



Friedrich-Schiller-Universität Jena



seit 1558

BIOREMEDIATION FOR HEAVY METAL CONTAMINATED BROWNFIELDS

DISSERTATION

provided in joint supervision for the academic degree

doctor rerum naturalium at Friedrich Schiller University Jena

and

doctor în știința mediului at Babeş-Bolyai University Cluj-Napoca

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Date of public defense:

Cluj-Napoca, 08.07.2016

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Abbreviations

BCF	bioconcentration factor
BCR	<i>Community Bureau of Reference</i>
DAPI	4',6-diamidino-2-phenylindole
ECM	ectomycorrhiza
EDX/EDS	energy dispersive X-ray spectroscopy
EGTA	ethylene glycol tetraacetic acid
FIB	focused ion beam
GSH	glutathione
HSP	heat shock protein
keV	kiloelectron-volt
MMNb	Modified Melin-Norkrans medium with casein
MT	metallothionein
PC	phytochelatin
PIPES	1,4-Piperazinediethanesulfonic acid
PMP	polymethylpentene
SEM	scanning electron microscopy
SEP	sequential extraction procedure
rcf	relative centrifugal force (g)
rpm	revolutions per minute
TEM	transmission electron microscopy
XAS	X-ray absorption spectroscopy
XRD	X-ray diffraction

Summary

Metals are natural constituents of the Earth's crust, some of them being essential elements for life. Human activity has altered the distribution and speciation of these metals, generating ecological imbalances by increasing toxicities in ecosystems prone to anthropogenic sources. The development of eco-friendly technologies for soil decontamination is of considerable importance in the achievement of environmental pollution control. Specifically, brownfields contaminated with heavy metals are causing concern, because the biogeochemical behavior of metals is influenced by the soil characteristics of the polluted sites. Heavy metals are considered persistent hazardous pollutants of environmental concern, the toxicity of which depends on the chemical speciation of the metal. For the prediction of ecotoxicological effects that might be exhibited by the presence of heavy metals is necessary to determine the bioavailability of the respective metals in the brownfield subjected to investigations.

With regard to this issue, a field site located in the North-Western part of Romania, close to a non-ferrous metallurgical plant, was chosen as study area. Soil samples were collected and a chemical assessment was conducted for the evaluation of environmental risks associated to metal contents in soil. The investigation revealed elevated metal concentrations which often exceeded the alert and intervention thresholds, and indicated concerning metal contents in the bioavailable fractions. In addition, the soil parameters were severely affected by the metal contamination, suggesting disturbances in soil function and quality. Since natural recovery requires extended periods of time and conventional methods are extremely expensive, the objective of the present study was to create a bioremediation system to promote development of a self-sustaining ecosystem under conditions present at the investigated site.

Ectomycorrhizal (ECM) symbiosis of metal resistant organisms can represent a beneficial association for the improvement of soil quality in order to re-establish vegetation in brownfields. The soil limitations in water and nutrient supply can be overcome by ECM fungi which are able to colonize the roots of the trees even in harsh

environments, while the host tree provides the required carbohydrates for the fungal growth. To anticipate the chances of such organisms to develop in the soil conditions of the investigated site, an biological experiment was designed. Pine (*Pinus sylvestris*) growth in the presence of the ECM fungi *Paxillus involutus* and *Pisolithus tinctorius* was tested in regard to exposure at different heavy metal concentrations. The organisms were chosen based on their reported ability to develop in heavy metal contaminated sites, and because they are natural ECM symbiosis partners. The main purpose of the experiment was to investigate the possible defense strategies involved in ECM metal tolerance. Different developmental features were observed in relation to differing metal concentrations, and the physiological modifications were linked to induced metal stress.

Bioremediation is an eco-friendly approach for decontamination of metal polluted environments. Initially, the decontamination strategies were focused on the removal of heavy metals from the soil using hyperaccumulator plant species, which are able to take up high contents of metals through phytoextraction abilities. Due to issues raised with biomass storage of hyperaccumulators, altering the chemical form of the metals to less mobile chemical forms are presently investigated as a better alternative. The goal of this remediation strategy is to decrease the metal toxicity in the soil by reducing the mobility and bioavailability of the heavy metals. Phytostabilization through metal resistant plant species can achieve these demands by restricting the metal movement in the rhizosphere and limiting the metal translocation to shoots.

Mycorrhized pine seedlings have been investigated by assessing lead uptake and distribution within plant tissues. Electron microscopy revealed metal accumulation on the surface of the colonized root, and a possible immobilization strategy by adsorption onto root surface, followed by metal precipitation. Metal accumulations were present also inside the root and inside the needle crown of the seedlings. The reduced number and sizes of metal deposits found in the needle compared to the ones found in the root was related to phytostabilization mechanisms, which have restricted the metal transport from root to shoot. An elemental microanalysis was performed to gain information on the composition of metal accumulations, and the results indicated a lead biomineralization as metal defense mechanism.

The biomineralization potential was discussed in terms of bioreduction, biosorption and bioaccumulation processes that might have been involved by the investigated organisms. The formation of biominerals were investigated by electron diffraction, which revealed pyromorphite minerals present within plant tissue. The structures were extracellularly embedded in the plant cell wall with indications for mineralization during cell wall growth. This study is the first evidence on biogenic chloropyromorphite mineralization as a defense mechanisms of pine seedlings exposed to lead. The identification of different phases of the minerals between root and needles of the same seedling were discussed with regard to tolerance and detoxification mechanisms. A hypothetical pathway was created in order to describe the transport of lead ions along the path from soil through water, mycorrhiza and plant biomass. This includes inter- and intracellular defense mechanisms towards lead. Since the bioremediation system proved its efficiency in the designed conditions, the ecological implications of ECM associations have been highlighted for afforestation strategies of metal polluted sites.

Zusammenfassung

Schwermetalle mit essentiellen oder toxischen Wirkungen auf Lebewesen sind in der Erdkruste häufig. Anthropogene Einflüsse ändern Verteilung und Spezierung der Metalle, so dass ökologische Ungleichgewichte entstehen, die die Toxizität erhöhen können. Ökologisch optimierte Sanierungsstrategien sind nötig, um Schäden in der Umwelt oder der Nahrungskette vorzubeugen. Altlasten mit Schwermetallkontamination sind dabei gesondert zu betrachten, da Metalle durch die biogeochemischen Veränderungen im Boden besonders beeinflusst werden. Die Ökotoxizität wird dabei durch die Bioverfügbarkeit der Schadstoffe bestimmt. Eine metallurgische Fabrik im Nordwesten Rumäniens mit hoher Belastung durch Blei in der Umgebung wurde ausgewählt und im Testfeld wurden Bodenproben zur chemischen Charakterisierung und Bestimmung der gesamten sowie der bioverfügbaren Metallgehalte gewonnen. Zusätzlich wurden Bodenparameter bestimmt, die eine starke Beeinträchtigung der Bodenfunktion und -qualität zeigten. Da eine natürliche Sukzession erhebliche Zeiten erfordert und konventionelle Sanierungsstrategien extrem kostenaufwändig sind, wurden hier Bioremediationsstrategien entwickelt, die die Wiederherstellung eines sich selbst regenerierenden Ökosystems unter den im Testfeld gegebenen Bedingungen unterstützen.

