically associated with Zn, we also present data on Cd extractability from soil and uptake into *P. tremula*.

Materials and Methods

Plant material

Populus tremula plantlets were grown from seeds collected from a metal-contaminated aspen stand near Arnoldstein (Carinthia, Austria) in spring 2006 (Krpata et al. 2009). That specific accession has previously been shown to accumulate large amounts of Zn and Cd in the above-ground biomass (Krpata et al. 2009; Unterbrunner et al. 2007). Seeds were surface-sterilized with H₂O₂ and germinated on nutrient agar (Langer et al. 2008). After 16 days seedlings had developed vigorous roots and were transferred to pots filled with perlite. Plantlets were cultivated in an incubation box under controlled conditions (at 25°C with a 16 h photoperiod) to allow moderate adaptation to decreasing air humidity. Pots were watered with a nutrient solution containing macro-nutrients (1 mM KNO₃, 0.5 mM Ca(NO₃)₂ × 4H₂O, 0.5 mM MgSO₄ × 7H₂O, 0.05 mM KH₂PO₄) and micro-elements (5 μM H₃BO₃, 0.2 μM H₂₄Mo₇N₆O₂₄ × 4H₂O, 10 μM MnSO₄ × 4H₂O, 2.5 μM CuSO₄ × 5H₂O, 0.25 μM NiSO₄ × 6H₂O, 50 μM Fe-EDDHA). ZnSO₄ (5 μM) was added once at the end of the pre-growing period to alleviate observed symptoms of Zn deficiency.

Fungal inoculum and growth substrate

The EcM fungal community native to the same metal-contaminated site near Arnoldstein was used for inoculation. The composition and abundance of EcM fungal species in soil and associated with European aspen at that site was characterized in earlier studies (Krpata et al. 2008; Oberkofler 2005). Krpata et al. (2008) identified 54 ectomycorrhizal fungi on the species level with *Cenococcum geophilum* and corticoid basidiomycetes dominating the mycorrhizal associations. Soil was collected from the upper 10 cm of the mineral horizon (below a 5-10 cm thick mor humus layer) and sieved to <5 mm (= soil A). Untreated soil was used in the mycorrhizal treatment (Nat-Myc), while soil was sterilized by γ-irradiation (⁶⁰Co source; 25.5 kGy min⁻¹) for use in the non-mycorrhizal treatment (Irr-NM). Both Nat-Myc and Irr-NM are further on stated as “mycorrhiza” treatments.

The experimental growth substrate (Table 1) used was a 1:2:2 (w:w:w) mixture of the contaminated soil A, an uncontaminated soil B, and quartz sand.

Soil A is a Calcaric Cambisol affected by former Pb, Zn and Cd smelter activities from the 15th century up to the 1990s (Friesl et al. 2006), containing 1960 mg kg⁻¹ total Zn, 24.3 mg kg⁻¹ total Cd and 3560 mg kg⁻¹ total Pb.

Soil B is a Calcaric Cambisol under agricultural use, chemically similar to soil A, but uncontaminated. It was added to reduce the total metal load of the growth substrate as the contaminated soil A was instantly toxic to individual plant seedlings. Hence it was not possible to start the dose-response experiment from the native metal concentration of soil A. Moreover, by spiking the uncontaminated soil B according to Barrow (1998) we avoided impairment of the natural microbial community hosted by soil A. Soil B was sieved (<5 mm) and sterilized by γ-irradiation to eliminate microbial activity. Portions of soil B were spiked with increasing amounts of Zn using aqueous solutions of ZnSO₄,

resulting in five levels of Zn addition (0, 10, 20, 40 and 80 mg Zn kg⁻¹ soil substrate mixture). Oven-dried soil portions were carefully mixed, moistened to 60% water holding capacity (WHC) with reagent grade-water (14 MΩ cm) and incubated at 60°C for 11 days. Choice of Zn addition levels was based on results from a pre-experiment on plant growth and plant Zn tolerance in response to elevated Zn supply (data not shown). Autoclaved quartz sand complemented the soil substrate mixture to ensure adequate aeration and water percolation.

Experimental setup

Five vigorous *P. tremula* plantlets of uniform size were selected and transferred to un-drained one-liter pots filled with 690 g of the experimental soil substrate. The soil surface was covered with plastic beads to minimize evaporation. Each treatment was replicated four times except treatments Zn 0 and Zn 40 which were replicated eight times to allow an intermediate harvest one month prior to the final harvest. The experiment was conducted in a green house at 22°C (17°C night temperature), with a 16 h photoperiod and 55% air humidity. Plants were irrigated (reagent grade-water) every third day by adjusting substrate moisture to 60% water holding capacity. After two months of growth, plants started to show symptoms of Fe deficiency. Hence FeCl₃ (0.14 mg pot⁻¹) was regularly supplied in solution immediately before watering the plants.

Harvests and analyses

P. tremula plants were grown for 57 days and 88 days respectively. At harvest plants were separated into leaves, stem and roots, carefully washed with running tap water and afterwards rinsed with reagent grade-water. Roots were further exposed for two minutes to 0.05 M CaCl₂ solutions in an ultrasonic bath to remove adhered soil particles and sorbed metal ions. Root subsamples were stored in 50% ethanol for determination of root length and mycorrhizal colonization and the rest was dried. The length of roots was measured by a gridline intersection method (Newman 1966). At the same time, the number of mycorrhizal root tips was counted in four preselected areas of the Petri dish covering 11 % of its area. Ectomycorrhizal root colonization was expressed as the number of mycorrhizal root tips per meter root length. Biomass of plant organs was determined after drying at 60°C for three days.

Aliquots of ground plant tissues (0.2 g) were digested in a 1:4 mixture of HNO₃: HClO₄ (Digester DK 42/26, Velp Scientifica). For analysis of extractable Zn and Cd fractions, subsamples of equal weight were taken from individual replicates within a treatment and pooled. These samples were sieved (<2mm) and extracted with 1 M NH₄NO₃ solutions (DIN-19730 1997). Replicate pots in treatments Zn 0 and Zn 40 were extracted individually and mean values were calculated. The extractable metal fraction is considered to represent labile forms that may become readily available to plants (Pulford and Dickinson 2005).

To evaluate potential side-effects of γ-irradiation pooled subsamples of the initial growth substrate were extracted with H₂O (ÖNORM-L-1092 1993) and dissolved organic carbon (DOC) analyzed (DIMA-TOC 100 analyzer, Dimatec).

A lab-internal soil standard and certified reference material CTA-OTL-1 (oriental tobacco leaves, Institute of Nuclear Chemistry and Technology; Warsaw; Poland) were used for quality control of soil extraction and plant tissue digestion, respectively. Concentrations of elements in plant digests (Zn, Cd, Fe, P,) and soil extracts (Zn,

Cd, Pb) were measured by means of ICP-MS (Elan 9000 DRCE, Perkin Elmer).

Total Zn and Cd contents in leaves, stems and roots were calculated from their respective weight multiplied by their metal concentration. Translocation factors (TF) were calculated for Zn (TF_{Zn}) and Cd (TF_{Cd}) as the concentration ratio between leaves and roots.

Statistical analyses

T-Tests were employed to evaluate significant ($p \leq 0.05$) differences between treatment means of extractable Zn and Cd in soil. Plant parameters were compared by analyses of variance (one-way ANOVA, main factor “Zn addition”, $p \leq 0.05$). If “Zn addition” turned out to affect plant growth and Zn (Cd) accumulation behavior the least conservative Post hoc test Fisher’s LSD ($p \leq 0.05$) and linear regression analyses ($p \leq 0.05$) were computed. Plant data were further analyzed by multifactorial ANOVA with “mycorrhiza” and “Zn addition” as the main factors ($p \leq 0.05$). Statistical analyses were conducted with the Statistica 6.0 software package.

Results

NH₄NO₃ extractable Zn and Cd concentrations in soil

The NH₄NO₃-extractable (labile) Zn, Cd and Pb fractions in soil at the beginning of the experiment and at the time of final harvest are given in Table 2. Initial labile Zn concentrations were significantly affected by the “mycorrhiza” treatment ($p = 0.002$). The mean of labile Zn in the Nat-Myc treatments was 2.31 mg Zn kg⁻¹, exceeding that in the Irr-NM treatment by 15.7 % (Table 2). The labile Zn fraction significantly ($p = 0.000$) decreased with experimental time approaching means of 1.09 and 1.04 in the Irr-NM and Nat-Myc treatments, respectively. The difference between the “mycorrhiza” treatments decreased during the experimental period and was not significant ($p = 0.392$) at the final harvest. Similar effects were observed for labile Cd fractions. The labile Pb fraction was even stronger affected by the “mycorrhiza” treatment ($p = 0.000$) with initial Pb concentrations being two-fold larger in the Irr-NM soil compared to the Nat-Myc treatment. Differences between the “mycorrhiza” treatments decreased with time but were still significant at the final harvest ($p = 0.000$) (Table 2).

Mycorrhizal colonization and root length

Roots in the Nat-Myc treatments formed 41 EM root tips per meter root length on average. The number of infected root tips formed in individual “Zn addition” treatments ranged from 29 to 58 m⁻¹ root length. The largest number of mycorrhized root tips was observed for plants grown at the two highest Zn concentration levels. However, Zn additions did not significantly affect mycorrhiza colonization of *P. tremula* plantlets (Table 3). In the Irr-NM treatments, γ-irradiation was very effective in eliminating EcM propagules with only 4 pots showing minor colonization (<2 EM root tips m⁻¹). Therefore we consider the Irr-NM treatments as being non-mycorrhizal. Root lengths were generally increased in the Irr-NM treatments exceeding those observed in the Nat-Myc treatments by at least four times. Zinc addition affected root length only significantly (one-way ANOVA, $p = 0.045$) in the Irr-NM treatments but we found no correlation between the two variables ($r^2 = 0.049$; $p = 0.362$).

Effects of “mycorrhiza” and “Zn addition” on plant growth

Biomass of leaves, stems and roots were significantly affected by the “mycorrhiza” treatments (Table 4). Mycorrhizal *P. tremula* yielded only one third of the non-mycorrhizal plant biomass on average (Fig. 1a). In the Nat-Myc treatment “Zn addition” had no effect on biomass production. The level of Zn addition only significantly affected dry-mass of non-mycorrhizal stems, which decreased with increasing Zn addition.

Foliar Fe and P concentrations

Populus tremula plantlets grown in the Nat-Myc treatments started to show severe symptoms of iron deficiency ahead of the first harvest. These plants had much lower foliar Fe concentrations than in the Irr-NM treatments (Table 5). Additional Fe supply from the first harvest onwards clearly reduced visible symptoms of Fe deficiency and improved foliar Fe concentrations of mycorrhizal plants at the lowest Zn application level (Nat-Myc Zn 0). However, foliar Fe concentrations of plants grown in the other treatments decreased. At the final harvest significant differences due to mycorrhiza inoculation could not be observed but foliar Fe concentrations were significantly affected and inversely correlated ($r^2 = 0.251$; $p = 0.024$) with “Zn addition”.

Foliar P concentrations were affected by both factors “mycorrhiza” and “Zn addition” (Table 5), with largest P concentrations up to 2520 mg kg⁻¹ in the Nat-Myc treatments. The foliar P concentrations in the Irr-NM plants were significantly lower, except at the highest Zn addition level (Zn 80). Foliar P concentrations and Zn concentrations were significantly correlated in the Nat-Myc treatments ($r^2=0.441$; $p=0.001$) but not in the Irr-NM controls ($r^2=0.301$; $p=0.012$).

Zn and Cd concentrations in plant tissues

Foliar Zn concentrations ranged from 1310 to 2260 and 1890 to 2740 mg Zn kg⁻¹ in Irr-NM and Nat-Myc treatments, respectively (Table 6). Thus foliar Zn concentrations were significantly affected by both factors “mycorrhiza” and soil “Zn addition” (Table 4). Moreover, in the Irr-NM treatments we found a positive correlation between foliar Zn concentrations and Zn addition ($r^2=0.680$; $p=0.000$).

Zinc concentrations in stems were also lower in the Irr-NM treatments, ranging from 171 to 258 mg Zn kg⁻¹ compared to mycorrhizal plants with 249 to 393 mg Zn kg⁻¹ (Table 6). The “mycorrhiza” treatment significantly affected Zn concentration in stems, whereas the effect of Zn addition was only significant in the Irr-NM treatment (Table 4).

Zinc concentrations in roots significantly differed between the “mycorrhiza” treatments (Table 4). In non-mycorrhizal roots, they ranged from 553 to 746 mg Zn kg⁻¹, and significantly exceeded values observed for the Nat-Myc treatments.

Populus tremula leaf tissues accumulated about 103 to 156 mg Cd kg⁻¹ (Table 6). Foliar Cd concentrations were not affected by the “mycorrhiza” treatment but varied significantly with Zn addition (Table 4). The observed foliar Cd concentrations in the Irr-NM treatments were positively correlated with the increasing soil Zn levels ($r^2=0.357$; $p=0.005$).