Ektomykorrhiza (ECM) ist eine Symbiose, die mittels schwermetallresistenter Partner auch auf Altlasten für eine Verbesserung der Bodenqualität sorgt und das Pflanzenwachstum befördern kann. Die Pilzpartner helfen der Baumwurzel in der Symbiose, Wasser und Nährstoffe unter den erschwerten Bedingungen der Schwermetallbelastung aufzunehmen, während der Baum den Pilz mit Kohlenwasserstoffen aus der Photosynthese versorgt. Um die Möglichkeiten der Symbiose unter den im Testfeld gegebenen Bedingungen zu untersuchen, wurden Kiefern (*Pinus sylvestris*) mit den Mykorrhizapilzen *Paxillus involutus* und *Pisolithus tinctorius* inokuliert und ihr Wachstum bei unterschiedlichen Metallkonzentrationen getestet. Die Auswahl der verwendeten Symbiosepartner beruhte auf der bekannten Fähigkeit, sich auch auf metallbelasteten Flächen durchsetzen zu können. Die Metalltoleranz der Symbiosepartner und die Entwicklung und Physiologie der Organismen bei unterschiedlichen Schwermetallkonzentrationen wurden untersucht.

Bioremediation ist eine ökologische Alternative zu technischen Sanierungsverfahren. Während zunächst insbesondere Hyperakkumulatoren eingesetzt wurden, die Metalle in die Pflanzenbiomasse aufnehmen und dort speichern, werden aufgrund der Probleme bei der Entsorgung dieser schwermetallhaltigen Pflanzen heute auch die Befriedung des Ökosystems durch Veränderung der Schwermetallmobilität und -spezifizierung untersucht. Dabei wird die Metalltoxizität im Boden durch Festlegen in nicht oder wenig bioverfügbare Fraktionen durch biologische Aktivität genutzt. Diese Phytostabilisierung wird durch schwermetallresistente Pflanzenarten erreicht werden, welche die Mobilität von Schwermetallen in der Rhizosphäre reduzieren und damit eine Aufnahme in oberirdische Biomasse verhindern. Mykorrhizierete Kiefern Sämlinge wurden untersucht, um die Aufnahme von Blei in die Pflanze durch Elektronenmikroskopie sichtbar zu machen. Dabei wurde auf der Oberfläche der kolonisierten Wurzel eine Immobilisierung durch Adsorption, gefolgt von der Bildung von Präzipitaten gezeigt. Metallanreicherungen konnten auch in der Wurzel sowie in den Nadeln der Krone nachgewiesen werden. Dabei waren die Zahl und Größe von Metallablagerungen in Nadeln deutlich geringer als in der Wurzel, was mit Mechanismen der Phytostabilisierung und reduzierten Metalltransport aus der Wurzel in die Pflanze korreliert. Mittels einer elementaren Mikroanalyse konnten die Präzipitate als Biominerale charakterisiert werden.

Damit ist die Biomineralisierung ein potentieller Weg, der durch Bioreduktion, Biosorption und Bioakkumulation zur Metalltoleranz beiträgt. Durch Röntgenabsorption wurden die Biominerale als Pyromorphit beschrieben, welches im Pflanzengewebe außerhalb der Zellen in der wachsenden Zellwand abgelagert wird. Damit ist dies die erste Beschreibung für biogene Chloropyromorphit-Mineralisation als Mechanismus zur Bleitoleranz bei Kiefern Sämlingen. Aus den unterschiedlichen Formen in Wurzel und Nadeln konnte ein Modell zur Detoxifizierung erstellt werden, in dem ein hypothetischer Weg vom Boden über das Wasser und den Mykorrhizapilz in die Pflanze für eine Anwendung in der Bioremediation von Blei beschrieben wird. Die involvierten inter- und intrazellulären Schutzmechanismen konnten unter den getesteten Bedingungen vorteilhaft eingesetzt werden. Daraus ergeben sich ökologische Alternativen für die

Nutzung der ECM-Symbiose für Wiederaufforstungen an schwermetallbelasteten Altlast-Standorten.

Rezumat

Metalele se regăsesc între componentele naturale ale scoarței terestre, o parte dintre acestea fiind catalogate elemente esențiale vieții. Activitățile desfășurate de oameni au generat dezechilibre în distribuția și forma chimică a acestor metale în mediul natural, astfel declanșând un grad ridicat de toxicitate în ecosistemele expuse la surse de poluare antropică. Dezvoltarea unor tehnologii ecologice pentru decontaminarea solurilor este un deziderat pentru controlul poluării mediului. Siturile industriale contaminate cu metale grele reprezintă o categorie specială ce întâmpină dificultăți aparte în procesele de decontaminare, deoarece comportamentul biogeochimic al metalelor este direct influențat de caracteristicile solului regăsit în situl poluat. Metalele grele sunt considerate poluanți periculoși persistenți de interes ecologic, a căror toxicitate depinde de forma chimică a metalului. Astfel, pentru estimarea efectelor ecotoxicologice care pot fi generate de contaminarea cu metale grele este necesară determinarea biodisponibilității metalelor prezente în siturile industriale supuse investigațiilor.

Cu privire la această problemă de mediu, o zonă de studiu a fost aleasă în partea de nord-vest a României. Probe de sol au fost prelevate de pe suprafața unui teren aflat în apropierea unei instalații metalurgice neferoase și o analiză chimică a fost desfășurată pentru evaluarea riscurilor de mediu asociate conținutului de metale grele. Rezultatele anchetei au evidențiat concentrații îngrijorătoare în fracțiile biodisponibile și au indicat conținuturi ridicate de metale în sol, adesea înregistrând depășiri ale pragurilor de alertă și intervenție. De asemenea, contaminarea cu metale grele a determinat o degradare semnificativă a parametrilor de calitate ai solului, astfel fiind preconizate dezechilibre ale funcțiilor aferente solului. Având în vedere că regenerarea naturală a mediului necesită perioade îndelungate de timp, iar metodele convenționale de decontaminare presupun costuri financiare ridicate, prezentul studiu a avut ca obiectiv crearea unui sistem de bioremediere care să susțină dezvoltarea unui ecosistem de sine stătător pentru condițiile regăsite în zona de studiu.