Cadmium concentrations in stems ranged from 26 to 51 mg kg⁻¹ and were not affected by “mycorrhiza” or “Zn addition”.

The Cd concentrations in roots were significantly affected by the “mycorrhiza” treatment, ranging up to 221 mg kg⁻¹ in the Nat-Myc

Zn 80 treatment, but did not vary significantly with soil “Zn addition”.

Metal translocation

Translocation factors for Zn and Cd are presented in Fig. 2. The Zn translocation factors (TF_{Zn}) were significantly influenced by the “mycorrhiza” treatment ($p = 0.007$) with a mean value for Irr-NM plants (2.91) exceeding the mean TF_{Zn} for mycorrhiza colonized plants by the factor 1.4. Zn translocation was not significantly affected by soil “Zn addition” ($p = 0.689$) showing nearly similar TF_{Zn} values for *P. tremula*, exposed to the lower Zn application levels. With increasing soil Zn levels Irr-NM TF_{Zn} tended to increase, whereas TF_{Zn} calculated for mycorrhiza colonized plants largely remained the same.

Translocation factors for Cd (TF_{Cd}), with means of 1.87 and 0.78 for Irr-NM and Nat-Myc treatments, respectively, were significantly affected by the “mycorrhiza” treatment ($p = 0.000$). In contrast, “Zn addition” had no significant effect on TF_{Cd} ($p = 0.926$).

Metal contents in plant tissues

The Zn and Cd contents in *P. tremula* tissues were higher in the Irr-NM treatments than those in the Nat-Myc treatments (Fig. 1b and 1c). The largest Zn contents were found in leaves, averaging 844 and 404 µg plant⁻¹ in the Irr-NM and Nat-Myc treatments, respectively. Zinc contents in roots were lower by a factor 5.2 in Irr-NM plants and 6.3 times in mycorrhizal plants. The lowest Zn contents were detected in stems with 53.8 (Irr-NM) and 16.7 µg plant⁻¹ (Nat-Myc), respectively. Multifactorial ANOVA (Table 4) calculated for leaves, stems and roots revealed significant differences due to the “mycorrhiza” treatment. “Zn addition” affected foliar Zn contents only, whereas it had no significant effect on the Zn content in other plant tissues. The “mycorrhiza” treatment significantly affected Cd contents in leaves, stems and roots, whereas increasing Zn addition had no effect on Cd contents in plant tissues.

Discussion

Plant growth

Biomass yield of the experimental plants severely differed between the “mycorrhiza” treatments with a three times larger biomass for non-mycorrhizal *P. tremula* grown in the Irr-NM treatment. Our results contrast observations on other species of the *Salicaceae* family such as *Populus canadensis* and *Salix viminalis* that showed no difference of biomass produced by ectomycorrhizal plants and in non-mycorrhizal controls (Sell et al. 2005). EcM formation by *S. dasyclados* (Baum et al. 2006) and AM formation by *P. alba* (Cicatelli et al. 2010) even enhanced plant growth in metal-contaminated environments. Our findings on *P. tremula* growth may primarily be explained by (1) an imbalance of the mutualistic symbiosis and (2) possible side effects of soil sterilization.

Mycorrhiza symbiosis is generally expected to improve plant vitality and growth (Smith and Read 1997), especially under nutrient (P) limitation (Schweiger et al. 2007; Smith and Read 1997) or environmental stress such as for example toxic effects in disturbed habitats (Jentschke and Godbold 2000; Leyval et al. 1997). However, there is also evidence that especially during the initial phase of mycorrhiza formation, the costs for the plant may exceed the benefits either induced by developmental or

environmental stimulants, or genetic properties (Johnson et al. 1997).

P. tremula biomass production also of the Irr-NM plants was generally low with maximum values around 1.2 g dry weight for the above ground plant parts. Differences to other *Populus* species such as *P. canescens* (Langer et al. 2009) or *P. canadensis* (Sell et al. 2005) grown in metal-enriched soil may be explained by species and clonal specificities as observed for a variety of *Salix* and *Populus* species (Castiglione et al. 2009; DosSantos-Utmazian et al. 2006), their provenance from a metal-affected habitat (Puschenreiter et al. 2010; Vyslouzilová et al. 2006), and the propagation method applied. Whereas *Populus* species typically regenerate from root-suckers in the field (Worrell 1995), experimental plants are mainly propagated by cuttings. However, the *P. tremula* accession from Arnoldstein regenerated poorly from twigs thus plantlets were grown from seeds (Langer et al. 2008). While this method successfully produces seedlings, it comes at the expense of slow development in the juvenile stage due to the lack of an endosperm (Borset 1954) and the possible impairment of seed quality on disturbed habitats (Fedorkov 1999). Thus the mycorrhiza colonization constituting the dominant carbon sink within the host-symbiont association may have substantially retarded juvenile growth of the fragile *P. tremula* seedlings.

Apart from *P. tremula* characteristics defining restrained growth and triggering an imbalance of the mycorrhiza symbiosis, the specific fungal community native to the metal-contaminated aspen stand may have reduced plant growth. Although studies on ectomycorrhizal *Salicaceae* species have not yet shown biomass responses to different fungal species/isolates (Baum et al. 2000; Sell et al. 2005), modified plant growth was repeatedly found for *Pinus sylvestris* (Bücking and Heyser 1994; Colpaert and VanAssche 1992; Colpaert and Assche 1993; Kozdrój et al. 2007). Particularly an early study of Colpaert and Van Assche (1992) on the provenance effects of fungal isolates demonstrated impaired plant development of *P. sylvestris* in symbiosis with *Suillus bovinus* from a metal-affected habitat as compared to an isolate from non-contaminated soil. Thus the natural inoculum in our experiment may have similarly retarded plant biomass for the benefit of enhanced mycorrhiza colonization and vigorous mycelial growth as observed in the latter study (Colpaert and VanAssche 1992). Amelioration of contaminated environments with mycorrhizal plants should therefore address the functional compatibility on the isolate level.

Moreover the microbial consortium co-occurring with ectomycorrhizal propagules in the natural inoculum may have impaired *P. tremula* biomass development, either by competition for nutrients or pathogen attacks.

Whereas juvenile growth in the Nat-Myc treatment was retarded, the biomass production of *P. tremula* was most likely promoted by γ -irradiation in the Irr-NM treatments which may explain the large difference between the “mycorrhiza” treatments. γ -Irradiation is known to reliably eliminate soil microbes and living propagules (Alpei and Scheu 1993) and has been used in numerous mycorrhizal studies. However, microbiological sterility comes at the expense of side effects such as the structural alteration of soil organic matter, enhanced availability of NH_4^+ and mineral P, and release of some nutrient elements into soil solution due to death and lyses of microbial cells (McNamara et al. 2003). The impact of γ -irradiation on the chemical properties of the experimental soil

substrate was confirmed by a twofold increase of DOC (dissolved organic carbon) in the Irr-NM treatment (data not shown). Moreover, the labile, potentially bioavailable fractions of Zn, Cd and Pb were significantly increased, probably due to the release from cell membranes or the cytoplasm and vacuoles of disrupted and devitalized microorganisms (Table 2).

Given the high susceptibility of the juvenile *P. tremula* plants to nutrient deficiencies in the presence of competitive mycorrhizal partners and other micro-organisms in the Nat-Myc treatments, the plants in the Irr-NM treatments may have benefited twofold: (1) from enhanced nutrient availability due to release from microbial cells and formation of labile organic nutrient complexes due to the increase of DOC; (2) from the lack of microbial competition for nutrient resources.

Increasing Zn addition did not significantly ($p > 0.05$) affect biomass of *P. tremula* tissues (except stem biomass in the Irr-NM treatment) although the tendency of non-mycorrhizal plants to reduce biomass production is evident (Fig. 1a). This trend is in line with findings of Todeschini et al. (2011) and Hermle et al. (2006) showing biomass reduction along with detrimental effects on *P. alba* / *P. tremula* photosynthesis in response to enhanced foliar Zn accumulation. The less pronounced response to increasing Zn doses in our experiment may be explained by the almost invariable labile Zn fraction irrespective to the amount of Zn added to the substrate (Table 2). The effective immobilization of added Zn is owing to the lime content and correspondingly high pH of the experimental substrate (Adriano 2001; Khan and Jones 2009)

Iron and P supply

At the final harvest visual inspection of the experimental plants revealed minor symptoms of Fe deficiency in the Nat-Myc treatment. However, analysis of mycorrhizal and non-mycorrhizal *P. tremula* leaves revealed Fe concentrations (Table 5) below critical values given for C3 plant species (Marschner 1995). Low Fe uptake and significant response of foliar Fe concentrations to the enhanced Zn supply can be explained by competitive uptake mechanisms of Zn, Cd and Fe due to congruent ion radii (Alcantara et al. 1994; Marschner 1995) and correspond well to other findings in metal affected soils (Vyslouzilová et al. 2006). The higher foliar Fe concentrations of non-mycorrhizal *P. tremula*, particularly detected at the first harvest (Table 5), are likely due to the formation of labile Fe complexes with dissolved organic compounds in soil, as indicated by the doubling of DOC upon γ -irradiation. This may have enhanced diffusive Fe supply to plant roots in the depleted rhizosphere (Degryse et al. 2009) even though the EDTA-extractable Fe (data not shown) did not vary with γ -irradiation.

In addition, soil micro-organisms in the Nat-Myc treatment may have contributed to the low foliar Fe concentrations by competing for essential nutrients such as Fe in the rhizosphere (Weber et al. 2006).

Mycorrhiza formation significantly increased foliar P concentrations of *P. tremula* which is in line with our expectations (Smith and Read 1997). However, the increased P accumulation is opposed by reduced biomass yields (Fig. 1). Accordingly, we suppose that the larger foliar P concentrations in the Nat-Myc treatments may additionally be explained by a “concentration” effect owing to the reduced biomass (Robinson et al. 2000).

Zn and Cd uptake, transfer and accumulation in plant tissues

The metal concentrations in *P. tremula* leaves were generally high (> 1300 mg Zn and 100 mg Cd kg^{-1} (Table 6) exceeding concentrations reported for *P. canadensis* (Langer et al. 2009), *P. canadensis* (Sell et al. 2005; Wang and Jia 2010), the hybrid poplar *P. tremula* \times *termuloides* (Migeon et al. 2009) or *Populus* \times *generosa* (Bissonnette et al. 2010), both in field studies and greenhouse experiments. In some treatments, the foliar Zn and Cd concentrations considerably exceeded those observed in *P. tremula* in Arnoldstein (Krpata et al. 2009), its original habitat and source of soil A and the EcM fungal community supplied in the current experiment. Enhanced HM concentrations in our pot experiment may partially be explained by the juvenile stage of the experimental plants as compared to the 25 years old *P. tremula* field stand. Wieshammer et al. (2007) found declining metal concentrations in *S. caprea* during a three year monitoring period. In our study, retarded plant growth in the Nat-Myc treatments may have further increased Cd and Zn concentrations in the plant tissues (Robinson et al. 2000).

Foliar Zn uptake in metal-tolerant members of the *Salicaceae* family typically increase in response to EcM formation with *Paxillus involutus*, *Cadophora finlandica* or AM formation with *Glomus mossae* and *G. intraradices* (Baum et al. 2006; DosSantos-Utmazian et al. 2007; Sell et al. 2005). In contrast, EcM formation has been repeatedly shown to decrease or not affect Zn uptake and translocation in *Pinus sylvestris* aboveground plant parts/needles (Adriaensen et al. 2006; Colpaert and VanAssche 1992; Krupa and Kozdroj 2007). Along with the results of these previous studies our results may suggest that mycorrhizae tend to enforce the uptake strategy of the host plant species either by enhancing element uptake of Zn “accumulators” or restricting transport to the aboveground plant parts in coniferous Zn “excluder” plants. However, transfer factors for Zn and Cd indicate barrier functions of the native EcM fungal community in symbiosis with the experimental plant *P. tremula*. Moreover mycorrhizal plants revealed the lowest $\text{TF}_{(\text{Zn})}$ value at the highest soil Zn addition level (Fig. 2) which pronounces the barrier effect of mycorrhizae with increasing soil Zn availability. Similar findings were obtained for *P. sylvestris* exposed to increasing Zn doses (Bücking and Heyser 1994). But, whereas *P. sylvestris* non-mycorrhizal controls similarly decreased Zn translocation in response to enhanced soil Zn addition, *P. tremula* plant internal Zn transfer slightly increased in accordance with the elevated soil Zn availability. This finding corresponds with results of non-mycorrhizal *P. canadensis* grown in a Zn dose-response study (Langer et al. 2009).

Populus tremula also accumulated more than 100 mg Cd kg^{-1} in leaves (Table 6) thus exceeding concentrations observed for numerous species of the *Salicaceae* family such as *P. canadensis*, *S. viminalis* or *S. dasyclados* (Baum et al. 2006; Sell et al. 2005), but did not attain the foliar accumulation potential of the highly accumulating species *S. caprea* and *S. smithiana* grown on metal-contaminated soil from the same general area near Arnoldstein (DosSantos-Utmazian et al. 2007). Thus our data also emphasize the impact of plant species characteristics on Cd uptake and accumulation behavior in leaf tissues. In contrast, EcM symbionts did not affect the foliar Cd concentration level of *P. tremula* (Table 6). This observation is well in line with findings of ectomycorrhizal *Salix* species (Baum et al. 2006; DosSantos-Utmazian et al. 2007). Contrasting results observed for *P. canadensis*, showing enhanced foliar Cd concentration in response to EcM inoculation (Sell et al.