Ectomicorizele (ECM) realizate între organisme rezistente la concentrații ridicate de metale ar putea contribui la refacerea vegetației în siturile industriale contaminate,

deoarece simbioza organismelor contribuie la îmbunătățirea calității solului. Deficitul de apă și nutrienți din sol poate fi depășit de fungii de ectomicorize care sunt capabili să colonizeze rădăcinile plantelor inclusiv în condiții de mediu nefavorabile, iar planta gazdă va asigura în schimb necesarul de carbohidrați pentru dezvoltarea fungilor. Pentru a anticipa șansele unor astfel de organisme de a se dezvolta în condițiile sitului aflat sub investigație, un experiment biologic a fost creat. Rolul acestuia a fost de a analiza, prin expunere la diferite nivele de concentrații de metale grele, modul în care poate fi afectată evoluția pinului (*Pinus sylvestris*) ce se dezvoltă în prezența fungilor de ectomicorize *Paxillus involutus* și *Pisolithus tinctorius*. Alegerea organismelor a avut în vedere capacitatea atribuită acestora de a se dezvolta în situri contaminate cu metale grele și faptul că sunt parteneri de simbioză ce se regăsesc în mod natural în mediul înconjurător. Scopul principal al experimentului a fost de a investiga posibilele strategii de apărare împotriva metalelor grele implicate de către partenerii unei simbioze ectomicorizice. Diferite stagii de dezvoltare au fost observate în funcție de variația concentrațiilor de metale, iar modificările fiziologice au fost atribuite efectului indus de prezența metalelor grele.

Bioremedierea mediilor poluate cu metale grele reprezintă o decontaminare ecologică a suprafețelor afectate. Strategiile de decontaminare abordate inițial se bazează pe extragerea metalelor din sol prin intermediul speciilor de plante hiperacumulatoare, care sunt caracterizate de abilități de fitoextracție ale unor cantități ridicate de metale grele. Depozitarea biomasei rezultate în urma utilizării hiperacumulatorilor a reprezentat o problemă dificil de gestionat, motiv pentru care modificarea formei chimice a metalelor în forme mai puțin mobile ale acestora este considerată o alternativă utilă. Scopul acestei strategii de remediere este de a diminua toxicitatea metalelor prin reducerea mobilității și a biodisponibilității acestora în sol, obiectiv ce poate fi realizat prin fitostabilizare cu ajutorul speciilor de plante rezistente la metale grele. Aceste plante asigură o restricționare a transportului de metale la nivelul rizoferei și blochează o mare parte a transferului de metale de la nivelul rădăcinii în părțile aeriene ale plantei.

Răsaduri de pin colonizate de fungi de micorize au fost supuse unor investigații pentru evaluarea conținutului de plumb acumulat de acestea și pentru stabilirea modului de distribuție a acestui metal în țesuturile plantei. Prin intermediul microscopiei electronice

au fost determinate acumulări de metal pe suprafața rădăcinii colonizate, astfel fiind emise premisele unor strategii de imobilizare a metalelor prin adsorbție pe suprafața rădăcinii, urmate de procese de precipitare a metalului. Acumulări ale plumbului au fost determinate și în interiorul rădăcinii și al acelor de pin, cele din urmă fiind prezente într-un număr mai restrâns și având dimensiuni reduse comparativ cu cele de la nivelul rădăcinii. Aceste diferențe au fost atribuite mecanismelor de fitostabilizare implicate de pin, care se presupune că au generat o reducere a transportului de plumb înspre părțile aeriene ale plantei. Pentru a obține informații referitoare la compoziția chimică a acumulărilor de metal a fost realizată o analiză microelementală, a căror rezultate au condus la ipoteza biomineralizării plumbului ca acțiune a mecanismelor implicate de plantă în apărarea împotriva metalelor grele.

Potențialul de biomineralizare a fost analizat din perspectiva proceselor de bioreducere, biosorbție și bioacumulare ce pot fi utilizate de organismele analizate. Formarea biomineralelor a fost investigată prin metode de difracție electronică care au determinat existența unor acumulări piromorfice în țesuturile plantei. Aceste structuri minerale au fost încorporate extracelular în peretele celular, localizare care presupune posibile procese de mineralizare în perioada de dezvoltare a peretelui celular. Acest studiu prezintă prima dovadă a transformării plumbului în piromorfit în urma mineralizării induse biologic ca mecanism de apărare al răsadurilor de pin cultivate în prezența plumbului. Având în vedere că mineralele de la nivelul rădăcinii prezintă o morfologie distinctă de cea a mineralelor de la nivelul acelor de pin, existența diferitelor mecanisme de detoxifiere și toleranță la metale grele au fost analizate. O cale de transport ipotetică a fost descrisă pentru deplasarea ionilor de plumb de-a lungul structurii biologice a plantei micorizate, urmărind ruta de transport a apei și posibilele mecanisme inter- sau intracelulare specifice care ar fi putut fi implicate de prezența plumbului. Având în vedere că sistemul de bioremediere propus și-a demonstrat eficiența preconizată, beneficiile ecologice implicate de ectomicorize în strategiile de revegetalizare și reîmpădurire a siturilor industriale poluate cu metale grele au fost evidențiate.

1 Introduction

1.1 Brownfield sites - general overview

According to the 2014 report from the European Commission's Joint Research Centre, about 1.2 million potentially contaminated sites have been identified in the European Union, representing only 45% of the total number of potentially contaminated sites assumed to exist in reality. 340 000 of the identified sites were already framed as contaminated sites, out of which only 15% have undergone remediation techniques. The complexity of environmental disturbances generated by soil contamination, together with the social and economic implications, has led to a deadlock in developing a Soil Directive at European Union level. In 2006 the European Commission adopted a Thematic Strategy for Soil Protection, including a Communication from the Commission to the other European Institutions (Comm(2006)231), a proposal for a Soil Framework Directive (Comm(2006)232), and an Impact Assessment (SEC(2006)1165 and SEC(2006)620), but after eight years of pending, in 2014 the proposal for Soil Framework Directive needed to be withdraw because it could not be agreed by a qualified majority in the EU Council.

The term "brownfield site" refers to real property, the expansion, redevelopment, or reuse of which may be complicated by the presence or potential presence of a hazardous substance, pollutant, or contaminant (US EPA, 2002). Usually, brownfields are associated with abandoned or underutilized commercial or industrial properties (Chen *et al.*, 2008; Cheng *et al.*, 2011). The concept of brownfield redevelopment is part of the overall framework for sustainable management of contaminated lands, and it is being seen as a future solution for urban development in terms of decreasing the consumption of greenfield sites and gain a higher protection for them (Ferber & Grimski, 2002). Brownfield redevelopment is considered an integrant component of environmental restoration, land use planning and economic policy, and the main objectives are represented by: revitalization of the former industrial site, remediation of the environment, and reintegration of the rehabilitated site into the economic cycle (Ferber & Grimski, 2002).

The need to ensure proper remediation strategies for contaminated lands is of important interest for the particular issue of brownfields contaminated with heavy metals, due to the biologically available fractions of metals that can pose ecotoxicological risks to the living organisms. Conventional remediation techniques often imply the use of technical methods like solidification/stabilization, vitrification, pneumatic fracturing, excavation and removal of contaminated soil layer, physical stabilization, or washing of contaminated soil using strong acids or heavy metal chelators (Bhargava *et al.*, 2012; Khan *et al.*, 2004). At European level, the most used remediation strategy reported by the European Commission until 2014 was the excavation of contaminated soil and its disposal as landfill (European Commission, 2014), also known as the "dig-and-dump" practice (Pulford & Watson, 2003). This strategy is related to *ex situ* remediation techniques, where the economical implications are increased by the high volumes of soil that need to be excavated, transported and stored for treatment application. Other drawbacks of the conventional methods are represented by the harsh treatments which are applied on the contaminated soils and generate adverse effects on physical and chemical parameters, contributing to the lost of soil structure and fertility, and disappearance of soil microorganisms (Dermont *et al.*, 2008). Therefore, *in situ* remediation techniques are preferred to conventional techniques, and bioremediation strategies would provide an eco-friendly approach for brownfields contaminated with heavy metals.