2005) may potentially be explained by considerably lower labile concentrations of both Cd and the essential nutrient Zn in the growth substrate or characteristics of the particular host-symbiont associations as proposed by Godbold et al. (1998).

General conclusions

Zn and Cd contents in *P. tremula* grown in the absence or presence of mycorrhiza and varied Zn levels in a pot experiment were generally low, particularly due to plant species specific growth properties. Thus this accession may not be suitable for phytoextraction purposes regardless of the mycorrhizal status.

The observed barrier properties in the mycorrhizal treatments suggest that mycorrhizal inoculation of *P. tremula* may be a promising strategy to enhance revegetation and phytostabilization of metal-polluted sites. However, early-stage growth of *P. tremula* may be limited by imbalances between host plant and fungal symbionts in such nutrient-deficient, toxic environments. Moreover the risk to increased topsoil Zn/Cd accumulation due to periodic leaf fall and decomposition has to be considered.

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Table 1 Chemical and physical properties of the substrate mixture at the zero level of Zn supply.

Property		Mean	SD
pH (CaCl ₂)		7.2	0.1
CaCO ₃	g kg ⁻¹	42.3	5.1
C _{org}	g kg ⁻¹	27.3	1.0
N _t	g kg ⁻¹	2.1	0.1
C/N		13.3	0.6
WHC	%	41.1	1.3
Texture			
Sand	g kg ⁻¹	547	
Silt	g kg ⁻¹	332	
Clay	g kg ⁻¹	121	
Heavy metal concentration (total; aqua regia digestion)			
Zn	mg kg ⁻¹	563	14.1
Cd	mg kg ⁻¹	9.3	0.5
Pb	mg kg ⁻¹	735	14.5

Table 2 Availability of Zn, Cd and Pb in the experimental soil substrates evaluated at the start of the experiment and the final harvest by means of 1 M NH₄NO₃ extracts.

Soil Zn addition		NH ₄ NO ₃ extractable Zn		NH ₄ NO ₃ extractable Cd		NH ₄ NO ₃ extractable Pb	
	mg kg ⁻¹	initial mg kg ⁻¹	final harvest mg kg ⁻¹	initial mg kg ⁻¹	final harvest mg kg ⁻¹	initial mg kg ⁻¹	final harvest mg kg ⁻¹
Irr-NM	Zn 0	2.53	0.99	0.24	0.14	0.74	0.27
	Zn 10	2.66	1.05	0.25	0.14	0.68	0.24
	Zn 20	2.72	1.07	0.25	0.14	0.66	0.25
	Zn 40	2.85	1.13	0.25	0.15	0.75	0.28
	Zn 80	2.92	1.23	0.25	0.14	0.64	0.23
Nat-Myc	Zn 0	2.40	0.95	0.24	0.14	0.43	0.18
	Zn 10	2.20	0.97	0.23	0.14	0.37	0.17
	Zn 20	2.10	1.03	0.21	0.14	0.33	0.17
	Zn 40	2.44	1.04	0.23	0.14	0.41	0.19
	Zn 80	2.39	1.20	0.21	0.15	0.34	0.17

Table 3 Mean values of mycorrhiza colonization and root length for non-mycorrhizal (Irr-NM) and mycorrhizal (Nat-Myc) *P. tremula* grown at five levels of soil Zn supply.

Soil Zn addition		Mycorrhiza colonization			Root length		
mg kg ⁻¹		mycorrhizal root-tips m ⁻¹ root length			m plant ⁻¹		
		mean value	SE _a	LSD _b	mean value	SE _a	LSD _b
Irr-NM	Zn 0	0	0		69.4	3.8	b
	Zn 10	1	0.5		91.5	14.1	ab
	Zn 20	0	0.2		76.9	10.4	b
	Zn 40	0	0		101.3	6.2	a
	Zn 80	0	0		65.6	11.4	b
Nat-Myc	Zn 0	39	10.9		17.1	0.6	
	Zn 10	29	2.3		17.6	0.7	
	Zn 20	33	8.5		13.8	4.2	
	Zn 40	58	18.5		13.6	3.3	
	Zn 80	46	16.0		14.2	1.9	
Effect		F-Ratio _c	p-Value _c		F-Ratio _c	p-Value _c	
Mycorrhiza		52.4	0.000*		269.1	0.000*	
Zn addition		0.8	0.519		2.9	0.040*	
Mycorrhiza x Zn addition		0.9	0.474		2.9	0.039*	

^a SE values indicate standard errors calculated on basis of four replicate pots.

^b Letters a-b indicate homogeneous groups identified by means of the Fisher's LSD test at the 95% confidence level (a>b). The Post hoc test was conducted after one-way ANOVA (main factor: "Zn addition") had shown significant differences between means ($p \leq 0.05$).

^c F-Ratios and P-Values result from multifactorial ANOVA.

* Asterisks denote significant differences between means ($p \leq 0.05$).

Table 4 Statistical results (F-Ratios and P-Values from multifactorial ANOVA, $p \leq 0.05$) calculated for plant parameters biomass, Zn (Cd) contents and Zn (Cd) concentrations separated into leaves, stems and roots.

Plant parameters	Effect	Leaves		Stems		Roots	
		F-Ratio	p-Value	F-Ratio	p-Value	F-Ratio	p-Value
Biomass	Mycorrhiza	282.3	0.000*	319.7	0.000*	139.2	0.000*
	Zn addition	1.0	0.424	3.0	0.032*	2.1	0.108
	Mycorrhiza x Zn addition	0.7	0.611	2.1	0.102	1.8	0.155
Zn contents	Mycorrhiza	152.1	0.000*	157.9	0.000*	77.4	0.000*
	Zn addition	3.9	0.012*	0.8	0.522	0.5	0.736
	Mycorrhiza x Zn addition	3.1	0.029*	0.5	0.72	1.3	0.283
Cd contents	Mycorrhiza	405.6	0.000*	274.7	0.000*	46.0	0.000*
	Zn addition	2.2	0.092	1.7	0.182	0.6	0.699
	Mycorrhiza x Zn addition	2.6	0.053	1.1	0.387	1.7	0.180
Zn concentrations	Mycorrhiza	37.2	0.000*	51.39	0.000*	21.1	0.000*
	Zn addition	9.1	0.000*	2.38	0.074	1.21	0.328
	Mycorrhiza x Zn addition	4.4	0.006*	1.76	0.163	0.7	0.596
Cd concentrations	Mycorrhiza	2.4	0.132	0.176	0.678	36.5	0.000*
	Zn addition	4.4	0.006*	2.2	0.093	1.59	0.203
	Mycorrhiza x Zn addition	2.3	0.083	2.316	0.080	0.82	0.522

* Asterisks denote significant differences between means.

Table 5 Foliar Fe and P concentrations for non-mycorrhizal (Irr-NM) and mycorrhizal (Nat-Myc) plants grown at five levels of soil Zn addition.

Soil Zn addition		Fe			Fe			P		
		Leaves			Leaves			Leaves		
		first harvest	SE _a	LSD _b	final harvest	SE _a	LSD _b	final harvest	SE _a	LSD _b
mg kg ⁻¹		mg kg ⁻¹			mg kg ⁻¹			mg kg ⁻¹		
Irr-NM	Zn 0	73.1	3.9		60.2	5.4	a	1590	54	bc
	Zn 10				41.2	2.3	bc	1530	86	c
	Zn 20				37.2	1.3	bc	1810	91	ab
	Zn 40	60.8	11.7		33.0	6.8	c	1720	76	abc
	Zn 80				46.9	2.8	b	1900	117	a
Nat-Myc	Zn 0	42.0	1.9		70.3	10.8	r	2090	118	st
	Zn 10				36.8	1.0	s	2090	108	st
	Zn 20				30.9	2.2	s	2520	153	r
	Zn 40	35.4	1.0		33.4	9.1	s	2250	194	rs
	Zn 80				27.1	9.9	s	1810	91	t
Effect		F-Ratio _c	p-Value _c		F-Ratio _c	p-Value _c		F-Ratio _c	p-Value _c	
Mycorrhiza		20.4	0.001*		0.9	0.358		36.7	0.000*	
Zn addition		2.3	0.155		9.2	0.000*		3.3	0.024*	
Mycorrhiza x Zn addition		0.2	0.658		1.6	0.192		3.6	0.017*	

^a SE values indicate standard errors calculated on basis of four replicate pots.

^b Letters a-c and r-s indicate homogeneous groups identified by means of the Fisher's LSD test at the 95% confidence level (a>b>c and r>s). Post hoc tests were conducted after one-way ANOVA had shown significant differences between means ($p \leq 0.05$) with "Zn addition" as the main factor.

^c F-Ratios and P-Values result from multifactorial ANOVA.

* Asterisks denote significant differences between means ($p \leq 0.05$).

Table 6 Zn and Cd concentrations in leaves, stems and roots for irradiated, non-mycorrhizal (Irr-NM) and natural, mycorrhizal (Nat-Myc) treatments at five levels of Zn addition.

Soil Zn addition		Zn									Cd								
		Leaves			Stems			Roots			Leaves			Stems			Roots		
		mean value	SE _a	LSD _b	mean value	SE _a	LSD _b	mean value	SE _a	LSD _b	mean value	SE _a	LSD _b	mean value	SE _a	LSD _b	mean value	SE _a	LSD _b
		mg kg ⁻¹			mg kg ⁻¹			mg kg ⁻¹			mg kg ⁻¹			mg kg ⁻¹			mg kg ⁻¹		
Irr-NM	Zn 0	1310	72	c	192	14	b	592	56		109	5	c	36	2		61	6	
	Zn 10	1520	72	bc	180	10	b	553	107		129	6	b	34	1		69	2	
	Zn 20	1760	105	b	198	12	b	746	193		137	6	ab	35	2		85	14	
	Zn 40	1570	53	bc	171	4	b	586	79		120	4	bc	31	1		64	6	
	Zn 80	2260	115	a	258	19	a	742	158		149	7	a	40	1		86	20	
Nat-Myc	Zn 0	1890	143	s	249	29		951	132		103	7		26	3		125	4	
	Zn 10	2090	182	s	349	33		1180	169		103	13		35	7		156	25	
	Zn 20	2740	253	r	393	61		1030	122		156	22		51	8		154	29	
	Zn 40	2070	110	s	338	40		1200	238		119	11		36	6		185	41	
	Zn 80	2160	76	s	342	38		1570	360		110	10		31	6		221	44	

^a SE values indicate standard errors calculated on basis of four replicate pots.

^b Letters a-c and r-s indicate homogeneous groups identified by means of the Fisher's LSD test at the 95% confidence level (a>b>c and r>s). Post hoc tests were conducted after one-way ANOVA had shown significant differences between means ($p \leq 0.05$) with "Zn addition" as the main factor.