Bioremediation is based on biological mechanisms used by microorganisms, fungi or green plants to destroy, transform or immobilize environmental contaminants. Natural bioremediation can be accomplished by specific plants which are able to colonize contaminated soils, a process in which soil fungi are often involved in the ability of plants to cope with ecotoxicological effects (Johansson *et al.*, 2008). Still, the remediation potential of fungi receives lesser attention in the decontamination process maybe due to the intimate association between fungus and plant which hampers the screening of fungi for resistance (Hartley *et al.*, 1997). For this reason, the term "phytoremediation" is mostly associated to bioremediation performed by plants, while the significant contribution of fungi in the soil colonization has led to the particularly studies of fungi under the concept of "mycoremediation" (Gadd, 2010; Singh *et al.*,

2015). Phytoremediation involves the use of specific plant species in the remediation process, and based on the plant's resistance mechanisms to heavy metals, phytoremediation techniques can be divided in phytostabilization and phytoextraction (Pulford & Watson, 2003). Initially, removal of heavy metals from polluted soils was the primary objective of phytoremediation strategies, and the reduction of metal contents was subjected to phytoextraction processes, achieved by hyperaccumulator plant species which were able to take up metals to concentrations more than 100 times higher than average plants (Wu *et al.*, 2010). Phytoextraction requires repeated cropping of plants because hyperaccumulators can absorb metals through their roots and translocate them into the shoots without being affected by the metal toxicity, but the aboveground biomass needs to be harvested when it reaches maturity (Bhargava *et al.*, 2012). Because the harvested biomass can be classified as a hazardous material due to the high accumulation of metals into the plant tissue, special attention needs to be carried out for proper disposal and appropriate utilization of the resulted biomass. Different approaches have been investigated as post harvesting treatments, among which composting and compaction, or combustion and gasification seemed to be more cost-effective for phytoextraction, but have not removed completely the environmental risks that can be generated by the hazardous biomass. (Garbisu & Alkorta, 2001; Lasat, 2002).

An alternative to the metal removal from contaminated soils is to reduce the mobility and bioavailability of the contaminant through phytostabilization. Metal tolerant plant species are used to reduce the underground leaching with the help of plant roots which act as sorption or precipitation surfaces for metal contaminants (Laperche *et al.*, 1997), and have the ability to minimize water percolation into the soil (Berti & Cunnigham, 2000). In contrast to phytoextraction, the translocation to shoots and metal accumulation in aerial biomass are being replaced by metal restriction at root level in the case of phytostabilization, where adsorption onto roots and precipitation within rhizosphere occurs due to root exudates (Brunner *et al.*, 2008), strategy which does not require harvesting of the plants anymore. In this way, the economical implications are kept minimal and the visual improvement of the landscape can be assured through the whole period of the remediation process. In addition, the dense root system of the metal

tolerant trees which can be used in phytostabilization brings physical stabilization to the contaminated land and prevents soil erosion, aims that cannot be assured by hyperaccumulators involved in phytoextraction, and which are usually lower plant species. Furthermore, the vegetation cover generated by trees enhances the soil litter and the aerobic environment in the rhizosphere, thus increasing the soil organic matter and promoting soil microorganism activity (Robinson *et al.*, 2006).

Phytoremediation strategies are site dependent, so they need to be adapted to the particular characteristics of each contaminated site, such as soil texture, pH, salinity, contaminant type, level of pollution etc. For brownfields contaminated with heavy metals, the plants used as remediation systems need to be selected in accordance to their metal resistance mechanisms to particular toxic effects of each metal.

1.2 Heavy metals

Metals are natural constituents of the Earth's crust, but their distribution and speciation into the soil was affected by anthropogenic activities since the industrial revolution (Wu *et al.*, 2010), and due to rapid urbanization without proper environmental protection regulations which have led to release of increased levels of contaminants into the environment (Margesin *et al.*, 2011). Some metals (e.g. B, Cu, Cl, Fe, Mn, Mo, Zn) are considered to be essential nutrients in low concentrations for plant growth, and exhibit toxic effects at high concentrations (Chibuike & Obiora, 2014), while other metals (e.g. As, Cd, Pb) have no known biological function and generate adverse effects to plants even at low concentrations (Marques *et al.*, 2009). Heavy metals are defined as metals with a density higher than 5 g cm^{-3} (Schützendübel & Polle, 2001) which constitute a group of inorganic chemical hazards, and where soil represents the major sink for their release into the environment. The persistence of these pollutants takes long periods of time because metals do not undergo microbial or chemical degradation (Wuana & Okieimen, 2011).

Copper (Cu), lead (Pb) and zinc (Zn) are among the most common heavy metals found in brownfields generated by smelting activities. Usually, Cu is seen as a particularly

toxic micronutrient, Zn is considered a hyperaccumulated metal, and Pb is often framed a non-essential metal for living organisms. Often these contaminants occur at the same site and their presence has a negative impact on the existing ecosystems, and generates a decrease of biodiversity within the affected area. The chemical form in which these metals are present in the soil is the main indicator for their potential toxicity to the environment. To predict the risks prone to living organisms is important to assess the metal mobility and bioavailability. The chemical speciation is directly related to the absorption potential by biota, while the mobility of metals depends upon several soil parameters as type of soil, soil pH, moisture content of the soil and water percolation (Miretzky & Fernandez-Cirelli, 2008).

Environmental risk assessment is the preliminary investigation that is required for an adequate remediation strategy. The current legislation regarding soil protection indicates the need to evaluate the total concentration of metals in soil for risk assessments, but the environmental science community indicates that total concentration is not an accurate predictor for an appropriate risk assessment, and recognize that bioavailable soil fractions would represent a more reliable indicator for potential environmental risks associated to heavy metals (Kötschau *et al.*, 2014; Traina & Laperche, 1999; Trajković *et al.*, 2014). Accepting that solubility of metal chemical forms can be linked to bioavailability, and bioavailability of metals can be linked to plant growth, allows the remediation strategies to be focused on inducing changes in the chemical form of a contaminant, thus decreasing the solubility and the corresponding ecotoxicological effects of a specific heavy metal (Traina & Laperche, 1999). Based on these principles, phytostabilization strategies seem to be more appropriate for remediation of brownfields contaminated with heavy metals, instead of complete removal of metals from the polluted soils.