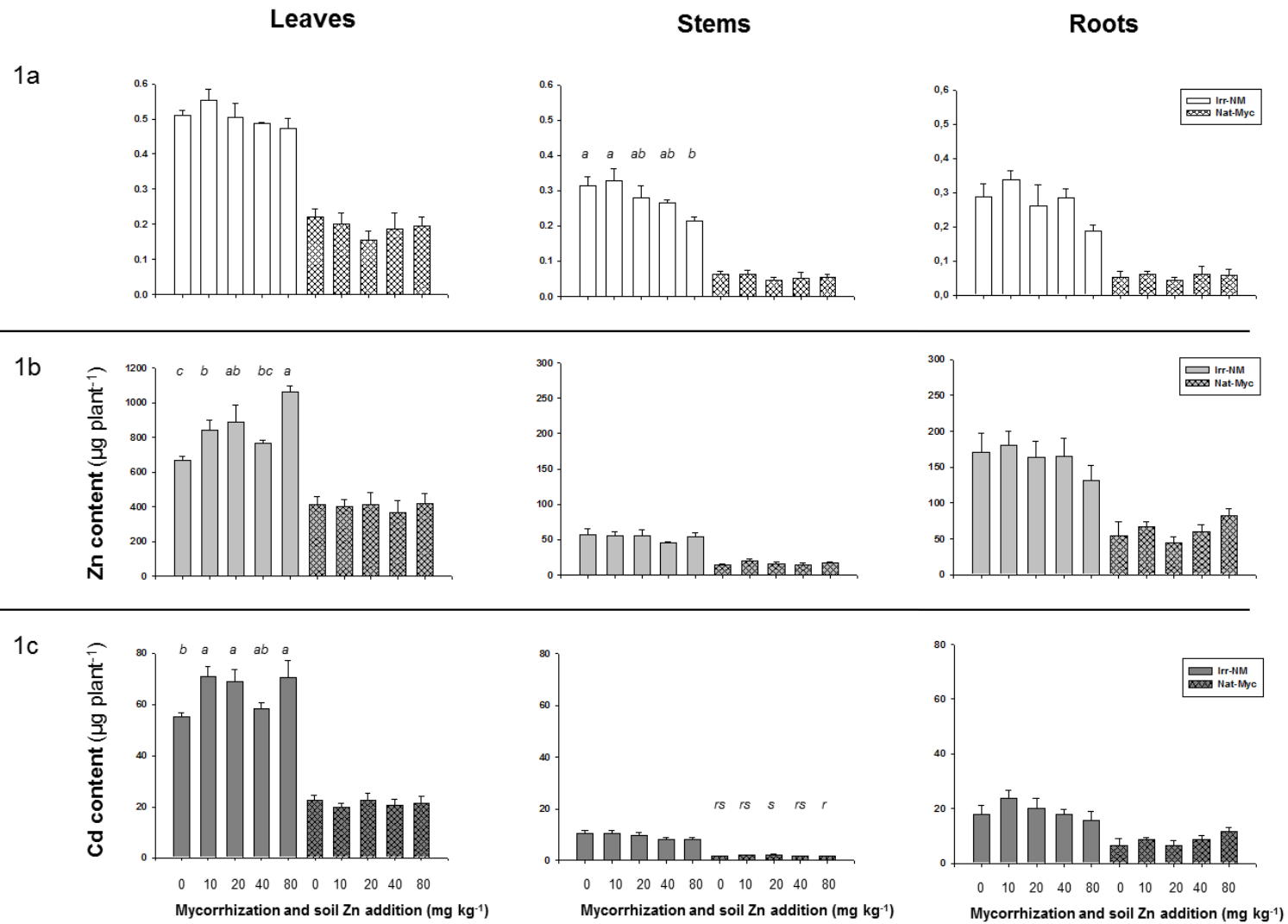


Fig. 1 The biomass (1a), Zn contents (1b) and Cd contents (1c) of *P. tremula* separated into leaves, stems and roots. Error bars give the standard errors calculated on basis of four replicate pots. Letters a-c and r-s indicate homogeneous groups identified by means of the Fisher's LSD test at the 95% confidence level ($a > b > c$; $r > s$). Post hoc tests were conducted after one-way ANOVA had shown significant differences between means ($p \leq 0.05$) with "Zn addition" as the main factor.

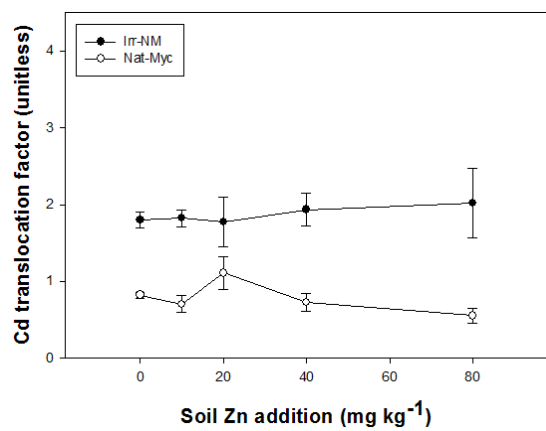
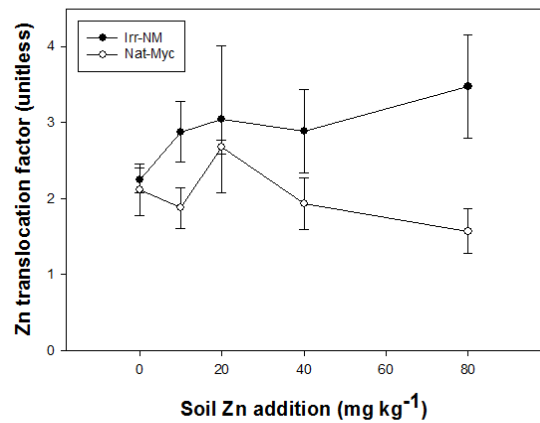


Fig. 2 Zn (Cd) translocation factors (root-to-shoot translocation) for the irradiated, non-mycorrhizal (Irr-NM; closed circles) and natural, mycorrhizal (Nat-Myc; open circles) treatments as affected by increasing levels of soil Zn supply. Bars give standard errors of measurements from four replicate pots.

**4.3 Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen
Populus tremula L.**

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Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula* L.

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Abstract The effect of various media formulations on in vitro ectomycorrhizal synthesis of identified fungal strains with European aspen (*Populus tremula* L.) was tested in Petri dishes. Pre-grown seedlings were transferred to various nutrient media and inoculated with *Paxillus involutus* isolates using modified sandwich techniques. Mycorrhiza formation was evaluated macroscopically and further confirmed by microscopic examination of semi-thin sections for anatomical features of the mantle and the Hartig net. Standard media formulations did not support successful ectomycorrhiza formation because of either very poor plant survival (below 20%) or impaired fungal growth. The inclusion of micronutrients and vitamins in a Melin Norkrans (MMN)-based medium increased plant survival rate to above 60% and supported successful mycorrhizal synthesis. *P. involutus* isolates formed mycorrhizas with a characteristic Hartig net restricted to the epidermis. Mantle density and thickness varied depending on the isolate. In a follow-up experiment, the adapted medium supported successful ectomycorrhiza formation by various *Laccaria* and *Hebeloma* isolates. Our results show that an exogenous supply of vitamins and micronutrients in the medium was a prerequisite for successful mycorrhization of *P. tremula* in vitro in Petri dishes.

Keywords Ectomycorrhiza · *Paxillus involutus* · *Populus tremula* (Poplar) · In vitro synthesis · Inoculation procedure · Symbiosis

Introduction

Species in the genus *Populus* have received general attention due to their use as energy crops in short rotation forestry (Dickmann 2006) and due to their great potential for carbon sequestration (Lemus and Lal-Referee 2005). *Populus* species have further attracted attention in contaminated land management based on their fast growth and considerable tolerance of increased soil heavy metal concentrations. Within, the genus, especially the European aspen *Populus tremula* L., has shown potential for use in the phytoremediation of contaminated sites (Robinson et al. 2000).

Populus tremula is one of the world's most widely distributed tree species, with its natural range extending throughout Europe to northeastern Asia and into northern Africa. It is a pioneer species that tolerates a wide range of climatic and soil conditions (Worrell 1995). *P. tremula* is also able to colonize variously disturbed habitats, including sites contaminated with heavy metals (Unterbrunner et al. 2007). Based on its ability to accumulate heavy metals in the aboveground biomass, *P. tremula* has been considered suitable for phytoextraction (DosSantos-Utmazian and Wenzel 2007).

Populus tremula generally grows in association with ectomycorrhizal (EM) fungi (Melin 1923; Krpata et al. 2008). These fungi affect heavy metal uptake by their host plants as well as within-plant heavy metal transport (Leyval et al. 1997). Only few studies examined the role of single fungal isolates on metal uptake by accumulator plants (Sell

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et al. 2005; Baum et al. 2006; DosSantos-Utmazian et al. 2007). However, in most of these studies, only very low levels of EM root colonization were observed. The extent of EM root colonization is greatly dependent on the establishment of the fungus on host plant roots during mycorrhizal synthesis. Thus, experimental work focusing on *P. tremula* requires an adequate inoculation procedure.

Numerous inoculation protocols have been published that resulted in successful establishment of functional ectomycorrhizas on various hosts (Molina and Palmer 1982; Peterson and Chakravarty 1991). Some of those protocols have proven successful with many different fungal as well as host plant species. Others have been developed for specific host plant–EM fungus combinations. Inoculation techniques reported for *Populus* species predominately focus on American poplars such as *P. trichocarpa* (Baum and Makeschin 2000; Baum et al. 2002; Selle et al. 2005), its hybrids (Heslin and Douglas 1986; Tagu et al. 2001) and *Populus tremuloides* (Fortin et al. 1983; Godbout and Fortin 1985; Cripps and Miller 1995;

Landhäuser et al. 2002). Further reports on mycorrhizal synthesis are available on hybrids between *P. tremuloides* and *P. tremula* (Hampp et al. 1996; Loewe et al. 2000; Selle et al. 2005). Inoculation protocols conducted with European *Populus* hybrids are scarce (Bücking and Heyser 2001; Gafur et al. 2004; Couturier et al. 2007; Langenfeld-Heyser et al. 2007). Moreover, single protocols again refer to procedures originally conducted with *P. tremula* × *tremuloides* (Gafur et al. 2004; Couturier et al. 2007). To our knowledge, *Populus tremula*, the European aspen, has not been used for studies on mycorrhizal synthesis since Melin in 1923.

In previous studies, we produced *P. tremula* plantlets by different plant propagation techniques (green cuttings vs. seedlings) and variously inoculated them with pre-grown mycelium (Perrin et al. 1996; Tagu et al. 2001; Sell et al. 2005), plugs or fungal suspensions (Landhäuser et al. 2002; Parladé et al. 2004). Plantlets were subsequently cultivated in diverse substrates such as soil, leca, sand, perlite, peat, and vermiculite. None of these inoculation

Table 1 Composition of media for cultures of EM fungi and inoculation procedures with *P. tremula* used in the present study

Compound		L-Knop ^a	MMN ^b	G-MMN ^c	L-MMN ^d
Macroelements (mg/l)	KH ₂ PO ₄	54.440	500.000	500.000	500.000
	KNO ₃	242.640			
	(NH ₄) ₂ SO ₄			250.000	
	(NH ₄) ₂ HPO ₄		250.000		250.000
	MgSO ₄ · 7H ₂ O	80.720	150.000	150.000	150.000
	CaCl ₂ · 2H ₂ O		50.000	50.000	50.000
	Ca(NO ₃) ₂ × 4H ₂ O	240.000			
	NaCl	0.400	25.000	25.000	25.000
Microelements (mg/l)	FeNaEDTA	2.936			
	FeCl ₃ · 6H ₂ O (1%)		12.000	12.000	12.000
	H ₃ BO ₃	0.572		15.458	15.458
	MnSO ₄ · 1H ₂ O			9.295	9.295
	MnCl ₂ × 4 H ₂ O	0.570			
	CuSO ₄ × 5 H ₂ O	0.015		1.310	1.310
	ZnSO ₄ × 7 H ₂ O	0.072		5.750	5.750
	CoCl ₂ × 6 H ₂ O	0.006			
	Na ₂ MoO ₄ × 2 H ₂ O	0.016		0.003	0.003
	myo-Inositol	100.000			100.000
Vitamins (mg/l)	Nicotinic acid	1.000			1.000
	Pyridoxine HCl	1.000			1.000
	Thiamine HCl	10.000	1.000	0.100	10.000
Carbohydrate source (g/l)	Glucose		2.5	5.0	5.0
	Sucrose	2.5			
	Malt extract		10.0	3.0	
Solidification agent (g/l)	Agar		9.0	9.0	9.0
	Gelrite	6.0			
pH		5.75	5.4	4.5	5.4

^a New medium composition based on the Knop nutrient solution (George 1993)

^b Modified Melin Norkrans medium (Brundrett et al. 1996)

^c Adapted modified Melin Norkrans medium following the protocol of Gafur (Gafur et al. 2004)

^d New medium composition based on the modified Melin Norkrans Medium

procedures, however, resulted in successful ectomycorrhizal synthesis.

The aim of the present study was therefore to develop an EM inoculation protocol specifically suitable for *P. tremula*. Synthesis experiments were conducted on *P. tremula* seedlings in vitro following several protocols in Petri dish systems. The first experiments focused on the composition of a medium, meeting the nutrient and vitamin demand of both *P. tremula* and *Paxillus involutus* isolates. Subsequently, a series of *P. tremula* inoculations was set up to confirm the suitability of the improved medium composition with *Laccaria*, *Hebeloma*, and *Paxillus* isolates.

Materials and methods

Plant material and seed germination

Populus tremula seeds were collected from a heavy metal contaminated *P. tremula* stand in southern Austria in spring 2005. *P. tremula* growing at that site has previously been shown to accumulate large amounts of both zinc and cadmium (Unterbrunner et al. 2007). In the laboratory, seeds were cleaned according to Latva-Karjanmaa et al. (2003) and stored at -18°C (Fechner et al. 1981). For the

experiments, seeds were surface sterilized with 30% H_2O_2 for 90 s and placed on a modified Knop medium (L-Knop medium; Table 1) in Petri dishes. The Knop medium (George 1993) was complemented with trace elements, vitamins (Gamborg B5 Vitamin mixture, Duchefa Biochemie B. V., The Netherlands), and sucrose (Table 1), and was solidified with 0.6% Gelrite (Duchefa Biochemie B. V., The Netherlands). Seed germination was carried out at room temperature (25°C) with a 16/8 h day/night cycle. After 10–14 days, seedlings had developed vigorous cotyledons and a root length of 4–5 cm.

Fungal inoculum

Four *Paxillus involutus* isolates, collected in Great Britain, Austria, and Switzerland were used in the experiments (Table 2). They were cultivated on modified Melin Norkrans medium (Table 1) lacking malt extract (MMN-m) and were transferred to fresh medium every 4 weeks. For the inoculation, 6×6 mm mycelial plugs were cut and pre-grown on fresh MMN-m agar until they were covered by actively growing mycelium.