1.3 Ectomycorrhiza

Mycorrhiza is a highly evolved symbiotic association between the roots of a vascular plant and soil fungi (Smith & Read, 1997). Through the mutualistic symbiosis which is created during the root colonization, the fungus receives photosynthetically derived

carbon (glucose and sucrose) becoming almost dependent on the plant, while the fungus enhances the plant's access to mineral nutrients and water in soil (Brundrett, 1991; Smith & Read, 2008).

Ectomycorrhiza (ECM) is of important interest because it improves the growth of plants on contaminated sites with heavy metals, by this promoting phytoremediation abilities. In an ectomycorrhizal symbiosis, the fungus forms a structure like a mantle or a sheath around the root of the plant, where the hyphae get in contact with the root hairs, become attached to the root surface, and begin to penetrate the root epidermis and cortex tissue to form a complex intercellular system - the Hartig' net, but do not penetrate into the root cells. For the Hartig' net formation the hyphae are changing their growth from apical extension to branching in the intercellular spaces of the epidermal and cortex cells (Smith & Read, 1997). The Hartig' net is considered the major site of nutrient exchange between the host plant and the fungus, and by this represents an important advantage of ectomycorrhizal symbioses in improving the plant growth. Another typical feature of ectomycorrhiza is the formation of short roots, which are roots reduced in apical growth and various in branching, similar in structure with long roots and different in slower rate of grow (Taylor & Alexander, 2005). In addition, ECM fungi form a mycelial network with the short lateral roots, thus enhancing the translocation of nutrients and water from soil to the host tree (Anderson & Cairney, 2007). Another role of ECM fungi is to provide plant protection against periods of drought and diseases, therefore promoting the ectomycorrhizal trees to be used for re-forestation of degraded lands (Gadd, 2010).

For predicting the ability of ectomycorrhizal trees to be used as remediation systems for brownfields contaminated with heavy metals, it is important to understand the detoxification and tolerance mechanisms that each symbiont partner can develop. The most discussed mechanisms for metal resistance in ECM fungi can be divided in extracellular and intracellular tolerance mechanisms, as presented in figure 1. Extracellular mechanisms restrict the metal access to the host roots through the formation of the hyphal mantle, which can absorb the bioavailable metal fractions and decrease their transport to vascular tissue due to the hydrophobicity of the fungal hyphae (Hall, 2002). At the same time, metals can be immobilized by adsorption onto

the external mycelia, or extracellular chelation through fungal exudates (Jentschke & Godbold, 2000). Chelation of metals can occur also inside the cell, in the cytosol, and is based on the action of ligands such as metallothionein (MT) and glutathione (GSH). Metallothioneins are a class of metal-binding peptides containing sulphur-based metal clusters which are involved in the homeostasis of essential trace elements and sequestration of toxic metals, while glutathiones are nonprotein thiols limiting the cell damages by sequestering the toxic metal ions (Bellion *et al.*, 2006). Transport mechanisms involve extrusion of toxic metal ions out of the cell or metal accumulation in the vacuolar compartments through protein mediation, but there are not enough constituent studies to prove these hypotheses (Williams *et al.*, 2000).

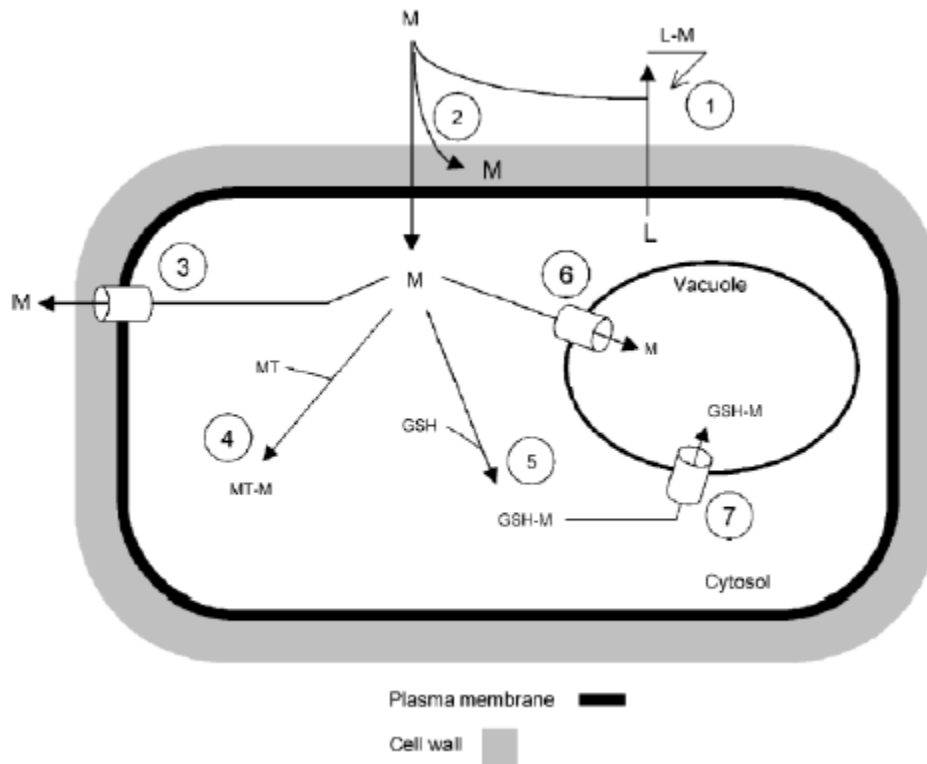


Figure 1, Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi. M, metal-ion; 1, extracellular chelation by excreted ligands (L); 2, cell-wall binding; 3, enhanced efflux; 4, intracellular chelation by metallothionein (MT); 5, intracellular chelation by glutathione (GSH); 6, subcellular compartmentation (vacuole or other internal compartments); 7, vacuolar compartmentation of GSH-M complex (Bellion *et al.*, 2006)

The survival mechanisms of lower and higher plants (Fig. 2) have been investigated in more detail compared to ECM fungi, which have remained relatively unclear due to incomplete or contradictory data (Meharg *et al.*, 1997). The maintenance of plasma membrane integrity has been indicated as the predominant defense strategy in metal resistant species of higher plants (Meharg, 1993). However, the cellular mechanisms for heavy metal detoxification and tolerance postulated for plants indicate multiple similarities with the ones of ectomycorrhizal fungi, avoidance and sequestration being the main strategies involved.