Fungal cultures of three *Hebeloma* and four *Laccaria* isolates (Table 2) were cultivated on 1/2 MMN medium (MMN with half amount of carbohydrates). Mycelial plugs

Table 2 Fungal taxa and abbreviations used in this study with details on fungal hosts and original habits

Abbreviation	Fungal taxa	Isolate provenance	Details of isolation, host and origin
Pax1	<i>Paxillus involutus</i> (Batsch: Fr.) Fr.	87.017	Isolated from a fruitbody in a coal waste with <i>Betula pendula</i> in Midlothian, Scotland ^a
Pax2	<i>Paxillus involutus</i> (Batsch: Fr.) Fr.	BOKU 04. M01	Isolated from a fruitbody under <i>P. tremula</i> on a heavy metal contaminated site in Carinthia, Austria ^b
Pax3	<i>Paxillus involutus</i> (Batsch: Fr.) Fr.	WSL #37.7	Isolated from a fruitbody from a <i>Salix</i> - and <i>Betula</i> stand in Switzerland ^c
Pax4	<i>Paxillus involutus</i> (Batsch: Fr.) Fr.	WSL #37.10	Isolated from a fruitbody on a heavy metal contaminated site in Switzerland ^d
Lac1-1	<i>Laccaria bicolor</i> (Maire) P.D. Orton	S-238a	Isolated from a fruitbody under <i>Tsuga mertensiana</i> stand, Crater Lake National Park, Oregon ^e
Lac1-2	<i>Laccaria bicolor</i> (Maire) P.D. Orton	CBS 560.96	
Lac2	<i>Laccaria proxima</i> (Boud.) Pat.	CBS 592.89	
Lac3	<i>Laccaria laccata</i> (Scop.) Berk. & Broome	CBS 377.89	
Heb1	<i>Hebeloma cylindrosporum</i> Romagn.	CBS 557.96	
Heb2-1	<i>Hebeloma crustuliniforme</i> (Bull.) Quél.	85.023	Isolated from spores from a fruitbody under <i>Picea sitchensis</i> ^f
Heb2-2	<i>Hebeloma crustuliniforme</i> (Bull.) Quél.	CBS 163.46	

^{a,e,f} (Finlay et al. 1992)

^b (Krpata et al. 2008)

^c (Sell et al. 2005)

^d Personal communication (I. Brunner (2005) WSL, Birmensdorf, Switzerland)

of these fungi were sub-cultured every 6 weeks and prepared for the inoculation procedure as described for *P. involutus*.

Testing of media for mycorrhizal symbiosis

Populus tremula plantlets were inoculated with two strains of *P. involutus* (Pax1, Pax3) using a modified sandwich technique (Peterson and Chakravarty 1991). Mycelial plugs, one plug per Petri dish (90 mm diameter), were placed on washed and autoclaved cellophane sheets, laid over either MMN-m or L-Knop-s (modified L-Knop medium without sucrose) media. Ten-day-old plantlets from the germination plate were placed on the cellophane with their roots arranged toward the expanding mycelium. The Petri dishes contained three plants on average and each fungus-medium combination was replicated twice. Dishes were sealed with Parafilm and incubated horizontally at 25°C with a 16-h light/8-h dark regime.

Additionally, *P. tremula* was inoculated on G-MMN medium (Gafur et al. 2004). For this experimental setup, five mycelial plugs were placed in two rows on sheets of cellophane. After 4 days plantlets were arranged in one row (five plants per plate), with their roots oriented toward the mycelial plugs (Burgess et al. 1996). Sealed Petri dishes were incubated in a slanted position under controlled conditions as described above. After 5 weeks, the survival rate of the plantlets, fungal growth and fungal habit were determined. The formation of mycorrhizas was evaluated macroscopically based on morphological characteristics, such as stimulation of lateral root growth, root ramification, shape and color of root tips.

Optimization of the inoculation experiment

Populus tremula plantlets were inoculated with the four *P. involutus* isolates on a further modified nutrient medium. Based on the results obtained on the different media, this L-MMN medium contained components of the Knop as well as the MMN medium (Table 1). Three plugs per plate were placed on cellophane sheets laid over the L-MMN medium and grown for 4 days. Five-week-old *P. tremula* plantlets were transferred to the dishes with their roots arranged around the inoculum. The shoots were left to stick out of the Petri dishes through openings, cut into the sidewall with a hot needle (Wong and Fortin 1988). Petri dishes were sealed with Parafilm and autoclaved silicon. They were further wrapped in aluminum foil and positioned vertically in an incubation box, which provided high humidity to the plant shoots (16 h light; 25 °C). The experiments were performed in triplicates. After 5 weeks, the survival rate of the plantlets, fungal growth and fungal habit were observed. The roots were evaluated macroscopically for mycorrhiza formation.

Preparation of EM root tips for microscopy

Single root tips showing morphological characteristics of mycorrhiza symbiosis were fixed in 2.5% glutaraldehyde in 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (0.1 M, pH 6.8). They were subsequently dehydrated using a graded ethanol series and infiltrated with Spurr resin. Semi-thin sections (1 µm) were cut, stained with toluidine blue and examined under a light microscope at ×400 magnification (Zeiss Axiovert 200M, Axiocam MRc5). Based on the presence of both mycelial sheath and Hartig net, the fungal isolates were classified as compatible or incompatible with *P. tremula*. Fungi that failed to form a characteristic Hartig net but formed a hyphal sheath were classified as intermediate. The anatomy of hyphal sheath and Hartig net formation were characterized according to Agerer (1990).

Evaluation of the general suitability of the medium composition

Plantlets were inoculated with *Hebeloma* and *Laccaria* isolates (Table 2) to evaluate the general suitability of the L-MMN medium for mycorrhizal synthesis on *P. tremula*. Based on the experiment described above, plantlets were also inoculated with *P. involutus* (Pax2) as a control treatment. The experimental setup followed the modified sandwich technique (Malajczuk et al. 1990). Mycelial plugs, one plug per Petri dish, were placed on cellophane and grown for 14 days. Thereafter, 2-week-old plantlets were arranged in the Petri dishes with their roots positioned on the expanding mycelial mats. Petri dishes, containing six plantlets each, were incubated in a horizontal position at room temperature (25°C) with a 16-h light/8-h dark regime. After 4 weeks, plant growth and fungal vitality were observed macroscopically. Single root tips showing characteristic features of mycorrhizal symbiosis were fixed and embedded in resin as described above. Semi-thin sections were cut and observed microscopically.

Results

Testing of media for mycorrhizal symbiosis

At least 50% of *P. tremula* plantlets inoculated on L-Knop-s nutrient medium survived, in contrast to only 8.5% and 20% of the plantlets treated on modified MMN media (Table 3). Plant survival was further determined by the fungal isolates, as plants inoculated with Pax1 generally had better survival rates than plants inoculated with Pax3. Plant survival was best (100%) when inoculated with Pax1 and grown on L-Knop-s medium.

Table 3 *Populus tremula* survival rate, fungal viability and mycorrhiza formation tested on three different nutrient media and with at least two *Paxillus involutus* isolates

Medium	Fungal isolate ^d	Number of plants inoculated	Plant survival rate ^e	Fungal viability ^f	Mycorrhiza formation ^g
MMN-m ^a	Pax1	6	17%	+	No
	Pax3	6	0%	+	No
			8.5% (mean value)		
L-Knop-s ^b	Pax1	6	100%	–	No
	Pax3	6	50%	–	No
			75% (mean value)		
G-MMN ^c	Pax1	10	20%	+	No
	Pax2	10	30%	+	No
	Pax3	10	10%	+	No
	Pax4	10	20%	+	No
			20% (mean value)		

^a Modified Melin Norkrans medium (MMN, Table 1) lacking malt extract

^b New medium composition based on the Knop nutrient solution (L-Knop, Table 1) lacking sucrose

^c Adapted modified Melin Norkrans medium following the protocol of Gafur (Gafur et al. (2004), G-MMN, Table 1)

^d Abbreviations of the *Paxillus involutus* isolates are listed in Table 2

^e Plant survival rate was specified by the percentage of living plantlets

^f Fungal viability was indicated positive (+) or negative (–) based on fungal growth and mycelial habit

^g Mycorrhizas were determined macroscopically by morphological root characteristics indicative of mycorrhiza formation

In addition to plant survival, their appearance and growth habit were observed. Plantlets inoculated on L-Knop-s medium showed uniform growth, with just occasional discolorations and single lesions. Shoot discolorations were only observed in combination with Pax3. In contrast, plants grown on G-MMN and especially MMN-m medium showed a severe reddening of leaves and dark lesions.

Fungal viability and habit were also affected by the inoculation medium (Table 3). *P. involutus* isolates grew very well on modified MMN media, while the L-Knop-s medium markedly inhibited mycelial growth and the color of the mycelium changed from bright cream to light brown.

No mycorrhiza formation was observed, regardless of medium composition and fungal isolates (Table 3).

Optimization of the inoculation procedure and description of plant performance and EM

Use of the modified L-MMN medium resulted in a plant survival rate >50% and in vital fungal growth (Table 4). Anatomical features typical for mycorrhizas were observed with all four *Paxillus* isolates.

Populus tremula plantlets inoculated with Pax1 on L-MMN medium were delicately built but developed a vigorous root system. Macroscopic evaluation of root tips indicated mycorrhizal symbiosis. Lateral root growth was stimulated and straight and unramified or monopodial ramified mycorrhizas were formed. Cross cuttings revealed a loosely woven (plectenchymatous) mantle of at least four to five layers and single hyphae penetrating between the

Table 4 Indications of a successful inoculation procedure (plant survival rate, fungal viability and fungal compatibility) for *P. tremula* with several *P. involutus* isolates conducted on L-MMN medium

Medium	Fungal isolate ^b	Number of plants inoculated	Plant survival rate ^c	Fungal viability ^d	Fungal compatibility ^e
L-MMN ^a	Pax1	6	67%	+	+
	Pax2	6	83%	+	+
	Pax3	6	50%	+	~
	Pax4	6	50%	+	+
			62.5% (mean value)		

^a New medium composition based on the modified Melin Norkrans Medium (L-MMN, Table 1)

^b Abbreviations of the fungal isolates are listed in Table 2

^c Plant survival rate was specified by the percentage of living plantlets

^d Fungal viability was indicated positive (+) or negative (–) based on fungal growth and mycelial habit

^e Fungi were classified as compatible (+) or incompatible (–) based on the presence of both mycelial sheath and Hartig net. Fungi which failed to form a Hartig net although enveloping the root tips with a mycelial mantle were termed intermediate (~).

epidermal cells (paraepidermal Hartig net). The cells of the epidermal layer were slightly enlarged but did not show radial elongation (Fig. 1).

Plants inoculated with Pax2 appeared robust with healthy green leaves and a vigorous root system. Mycorrhizal symbiosis was characterized by stimulated lateral root growth and the formation of simple and unramified mycorrhizas. Mycorrhizal root tips were stout and dark-colored. Cross sections revealed a dense mantle (pseudoparenchymatous mantle) and the formation of a paraepidermal Hartig net. No enlargement and radial cell elongation was detected within the epidermal cell layer.

Populus tremula plantlets in combination with Pax3 had extensive root growth and were robust in general, with few discolorations of shoots. Root systems were characterized by an enhanced production of lateral roots and dark and swollen root tips forming straight and unramified mycorrhizas. Cross sections cut near the tip proved the formation

of a characteristic sheath interweaving single cells of the calyptra. Successional sections revealed hyphae of the inner mantle layer penetrating epidermal cells and growing towards the root cylinder. Thus, the symbiosis lacked a characteristic Hartig net (Table 4).

Inoculation with Pax4 resulted in poor shoot and root growth. Root systems consisted of few lateral roots with slightly clubbed and dark-colored unramified mycorrhizas. This fungus formed a very thick and dense mantle (pseudoparenchymatous) and a paraepidermal Hartig net. At tip bases, hyphae of the inner mantle were seen to penetrate cells of the epidermal layer, the cortex and the central root cylinder (Fig. 1).

Evaluation of the medium composition on the performance of other fungal inocula

The L-MMN medium was used for inoculation experiments with various *Laccaria*, *Hebeloma*, and *Paxillus* isolates. Plantlets, completely enclosed in the Petri dishes, achieved a survival rate of 100%. Isolates of each fungal genus were able to form mycorrhizas with *P. tremula* (Table 5). However, there was considerable variation in extent of hyphal sheath and Hartig net formation.

Populus tremula plantlets inoculated with Lac1–1 grew well and formed dark and slightly clubbed mycorrhizas. In semi-thin sections, a dense and voluminous pseudoparenchymatous mantle was visible near the root apex enclosing cells of the root calyptra. In older parts, the hyphal sheath became thin and loose. No characteristic Hartig net was observed and the epidermal cells remained small and tightly packed (Table 5 and Fig. 2)

The strain Lac1-2 severely stressed *P. tremula*. It depressed plant growth and induced distinct leaf discolorations and numerous lesions. Mycorrhiza formation could not be detected macroscopically (Table 5), whereas microscopic evaluation confirmed the formation of shortened and dark lateral roots. The screening of cross sections revealed the formation of a cohering but shallow and loosely woven sheath (plectenchymatous mantle) and the presence of a paraepidermal Hartig net (Fig. 2).