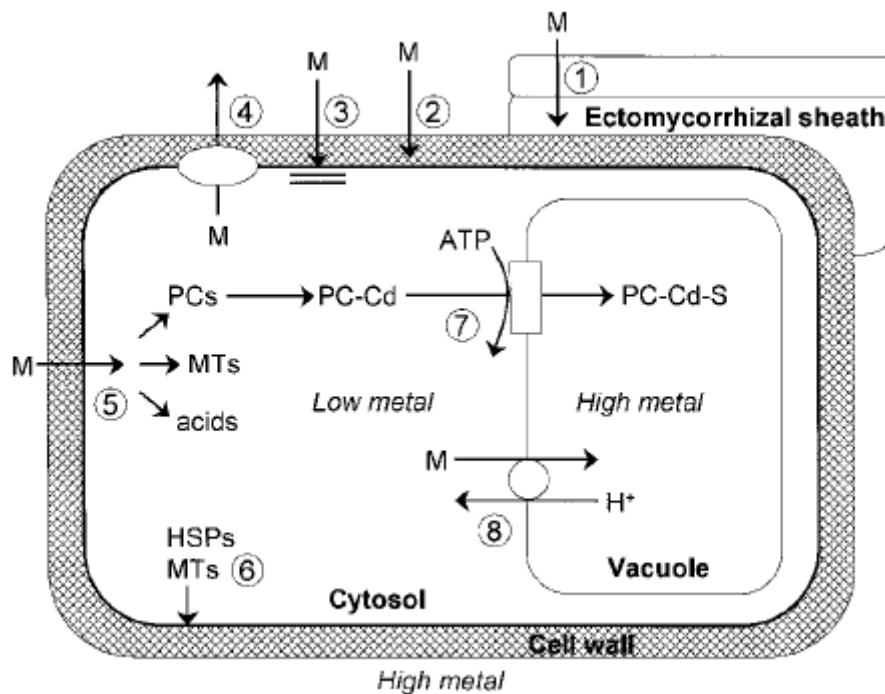


Figure 2, Summary of potential cellular mechanisms available for metal detoxification and tolerance in higher plants. 1. Restriction of metal movement to roots by mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reduced influx across plasma membrane. 4. Active efflux into apoplast. 5. Chelation in cytosol by various ligands. 6. Repair and protection of plasmamembrane under stress conditions. 7. Transport of PC-Cd complex into the vacuole. 8. Transport and accumulation of metals in vacuole. (Hall, 2002)

Ectomycorrhiza by its own is being considered a metal restraining mechanisms used by higher plants, and it is dependent on metal specificity. The species of organisms that

may be involved in the symbiosis influence the way in which the metal tolerance can be achieved (Hütterman *et al.*, 1999). Plants also have the ability to release exudates through their roots and bind metals to their cell wall like ectomycorrhizal fungi, thus enhancing the overall external tolerance mechanism of a mycorrhized plant. Other similarities with ECM fungi are achieved by metal efflux from cytosol (either into the apoplast, either into the vacuole) and metal chelation inside de cytosol. Chelation as a heavy metal detoxification mechanism of plants relies on ligands in the form of metallothioneins and phytochelatins, and possibly on amino acids and organic acids (Clemens, 2001; Rauser, 1999). Particular to plants are the phytochelatins (PCs), which are metal-complexation peptides that can be rapidly induced in plants by metal presence. Also heat shock proteins (HSPs) may act as repair mechanisms under heavy metal stress conditions, contributing to a more resistant plasma membrane (Hall, 2002). Unfortunately, the literature in this research field is restricted to some particular studies, reason for which generalization to a wide range of ECM plants or fungi would not be indicated since metal resistance mechanisms depend also on specific toxicological effects that may be exhibited by heavy metals.

1.4 Self-sustaining ecosystems for ecological remediation

From an ecological point of view, the mobile fractions of metals constitute an ecotoxicological risk because these concentrations are considered to be bioavailable to living organism found in the soil, so they can be taken up even by plants. The toxicity of a contaminant is given by its chemical speciation found in the soluble or easy soluble phases, when considering that contaminant uptake occurs through the solution phase (Traina & Laperche, 1999). In terms of bioremediation through phytostabilization, the chemical form of the metals present in the bioavailable fractions would be changed to other less soluble forms, either in the soil, or either after absorption into the plant tissue.

Soils contaminated with heavy metals are deficient in nutrients and organic carbon, therefore a self-sustaining ecosystem could represent a viable solution if the organisms constituting such an ecosystem can adapt to the induced metal stresses, and

furthermore can develop in the disturbed conditions of the soil. This goal could be achieved by metal tolerant plant species that are able to form mutualistic symbiosis with ectomycorrhizal fungi, thus the created ecosystem being characterized by tolerance and detoxification mechanisms from both symbiont partners. As presented before, plants can release root exudates which are involved in the immobilization of metals found in the rhizosphere and precipitation onto the root surface. Root exudates are substances variable in composition, mainly represented by acetic acid, oxalic acid and amino acids, and are often found in *Pinus* species (Smith, 1969). Pine trees are known for their ability to develop in metal contaminated sites as pioneer populations, but the development of primary succession relies on their associations with mycorrhizal fungi (Colpaert *et al.*, 2011).

Pinus sylvestris (Scots pine) is an eurytopic organism able to develop in a wide range of habitats characterized by different ecological conditions, including high metal concentrations. Thus, it is often used for the reclamation of metal polluted sites (Stefanowicz *et al.*, 2010), and it is considered a reliable bioindicator due to its sensitivity to industrial pollution, being often used as a model for studies involving anthropogenic stresses (Pietrzykowski *et al.*, 2014). Tree species characterized by fast growth and large biomass that are able to stabilize the soil surface, such as *P. sylvestris*, are considered suitable candidates for phytoremediation (Pulford & Watson, 2003). *P. sylvestris* is known to form ectomycorrhizal symbiosis and it is host specific for different basidiomycetes. *Paxillus involutus* and *Pisoulithus tinctorius* are symbiont partners found in natural ecosystems, and can be framed as early- or late-stage fungi depending on different physiological characteristics of the host tree (Keizer & Arnolds, 1994), classification which enables the understanding of primary succession in regard to land bioremediation. Through fungal root colonization the pine tree gets protection against direct contact to soil contaminants, and the metal uptake is being decreased by additional metal immobilization generated by the fungal exudates and precipitation onto the external mycellia. Based on these primary reasons, *P. sylvestris* in association with *P. involutus* and *P. tinctorius* were selected as constituent members of a bioremediation system for the development of a self-sustaining ecosystem.

1.5 Aims of the study

The remediation of brownfields contaminated with heavy metals is site specific, therefore the decontamination techniques need to be adapted to the soil characteristics of the site and to the pollution degree. Therefore, an investigation area was chosen and the bioremediation strategy was designed based on its specific conditions.

The main objective of the present study was to elucidate the involvement of representative structures from plant-fungi associations in the phytostabilization process of specific metals. The approaches used considered the following research lines:

1) Biomineralization potential has been indicated as a defense mechanism of metal resistant plants. It is important to distinguish between immobilization of metals in the rizhosphere or inside the plant. The latter requires the determination of whether plant tissue and organs can provide metal storage sites, with transformation into stable chemical phases of the metals in question. Therefore, the localization of possible metal deposits and the identification of their chemical form within specific plant tissues (e.g. vacuoles or the cell wall) is an important step in order to consider possible mechanisms involved in the metal resistance of mycorrhized pine trees.

2) Phytostabilization involves metal storage in roots, and poor translocation into shoots. Therefore, possible tolerance and detoxification mechanisms that might be implied by mycorrhized seedlings grown in metal stress conditions need to be evaluated in order to prevent unwanted consequences of the proposed bioremediation system. It is believed that various mechanisms can be involved simultaneously, and they can differ depending on the plant structure (e.g. root, stem, leaf).