Lac2 inoculated plantlets grew vigorously, but did not form any mycorrhizas, with no hyphal sheath and no Hartig net (Table 5).

Populus plantlets inoculated with Lac3 developed poorly. Plant shoot and root growth were reduced and leaves showed severe red discolorations. No mycorrhizas were formed; however, hyphal clusters were detected within the roots growing within the vascular bundle and the endodermis (Table 5).

Heb1-inoculated *Populus* plantlets developed vigorously and produced short and distinctly clubbed lateral roots with silvery appearance. Semi-thin sections confirmed the

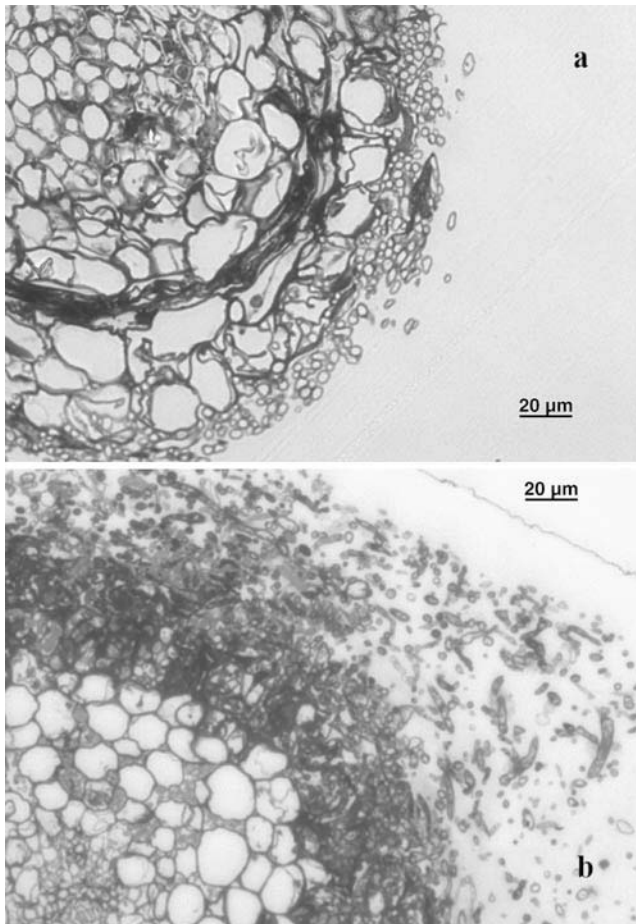


Fig. 1 *Paxillus involutus* isolates forming mycorrhizas with *P. tremula* (bars: 20µm). **a** Isolate Pax1 forming a loosely woven mantle and a paraepidermal Hartig net. **b** Cross section at the bases of isolate Pax4 mycorrhiza showing hyphal proliferation toward the root cylinder

Table 5 Indications of a successful inoculation procedure (plant survival rate, fungal viability and fungal compatibility) for *P. tremula* with several ectomycorrhizal fungi conducted on L-MMN medium

Medium	Fungal isolate ^b	Number of plants inoculated	Plant survival rate (%) ^c	Fungal viability ^d	Fungal compatibility ^e
L-MMN ^a	Lac1-1	6	100	+	~
	Lac1-2	6	100	+	+
	Lac2	6	100	–	–
	Lac3	6	100	+	–
	Heb1	6	100	+	+
	Heb2-1	6	100	+	–
	Heb2-2	6	100	+	+
	Pax2 (control)	6	100	+	+
			100 (mean value)		

^a New medium composition based on the modified Melin Norkrans Medium (L-MMN, Table 1)

^b Abbreviations of the fungal isolates are listed in Table 2

^c Plant survival rate was specified by the percentage of living plantlets

^d Fungal viability was indicated positive (+) or negative (–) based on fungal growth and mycelial habit

^e Fungi were classified as compatible (+) or incompatible (–) based on the presence of both mycelial sheath and Hartig net. Fungi that failed to form a Hartig net although enveloping the root tips with a mycelial mantle were termed intermediate (~).

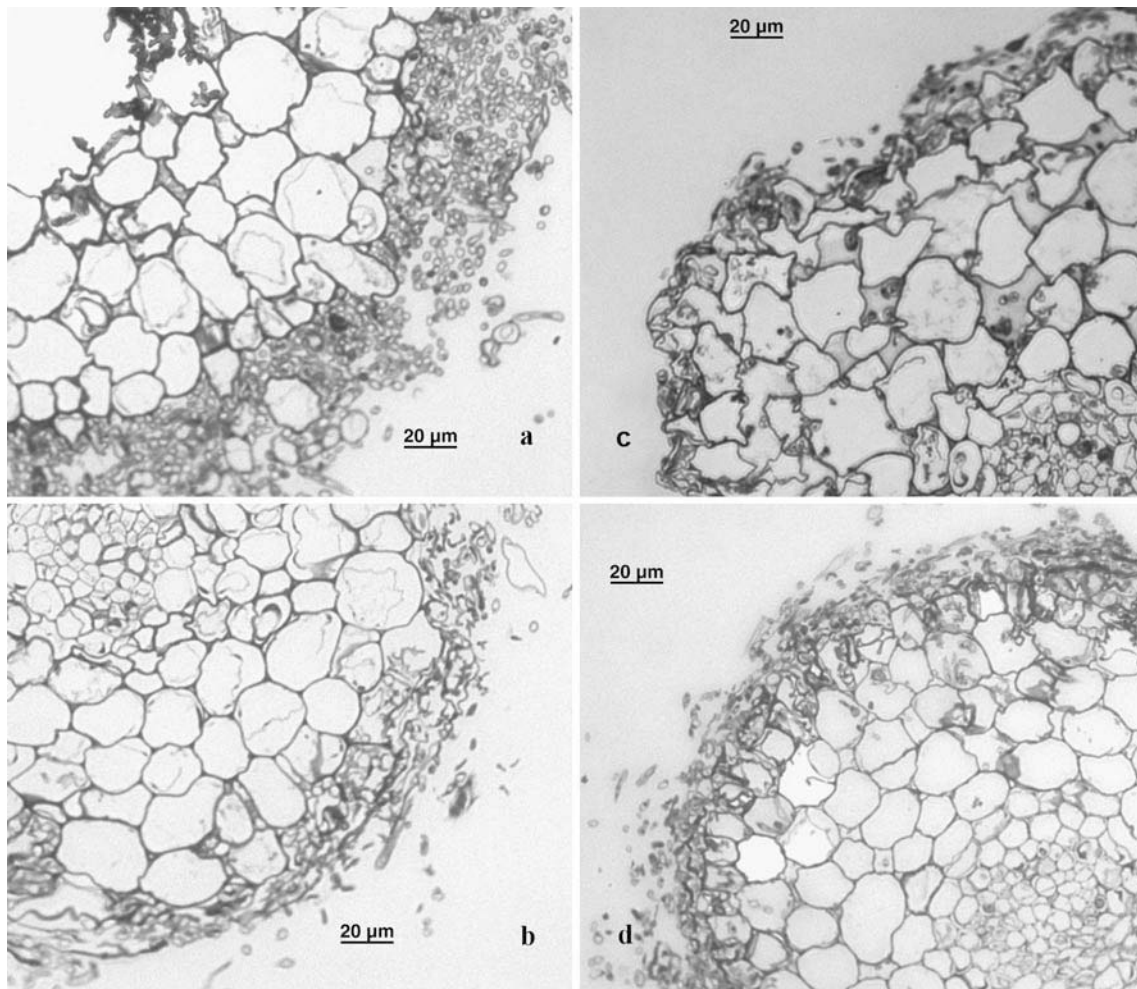


Fig. 2 *Populus tremula* mycorrhizas formed with *Laccaria bicolor* and *Hebeloma crustuliniforme* (bars: 20 µm). **a** Lac1-1 mycorrhiza showing a dense mantle but lacking a characteristic Hartig net. Epidermal cells remain small and tightly packed. **b** Lac1-2 forming a shallow and loosely woven sheath and a paraepidermal Hartig net. **c**

Hyphae of Heb2-1 growing adhered to the root surface and within the root cortex. Cortex cells are loosely interconnected. **d** Heb2-2 mycorrhiza cut at the base of the root tip showing a shallow sheath and a para- to periepidermal Hartig net. Hyphae start penetrating between cortex cells toward the endodermis

presence of mycorrhizas with an extremely thick and dense hyphal sheath (pseudoparenchymatous mantle) and a paraepidermal Hartig net (Table 5).

Plantlets inoculated with the fungal strain Heb2-1 did not form mycorrhizas. In semi-thin sections, loose hyphae were seen to adhere to the epidermis and single hyphae penetrated between cells of the epidermal layer, the cortex, and the vascular cylinder. Cortex cells were interconnected loosely (Table 5 and Fig. 2).

A further strain of *Hebeloma crustuliniforme* (Heb2-2) negatively affected plant growth. Plant roots appeared very thin and reddish and showed untypical ramifications. Individual short and darkened lateral roots were observed. Their mycorrhizal status was confirmed by a plectenchymatous mantle and hyphae partly enveloping the epidermal cells (paraepidermal/ periepidermal Hartig net). In older parts of the tips, also this fungus started to penetrate between cortical cell layers toward the endodermis (Table 5 and Fig. 2).

Pax2 was confirmed as a compatible fungus for the mycorrhization of *P. tremula*. Plants inoculated with this *Paxillus* isolate appeared robust and had a well-developed root system. Microscopic evaluation confirmed mycorrhizal symbiosis (Table 5).

Discussion

Interest in inoculation of *Populus* species with ectomycorrhizal fungi has recently increased, mainly due to the use of poplars for biomass production and application in phytoremediation. Therefore, suitable information on mycorrhiza synthesis with poplar species such as *Populus tremula* is highly valuable. In the present study, *P. tremula* seedlings were variously inoculated with ectomycorrhizal fungi in Petri dishes in vitro. Synthesis experiments were conducted on several nutrient media, which clearly affected the formation of functional mycorrhizas.

The first series of experiments did not result in successful mycorrhiza synthesis mainly due to very poor plant survival on media based on the widely used MMN formulation. Only 8.5% of *P. tremula* plantlets survived on MMN-m medium whereas mycorrhiza formation on similar media was documented with *Pinus sylvestris* (Niemi et al. 2007) and *Betula pendula* (Brun et al. 1995; Blaudez et al. 1998). Modified MMN media, additionally supplemented with trace elements, facilitated plant growth and mycorrhiza formation with both gymnosperm *Larix* and *Pinus* (Wong and Fortin 1988) and broad-leaved *Eucalyptus* (Malajczuk et al. 1990) species. Moreover mycorrhiza formation was confirmed with *Populus* hybrids *P. tremula* × *tremuloides* (Hampp et al. 1996; Selle et al. 2005) and *P. tremula* × *alba* (Gafur et al. 2004).

In contrast, present experiments conducted on the G-MMN medium (Gafur et al. 2004) did not prove suitable for *P. tremula*. This may indicate the specific nutrient demand of the plantlets during mycorrhizal synthesis. Plant survival increased to 75% or more by the addition of a vitamin mixture (nicotinic acid, pyridoxine, myo-Inositol, and thiamine), usually supplemented to plant tissue culture media (George 1993). Normally, the addition of thiamine meets the vitamin requirements of plants during mycorrhizal synthesis. Malt extract, which contributes some vitamins, amino acids and growth regulants (George 1993), could not compensate for the lack of vitamins in the G-MMN medium.

It seems that *P. tremula* seedlings were not able to produce sufficient amounts of vitamins, required for normal growth and development. The demand of *P. tremula* for additional micronutrients and especially vitamins may be due to the very low seed weight of approximately 0.01 g. This is less than 10% of the average seed size of species such as *Populus alba* or *Betula pendula* (Bärtels 1989). *P. tremula* seeds lack the endosperm (Borset 1954), which markedly influences juvenile plant growth. In general, *P. tremula* mainly reproduces by root suckers and only to a much less extent by seeds (Worrell 1995). Moreover, seed quality varies considerably (Worrell 1995). Seed quality may be further impaired by environmental stress such as for example heavy metal soil contamination of the collection area (Fedorkov 1999).

Common media based on the MMN formulation did not favor survival of *P. tremula*, but promoted EM fungal growth. The L-Knop-s medium, on the other hand, promoted plant growth but failed to feed the EM fungus *P. involutus*. This fungus is in general easily cultured, and has been extensively used in studies on mycorrhizal functioning (Wallander and Söderström 1999). The failure may be due to differences between the media in carbon, nitrogen, and phosphorus supply. The L-Knop-s medium does not contain any sucrose or malt extract. The only carbon source within the inoculation procedure derives from the glucose leftover in the mycelial plug.

Contrary to our results, mycorrhiza formation has been observed on sugar-free media with several EM fungi on *Populus* (Selle et al. 2005), *Larix*, and *Pinus* species (Wong and Fortin 1988). The use of exogenous supply of glucose for mycorrhizal studies has repeatedly been disputed in the past. Duddridge (1986) cautioned against exogenous glucose supply, as mycorrhizas may be formed by partners that would be regarded incompatible in nature. High levels may also lead to the formation of an unusual host–fungus interface (Duddridge and Read 1984). However, Hutchison and Piché (1995) observed that plant infections were not necessarily increased with rising glucose concentrations, but that glucose may enhance mycelial growth. The glucose

content of the inoculum applied in our experiments obviously did not satisfy the fungal carbon demand until mycorrhizas were established.

The L-Knop-s medium contains nitrate, whereas ammonium is the predominant nitrogen source for EM fungi in soil and is supplemented to the most common media. Studies on the ability of EM fungi to utilize nitrate found generally much better growth on ammonium, although results differed considerably between fungal species and isolates (Finlay et al. 1992). None of the tested fungi, however, completely stopped growth on nitrate-supplemented media as observed in our experiment. *Paxillus involutus* isolate Pax1 was one of the best performing fungi on nitrate-supplemented medium. However, mycelial development was restricted to half of the normal growth within the first 2 months (Finlay et al. 1992). Thus, nitrate application may have contributed to depress fungal growth in our first experimental setup.

Whereas nitrogen supply of the L-Knop-s medium differs in the nitrogen compound added, phosphorus application differs in the concentration, which is reduced to one tenth normally supplied with the MMN medium. Such a reduction in phosphorus concentration was previously found to reduce the percentage of mycorrhizal root tips formed by late-stage fungi; however, it did not clearly affect linear extension of all fungi tested (Gibson and Deacon 1990).

The L-MMN-medium supported successful mycorrhizal synthesis of *P. tremula* with several EM fungi. First experiments conducted with *P. involutus* isolates revealed the compatibility of three strains, whereas one isolate failed to form a characteristic Hartig net. These differences among isolates are commonly observed (Cairney 1999) and agree with results from investigations on various *Paxillus* strains tested for the ability to form mycorrhizas with *Populus canescens* (Gafur et al. 2004).

In our study, *Paxillus involutus* isolates compatible with *P. tremula* formed a mantle of variable density and thickness. The Hartig net was consistently paraepidermal, formed by single hyphal rows within the epidermal layer. Characteristics of the mantle and the Hartig net observed here correspond to those of mycorrhizas formed by *P. tremuloides* and *P. canescens*, respectively (Godbout and Fortin 1985; Gafur et al. 2004). We did not observe radial elongation of epidermal cells, although this is frequently detected in mycorrhizas formed by angiosperms, where the Hartig net is restricted to the root epidermis.

Similar to *P. involutus*, isolates of *Laccaria bicolor* and *H. crustuliniforme* differed in their ability to form mycorrhizas with *P. tremula*. Also *L. bicolor* and *Hebeloma* spp. mycorrhizas were characterized by a paraepidermal Hartig net. This mycorrhizal characteristic is consistent with 32 EM fungi, with *P. tremuloides* as the host symbiont (Godbout and Fortin 1985).

In the last experimental setup *H. crustuliniforme*, however, seemed to enclose epidermal cells with Hartig net hyphae (periepidermal Hartig net). This agrees with investigations on *P. tremuloides* forming mycorrhizas with several fungal species (Godbout and Fortin 1985) and may be explained by the rate of fungal growth in general, the time roots are examined and the inoculation system used. However, cross sections through parts of roots with elongated epidermal cells angularly orientated toward the root apex may give the impression of several cell layers and thereby falsely be interpreted as periepidermal Hartig net.

In the present study, hyphae of the inner mantle occasionally penetrated cells of the root epidermis and grew within the cortex toward the root cylinder. Such hyphal proliferation is not typical in stable mycorrhizas but may be explained by the saprobic ability of individual EM fungi. This has particularly been found for *P. involutus* (Wallander and Söderström 1999) or single *Hebeloma* species (Marmeisse et al. 1999). Modifications of mycorrhizal anatomy generally indicate an imbalance of the symbionts based on the inoculation system utilized. Investigations by Duddridge (1986) demonstrated the impact of glucose on *Suillus grevillei* mycorrhiza ultrastructure, which underlines the relevance of a balanced nutrient supply as discussed above. A balanced mycorrhiza may further be attributed to conditions in the synthesis chamber not favoring one or the other of the symbionts (Duddridge 1986). The enclosure of only the plant roots in the last experiment may have strengthened *P. tremula* development and thereby affected the ability of the various fungi to successfully form mycorrhizas.

The inoculation systems used in our experiments mainly differed in the selective enclosure of the roots and the use of plantlets of different age. *P. tremula* inoculated in Petri dishes with their shoots sticking out had a survival rate of 62.5% on average. This relatively low value compared to the 100% observed for plantlets completely enclosed in Petri dishes may be due to the lower air humidity to which the shoots of these plants were exposed. This agrees with several observations on the high sensibility of *Populus* species to low air moisture (Hampp et al. 1996; Gafur et al. 2004). Plantlets once adapted to these environmental conditions developed vigorous shoots and a prolific root system. These benefits may be attributed to the use of more mature plantlets and to the fact that plants did not suffer from potential CO₂ deficiency or accumulation of volatile substances such as possible in a fully enclosed Petri dish system (Peterson and Chakravarty 1991).

To summarize, our results show that *P. tremula* may form mycorrhizas with EM fungi in Petri dishes in vitro comparable to American poplars and several *Populus* hybrids. However, *P. tremula* needs special nutrient support in the synthesis medium. A balanced micronutrient supply

and the addition of a number of vitamins are vital for plant growth and successful mycorrhizal synthesis.

The new medium composition used with sandwich techniques in the presented study may also be considered for other inoculation procedures. Several sterile and non-sterile inoculation techniques utilize substrates such as sand, perlite, peat, and vermiculite moistened with common nutrient solutions. These substrates may be complemented with the adapted medium composition and may thereby increase the proportion of successful EM synthesis on *P. tremula*. Apart from use in Petri dishes, it may further be employed with syntheses techniques carried out in growth pouches, jars, or containers.

Finally, ectomycorrhizal *P. tremula* plantlets may subsequently be used in studies on plant–fungus interactions such as heavy metal uptake by this accumulator plant species and within-plant heavy metal transport as affected by mycorrhizal status.

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5 Summary & Conclusion

The European poplar species *P. canescens* was grown in two dose-response experiments. Its growth was not affected by NH_4NO_3 -extractable soil Zn concentrations up to 60 mg Zn kg^{-1} soil, but it was completely inhibited at extractable concentrations above 90 mg Zn kg^{-1} soil. From this data an effective concentration of $68.5 \text{ mg extractable Zn kg}^{-1}$ soil was calculated at which plant growth was reduced by 50%. Foliar Zn concentrations increased with increasing extractable soil Zn to just above $2000 \text{ mg Zn kg}^{-1}$ dry mass. Concentration values coincide with results evaluated for the same clone, grown on a metalliferous field habitat in Arnoldstein. Calculated accumulation factors ($\text{AF} = \text{leaf Zn concentration} : \text{soil Zn concentration}$) decreased linearly with increasing extractable soil Zn concentrations when plotted on log-log scale. Such relations indicate the decreasing relative extraction efficiency with increasing soil Zn concentrations. Zn transfer factors ($\text{TF} = \text{leaf concentration} : \text{root concentration}$) in the second experiment increased from below 1 at the native Zn status of the soil up to 2.1 at an extractable soil Zn concentration of approximately 3 mg Zn kg^{-1} soil. Zn TFs were more variable in the first experiment where they reached a value of 2.1 at approximately 65 mg Zn kg^{-1} soil. Values for TF did not increase any further and were finally markedly decreased when plant growth was reduced by toxic soil Zn concentrations. Leaf dry mass production of *P. canescens* was reduced at foliar Zn concentrations above $1300 \text{ mg Zn kg}^{-1}$. This concentration was measured in a plant grown at 95 mg kg^{-1} extractable soil Zn. Physiological processes in leaves of various *Populus* species are already negatively affected by lower Zn concentrations.

The European aspen *P. tremula* was grown in soil substrate initially characterized by NH_4NO_3 -extractable Zn concentrations of $2.1 - 2.9 \text{ mg Zn kg}^{-1}$ and $0.2 - 0.3 \text{ mg Cd kg}^{-1}$. Compared to the latter *Populus* species, *P. tremula* biomass yield was low and foliar dry-weight did not significantly differ with increasing soil Zn addition.

However the foliar Zn (Cd) concentrations exceeded accumulation values observed for numerous *Salix* and *Populus* species showing maximum concentrations of $2740 \text{ mg Zn kg}^{-1}$ ($156 \text{ mg Cd kg}^{-1}$) dry-weight. Foliar metal concentrations significantly differed with increasing soil Zn availability. Calculated transfer factors for Zn and Cd ranged from 1.4 - 3.0 and 0.5 - 1.9 respectively. Maximum values of plant-internal Zn transfer considerably exceeded transfer factors of *P. canescens*. Moreover calculated translocation factors for *P. tremula* were significantly affected by ectomycorrhiza colonization.

The natural community of ectomycorrhizal fungi successfully formed mycorrhizas with *P. tremula* showing 41 EM root tips per meter root length on average. In control treatments formation of functional ectomycorrhizas was efficiently inhibited by γ -irradiation. In addition, γ -irradiation increased dissolved organic carbon (DOC) in the substrate and affected the extractability of Zn and Cd by 1M NH_4NO_3 . We found three times larger biomass and more than four times increased root lengths in the Irr-NM compared to the Nat-Myc treatments which may be explained by the doubled DOC concentrations and related Fe mobilization due to formation of labile complexes in the irradiation treatment and the absence of microbial competitors for (nutrient) resources. Our results indicate an imbalance of the normally mutualistic symbiosis between mycorrhizal fungi and the host at early growth stage, possibly further enhanced by the high susceptibility of the *P. tremula* seedlings obtained from a contaminated site. Foliar Zn concentrations were generally larger in the Nat-Myc treatments and exceeded those reported for numerous *Salix* and *Populus* species. While the Zn concentrations increased with increasing Zn additions, Zn translocation to shoots was inhibited at high Zn levels in the Nat-Myc treatments, indicating a barrier function of the mycorrhizal consortium.

In a final series of experiments pre-grown seedlings of *P. tremula* and fungal inocula of *Paxillus involutus* isolates were grown in Petri dishes *in vitro* to establish a standardized inoculation procedure for ectomycorrhiza formation. Common nutrient media based on the MMN formulation did not favor survival of *P. tremula*, but promoted fungal growth. On the other hand plant growth assisting media did not satisfy the fungal carbon demand until mycorrhizas were established. Survival of *P. tremula* seedlings increased with the addition of a set of vitamins (nicotinic acid, pyridoxine, myo-Inositol, thiamine), usually supplemented to plant tissue cultures whereas balanced ammonium and glucose supply promoted adequate fungal growth.

Experiments conducted with the new L-MMN medium revealed the compatibility of three *P. involutus* strains, whereas one isolate failed to form a characteristic Hartig net. Similar to *P. involutus*, isolates of *Laccaria bicolor* and *Hebeloma crustuliniforme* differed in their ability to form mycorrhizas with *P. tremula*. These differences among isolates are commonly observed and agree with results from investigations on various *Paxillus* strains tested with the European poplar species *P. canescens*. Mycorrhizas associated with *P. tremula* mainly showed a mantle of variable density and thickness and a paraepidermal Hartig net consistently formed by single hyphal rows within the epidermal layer. Radial elongation of epidermal cells was not observed, although this is frequently detected in mycorrhizas formed by angiosperm plant species.

Our results show that *P. tremula* may form mycorrhizas with EM fungi in Petri dishes *in vitro* comparable to American poplars and several *Populus* hybrids. However, a balanced macro- and micro-nutrient supply and the addition of a number of vitamins are vital for plant growth and successful mycorrhizal synthesis.

Based on the current study it can be concluded that the examined *P. canescens* accession may be used in phytoextraction processes, most suitable for planting on sites that contain up to 30 mg extractable Zn kg⁻¹ soil, where foliar Zn concentrations reached 1000 mg Zn kg⁻¹. The suggested upper margin of 30 mg extractable Zn kg⁻¹ soil is higher than measured in the vast majority of contaminated field sites.

P. tremula Zn and Cd contents were generally low, particularly due to plant species specific growth properties. Thus this accession may not be suitable for phytoextraction purposes regardless of the mycorrhizal status. The observed barrier properties in the mycorrhizal treatments suggest that mycorrhizal inoculation of *P. tremula* may be a promising strategy to enhance revegetation and phytostabilization of metal-polluted sites. However, early-stage growth of *P. tremula* may be limited by imbalances between host plant and fungal symbionts in such nutrient-deficient, toxic environments. Moreover the risk to increased topsoil Zn/Cd accumulation due to periodic leaf fall and decomposition has to be considered.