3) Since the evidence on effects of metal induced stresses on the mycorrhizal symbiosis are rather limited, the development of young seedlings of Scots pine in axenic environments contaminated with Cu, Pb and Zn was investigated in the presence of *Paxillus involutus* and *Pisoulithus tinctorius*. It is assumed that the presence of the mycorrhizal fungi will enhance the plant's abilities to cope with ecotoxicological effects. The mechanisms induced by the mycorrhizal association can provide ecological improvements for the polluted environments.

2 Material and methods

2.1 Investigation site and soil sampling

The study was conducted on a 12000 m² investigation site located in the North Western part of Romania, close to a non-ferrous metallurgical plant from the Baia-Mare town, where the field features positive relief between 290 m and 340 m (Fig. 3). The area is known for environmental pollution due to dust emissions and metallurgical wastes generated by industrial activities, such as mining exploitations and non-ferrous processing plants (Frentiu *et al.*, 2008b; Senila *et al.*, 2001).

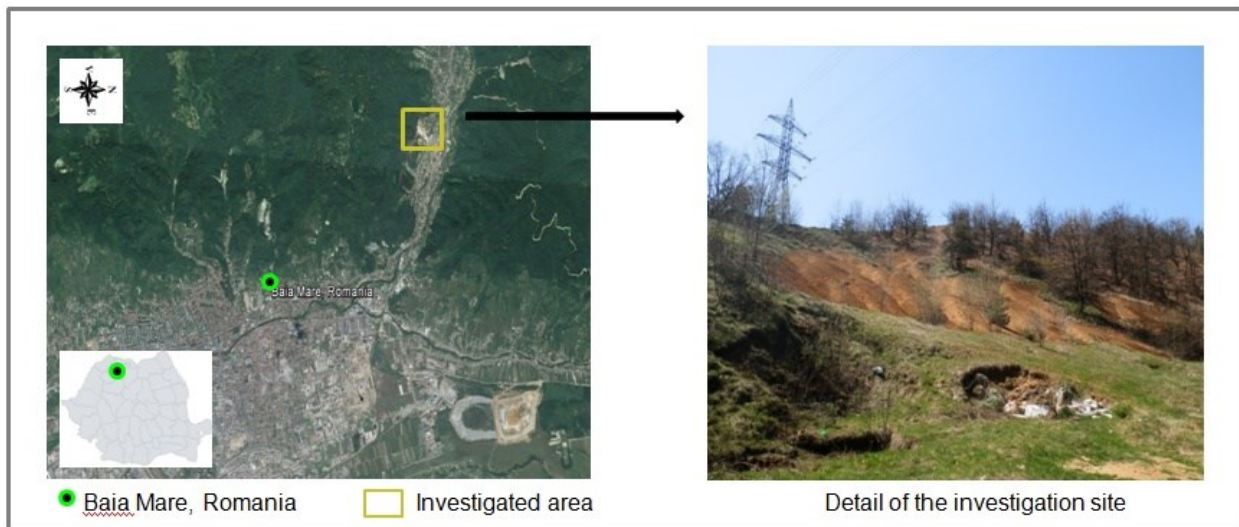


Figure 3, Localization of the investigation site where the study was conducted

A number of 15 sampling points (S1-S15) were selected covering the area of the former smokestack belonging to the plant (Fig. 4). An additional sampling point (R) was chosen as reference from an area considered to be unpolluted at the shore of Firiza Lake, which is the storage reservoir used for water supply of Baia-Mare town, and is located at 15 km distance from the investigation site. Soil samples were collected from ranges between 10-30 cm and 40-60 cm depth from each sampling point, except S6 and S15, where the very compact soil texture allowed drilling only at 10-30 cm depth. Due to demolition of the former smokestack, the soil samples from S1, S2 and S3 points had a heterogeneous texture, being mixed with ash, pieces of brick and unidentified waste

rocks, and the color of the soils ranged from different shades of grey to black color. The other soil samples were characterized by a more homogeneous texture specific to clay soils, and by a brown or orange-brown soil color.

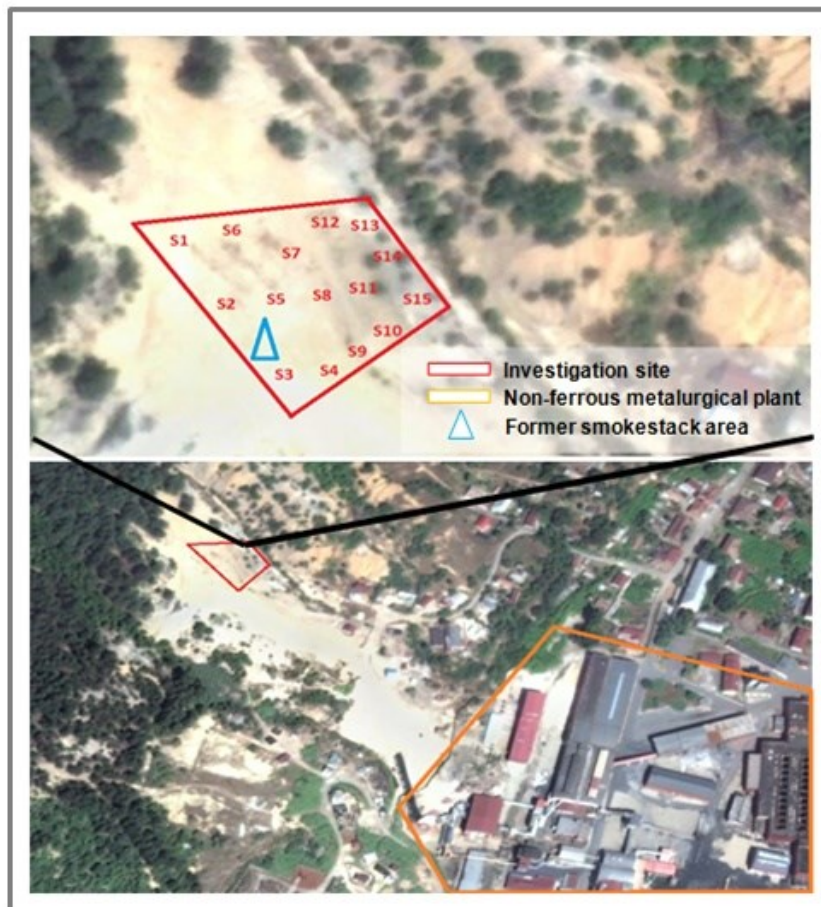


Figure 4, Sampling points from the investigation site in Baia-Mare, NW Romania

2.2 Chemical assessment of the soil samples

The soil parameters chosen to be investigated were pH, which is of high importance being related to bioavailability of metals, total carbon (TC) and dissolved organic carbon (DOC), which indicate the quality of the soil and the development potential of soil organisms. Regarding the pollution, a chemical assessment focused on the identification of toxic elements present in the soil and their concentrations in different metal fractions. Due to the smelting activities that took place at the non-metallurgical plant, Cu, Pb and Zn were selected for the metal investigations.