The new medium composition successfully promoting mycorrhiza formation of the European poplar *P. tremula* with individual EcM fungi in Petri dishes *in vitro* may also be considered for use in non-sterile inoculation techniques. These techniques make use of growth substrates such as sand, peat and vermiculite or soil-based growth substrates applied in growth pouches, jars or containers and promise more vigorous mycorrhizal plants for use in revegetation and phytoremediation processes in the field.

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7. List of Publications

7.1. Publications in SCI-Journals

12. Langer, I; Santner, J; Krpata, D; Fitz, W; Wenzel, W.W; Schweiger, P (2011): Ectomycorrhizal impact on Zn accumulation of *P. tremula* L. grown in metalliferous soil with increasing Zn concentration. Submitted (Plant and Soil)
11. Vaculník, M; Langer, I; Windhager, C; Adlassnig, W; Puschenreiter, M; Lux, A; Hauser, M.T (2011): Anatomical differences of roots correlate with Zn and Cd accumulation capacities of *Salix caprea* isolates. Submitted (Environmental Pollution)
10. Langer, I; Syafruddin, S; Steinkellner, S; Puschenreiter, M; Wenzel, W.W (2010): Plant growth and root morphology of *Phaseolus vulgaris* L. grown in a split-root system is affected by heterogeneity of crude oil pollution and mycorrhizal colonization. Plant and Soil. 332:339–355.
9. Syafruddin, S., Wieshammer, G., Puschenreiter, M., Langer, I., Wieshammer-Zivkovic, M., Wenzel, W.W. (2010): Effect of N and P fertilization and aeration on biodegradation of crude oil in aged hydrocarbon contaminated soils. Plant Soil Environ.56: 163-167.
8. Krpata, D; Fitz, W; Peintner, U; Langer, I; Schweiger, P (2009): Bioconcentration of zinc and cadmium in ectomycorrhizal fungi and associated aspen trees as affected by level of pollution. ENVIRON POLLUT. 157(1): 280-286.
7. Langer, I; Krpata, D; Fitz, WJ; Wenzel, WW; Schweiger, PF (2009): Zinc accumulation potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone in a dose-response study. ENVIRON POLLUT. 157(10): 2871-2877.
6. Lenzemo V., Kuyper T.W., Urban A., Vegvari G., Puschenreiter M., Schickmann S., Langer I., Steinkellner S., Vierheilig H. (2009): The arbuscular mycorrhizal host status of plants cannot be linked with the *Striga* seed-germination-activity of plant root exudates. J PLANT DIS PROTECT, 116, 86-89;
5. Krpata, D; Peintner, U; Langer, I; Fitz, WJ; Schweiger, P (2008): Ectomycorrhizal communities associated with *Populus tremula* growing on a heavy metal contaminated site. Mycol Res. 112(9):1069-1079
4. Langer, I; Krpata, D; Peintner, U; Wenzel, WW; Schweiger, P (2008): Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula* L. MYCORRHIZA. 18(6-7): 297-307

3. Steinkellner, S; Lenzemo, V; Langer, I; Schweiger, P; Khaosaad, T; Toussaint, JP; Vierheilig, H (2007): Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules*. 2007; 12(7):1290-1306
2. Steinkellner, S., Langer, I. (2004): Impact of tillage on the incidence of *Fusarium* spp. in soil. *Plant and Soil*, 267, 13-22
1. Steinkellner, S., Shala-Mayrhofer, V., Langer, I. (2002): Influence of tillage on *Fusarium* spp. in different crop rotation systems. *Mycotoxin Research*, 18, 11-15

7.2. Presentations at international conferences

- Langer, I; Riedmann, P; Puschenreiter, M; Wenzel, W.W. (2011) *Thlaspi goesingense* Hálácsy root plasticity in serpentine soils contradicts the metal foraging behavior. [1. Tagung der Österreichischen Gesellschaft für Wurzelforschung; 13-14. Sept.2011; Gumpenstein]
- Langer, I; Riedmann, P; Puschenreiter, M; Wenzel, W.W. (2011) *Thlaspi goesingense* root growth and morphology as affected by heterogeneity of serpentine soils. [11th International Conference on the Biochemistry of Trace Elements; July 3-7, 2011; Florence]
- Reichert S, Horner A, Hage-Ahmed K, Vierheilig H, Langer I, Steinkellner S (2010): The interaction of the plant pathogen *Fusarium oxysporum* f.sp. *lycopersici* and the arbuscular mycorrhizal fungi *Glomus mossae* in tomato roots. [Poster] [IMC 9 – The biology of fungi, Edinburgh, UK, August 1-6, 2010]
- Horner, A., Hage-Ahmed, K., Langer, I., Steinkellner, S. (2010): Alteration of root morphology of tomato roots due to *Fusarium oxysporum* f. sp. *lycopersici*, arbuscular mycorrhizal fungi and phosphorous supply. In: Gabriele Berg, Ilaria Pertot, Biological control of fungal and bacterial plant pathogens. Climate change: challenge or threat to biocontrol [Poster] [Biological control of fungal and bacterial plant pathogens. Climate change: challenge or threat to biocontrol, Graz, Austria, June 7-10]
- Langer, I., Syafruddin, S., Schweiger, P., Steinkellner, S., Puschenreiter, M., Wenzel, W. W. (2009): Arbuscular mycorrhizal fungi systemically compensate crude oil effects on root biomass. In: Margarita Himmelbauer, Willibald Loiskandl Eds., 7 th ISRR Symposium Root Research and Applications , p. 105 [International Symposium RootRAP, Vienna, Austria, SEPT 2-4, 2009]
- Santner, J., Langer, I., Wenzel, W.W., Schweiger, P. (2009): Ectomycorrhization decreases the ratio of Cd/Zn translocation from roots to leaves of *Populus tremula* plants. [10th ICOBTE - Frontiers in Trace Elements - Research and Education, Chihuahua, MEXICO, JUL 13-16, 2009]

- Vaculík M, Lux A, Puschenreiter M, Langer I, Windhager C, Adlassnig W, Lichtscheidl I, Hauser M-T (2009): Root anatomical responses of two *Salix caprea* isolates growing in different environmental conditions. . In: Himmelbauer M, Loiskandl W, RootRAP 7th International Symposium Root Research and Applications (ISRR) [Poster] [RootRAP 7th International Symposium Root Research and Applications (ISRR), Vienna, AUSTRIA, SEP, 2-4]
- Windhager C, Türktas M, Langer I, Puschenreiter M, Hauser M-T (2009): Does Heavy Metal Induced Gene Expression Correlate with Zn and Cd Accumulation Capacities of the Willow *Salix caprea*? . In: COST Action 859, Phyto2009: “Phytotechnologies to promote sustainable land use and improve food safety” [Poster] [COST Action 859 – Phyto2009: “Phytotechnologies to promote sustainable land use and improve food safety”, Ascona, Switzerland, OCT, 12–16]
- Syafruddin S, Langer I, Schweiger P, Puschenreiter M, Wenzel W.W (2008): Crude oil contamination and arbuscular mycorrhiza differentially affect *Phaseolus vulgaris* root morphology [Poster] [International Symposium: Land Use after the Tsunami – Supporting Education, Research and Development in the Aceh Region; Indonesia, November 4-6, 2008]
- Langer I., Santner J., Wenzel W.W., Schweiger P. (2008): Influence of ectomycorrhizae on heavy metal uptake by *Populus tremula*. In: Blum W.E.H., Gerzabek M.H., Vodrazka M. (Eds.), Book of Abstracts, 186; ISBN: 978-3-902382-05-4 [Poster] [Eurosoil 2008, Vienna, AUSTRIA, August 25-29, 2008]
- Langer I., Santner J., Wenzel W.W., Schweiger P. (2008): The impact of ectomycorrhizas on *Populus tremula* heavy metal uptake in response to elevated zinc supply. In: Turnau K., Oleszek W. (Eds), Plant-Microbial-Interactions 2008 Book of Abstracts and Program, 47-48 [Plant-Microbial-Interactions, Krakow, POLAND, July 2-6, 2008]
- Langer I, Schweiger P (2006): Improved method for inoculation of *Populus tremula* with ectomycorrhizal fungi. In: Estacion Experimental del Zaidin (Hrsg.), 5th International Conference on Mycorrhiza "Mycorrhiza for Science and Society", p 228, International Conference on Mycorrhiza, 23.-27.7.2006, Granada; ISBN: Gr-1546-06 [International Conference on Mycorrhiza, Granada, 23.-27.7.2006]

7.3. Research reports

- Glauning, J., Langer, I., Shala-Mayrhofer, V., Lanzer, B., Liebhard, P. (2002): Untersuchungen zur Beikrautflora und zu ausgewählten Pflanzenschutzproblemen bei unterschiedlicher Bodenbearbeitung. Studie im Auftrag des Bundesministeriums für Land- und Forstwirtschaft Wien

Steinkellner, S., Glauninger, J., Langer, I., Shala-Mayrhofer, V., Liebhard, P., Schausberger, P., Grabenweger, G., Lanzer, B., Lawson-Balagbo, E. (2002): Auswirkungen konservierender Bodenbearbeitungsmaßnahmen auf das Auftreten von Pflanzenkrankheiten, Schädlingen und Beikräutern in österreichischen Fruchtfolgesystemen. Abschlußbericht Forschungsprojekt, Nr. 1181, 117 S; im Auftrag des BM:LFUW

8. Curriculum Vitae



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First Language: German

Foreign Language: English

Education

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|-----------|--|
| 1986-2000 | BOKU-University of Natural Resources and Applied Life Sciences; Studies of Agricultural Sciences; Graduation to Master of Engineering (Dipl.-Ing.) |
| 2004-2011 | Doctoral Thesis at the BOKU-University of Natural Resources and Applied Life Sciences; Title: "Evaluation for a plant-assisted bioremediation approach: Zn (Cd) accumulation properties of indigenous poplar species and the impact of ectomycorrhizas on phytoextraction characteristics" |

Career History

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|-----------|--|
| 2009-2010 | BOKU-University of Natural Resources and Life Sciences; Department of Forest and Soil Sciences: Evaluation of heavy metal responses in <i>Salix caprea</i> for the improvement of phytoextraction strategies (FWF project L433 B17) |
| 2008-2009 | BOKU-University of Natural Resources and Life Sciences; Department of Forest and Soil Sciences: The Re-establishment of Human Resources, Curricula, System and Institutions at the Agricultural Faculty of the Syiah Kuala University in Aceh (Asia Link Project 0800/110-005) |
| 2004-2008 | BOKU-University of Natural Resources and Life Sciences; Department of Forest and Soil Sciences: Mycorrhizal associations of Zn/Cd-accumulating poplars: perspectives for phytoremediation? (FWF project P17012-B06) |

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|-----------|--|
| 2003-2004 | BOKU-University of Natural Resources and Life Sciences; Department of Applied Plant Sciences and Plant Biotechnology: Making plants resistant to plant parasitic nematodes (NONEMA –QLK5-CT) |
| 2001-2003 | BOKU-University of Natural Resources and Life Sciences; Department of Applied Plant Sciences and Plant Biotechnology: Auswirkungen konservierender Bodenbearbeitungsmaßnahmen auf das Auftreten von Pflanzenkrankheiten, Schädlingen und Beikräutern in österr. Fruchtfolgesystemen. (BM:LFUW Project 1181, 117 S) |

Current research areas

- Rhizosphere interactions affecting plant growth, the uptake, translocation and accumulation of toxic trace elements by accumulator and hyperaccumulator plants
- Root morphology in response to abiotic stresses (soil pollutants) and mycorrhizal symbiosis in heterogeneous soil systems
- Ecto- and arbuscular mycorrhizas as affected by toxic trace elements and hydrocarbon pollutants
- Mycorrhizal impact on host plants and signalling pathways

Affiliation with professional societies

- Austrian Society of Root Research (ASSR)
- International Mycological Association (IMA)

Academic service

- Reviewer for several scientific journals (Plant and Soil, Soil Biology and Biochemistry, New Biotechnology, Phytochemistry, International Biodeterioration & Biodegradation, Journal of Hazardous Materials, Sydowia,)

Teaching Activities

- | | |
|---------|--|
| SS 2010 | Soil - Microbe - Plant Interactions: Fundamentals and Applications (cooperation) |
| SS 2009 | “Experimental techniques on root morphology and mycorrhization” (Practical course) in: “Soil-Microbe-Plant Interactions: Fundamentals and Applications” (IP SOKRATES – Summerschool) |
| SS 2008 | “Experimental Techniques to Investigate Mycorrhiza” (Practical course) in: “Soil-Microbe-Plant Interactions: Fundamentals and Applications” (IP SOKRATES – Summerschool) |

- SS 2007 “Experimental Techniques to Investigate (Arbuscular-) Mycorrhiza” (Practical course) in: “Soil-Microbe-Plant Interactions: Fundamentals and Applications” (IP SOKRATES – Summerschool)
- SS 2007 „Ectomycorrhiza“ in: „Symbionten und Pathogene in der Rhizosphäre“ (invited lecture)