The soil samples were oven dried at 105°C until constant weight, followed by grinding and sieving at 2 mm particle size. The fraction below 2 mm was collected, homogenized and stored in polyethylene bags for further analyses.

In accordance with the Romanian regulations (MO, 756/1997) the total metal content needed to be determined in order to evaluate the level of pollution in the chosen site. According to ISO 11466:1995 digestion protocol, 1 g of soil was introduced into 50 mL reaction flask and extracted in *aqua regia* (21 mL of 12 mol l⁻¹ of HCl and 7 mL of 15.8 mol l⁻¹ HNO₃). The solution was left to mineralize at room temperature overnight, followed by 2 hours of boiling on sand bath. The extracts were filtered and brought to 50 mL with ultrapure water (Millipore, Molsheim, France). The metal concentrations were determined through flame atomic absorption spectroscopy (FAAS) performed by ZEE nit 700 spectrometer, using an acetylene-air (C₂H₂-air) flame and the adequate cathode lamps.

For determining the bioavailable metal fractions and to establish the mobility of metals present in the investigated site, a modified four steps sequential extraction procedure (SEP) after the *Community Bureau of Reference (BCR) Procedure* was applied on the collected soil samples. The modified scheme after Žemberyová *et al.* (2006) and Zimmerman & Weindorf (2010) is presented in table 1.

Table 1, Modified BCR four steps sequential extraction procedure

Step	Fraction	Reagent	Target phase(s)
I	Acid extractable	CH ₃ COOH (0.11 mol l ⁻¹)	Exchangeable, carbonates
II	Reducible	NH ₂ OH*HCl (0.1 mol l ⁻¹)	Iron and manganese oxides
III	Oxidisable	H ₂ O ₂ (8.8 mol l ⁻¹), NH ₄ OAc (1 mol l ⁻¹)	Organic matter and sulfides
IV	Residual	<i>Aqua regia</i> (21 ml of 12 mol l ⁻¹ HCl and	Remaining, non-silicates

7 ml of 15.8 mol l⁻¹ HNO₃)

The sequential extraction was performed on 1 g of soil sample placed into a 50 mL centrifuge tube and consecutively exposed to different reagents. The acid-extractable fraction (AE) was extracted in 40 mL of 0.11 mol l⁻¹ acetic acid for 16 h at 20 ± 5°C under continuous shaking (15 rpm) using a Heidolph Reax 20/8 overhead shaker. The extract was centrifuged at 3120 rcf for 10 minutes, filtered in new 50 mL centrifuge tubes and analyzed. To the left residue 40 mL of 0.1 mol l⁻¹ hydroxylamine hydrochloride (pH 2 adjusted with HNO₃) were added, and kept for 16 h under continuous shaking conditions at 20 ± 5°C. The extraction procedure for the reducible fraction was performed as described before. For the oxidisable fraction (OX) 10 mL of 8.8 mol l⁻¹ hydrogen peroxide (pH 2 adjusted with HNO₃) were added to the left residue from the previous fraction and digested for 1 h at room temperature with occasional manual shaking. Then, another 10 mL of 8.8 mol l⁻¹ hydrogen peroxide were added and the solution was heated to 85°C in a water bath, until the volume was reduced to approximately 1 mL. 50 mL of 1 mol l⁻¹ of ammonium acetate (pH 2 adjusted with HNO₃) were added to the cool moist residue and the slurry was stirred for 16 h under continuous shaking conditions at 20 ± 5°C, followed by separation of extract as in the previous steps. The residual fraction (RES) was determined by digestion in 28 mL of *aqua regia* after drying and collecting the residue from the oxidisable fraction in a 100 mL reaction flask. The solution was left to mineralize overnight and then heated under reflux conditions for 2 hours. The extract was filtered and diluted to 50 mL with 0.5 mol l⁻¹ nitric acid. The Cu, Pb and Zn concentration of the extracts were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using an OPTIMA 3500 DV spectrometer (Perkin Elmer, USA).

The soil pH of the samples was determined in a suspension 1/5 (w/v) soil to water extract using a 350i multiparameter (WTW, Germany). TC was measured by combustion and infrared detection of CO₂ using the solide module of a Multi N/C 2100S Analyzer (Analytic Jena, Germany). Approximately 0.5 g of soil were weighted in porcelain boat (handled with tweezers to avoid hand touching) from each sample and

placed in the combustion unit of the analyzer. For determining the DOC of the investigation area, the suspension of 1/10 (w/v) soil to water extract for each sample was kept for 24 h in continuous overhead shaking conditions (15 rpm) at $20 \pm 5^\circ\text{C}$, and then filtered through 0.45 μm pore size PTFE membrane filters. The resulted extract was analyzed by thermo-catalytic high temperature oxidation and infrared detection using the liquid module of a Multi N/C 2100S Analyzer (Analytic Jena, Germany).

2.3 Axenic cultivation

To investigate the metal resistance of the proposed bioremediation system, co-cultures on germination medium with piperazine (Chilvers *et al.*, 1986) inoculated with different levels of heavy metal solutions were grown for twelve weeks. All substances and solutions which were used are from Boehringer (Mannheim), Difco (Michigan, USA), Fisher Scientific (Loughborough, U.K.), Merck (Darmstadt), Riedel de Haen (Seelze), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (Steinheim). *Pinus sylvestris* (Scots pine) was selected as investigation tree and the seeds were provided from Thüringer Forstamt Schmalkalden, Germany. *Paxillus involutus* RK081020 and *Pisolithus tinctorius* MG090923_06 were chosen as symbiont partners, and the ectomycorrhizal fungi originated from the strain collection of Microbial Communication, Friedrich Schiller University Jena, Germany.

The design of the experiment aimed to simulate the soil conditions found in the investigation site. For metal resistance, the bioremediation system was tested in regard to bioavailable metal fractions. Therefore, the metal concentrations determined in step I (mobile fraction) and step II (easy mobilizable fraction) of the SEP were summed up and the investigation site was divided in three different regions, based on the resulted levels of metals at each sampling point. An area covering high metal concentrations, one area with medium metal concentrations and another which gathered low metal concentrations were defined, and from each the average concentration was calculated. The resulted metal concentrations were individually inoculated into the germination medium under the form of CuCl_2 , PbCl_2 and ZnCl_2 , generating ten different growing conditions to be tested for the bioremediation system (Tab. 2).

In order to provide enough sterile space for the development of pine seedlings, the co-cultures were created in Weck jars (Fig. 5A), a system composed of a glass jar (20 cm height and 9.5 cm diameter) closed with a glass lid and sealed with parafilm. The addition of heavy metal stock solutions took place through sterile filtering after the sterilization process (30 minutes at 121°C and 1 bar) of the germination media, while the medium was around 80°C. Each Weck jar contained 300 mL of medium providing different growing conditions and twenty seeds of pine. The seeds were watered overnight, sterilized in 30% H₂O₂ for 1.5 h at constant shaking conditions at room temperature, rinsed three times with distilled water and inoculated on the germination medium. 20 pine seeds were used for each co-culture. Two MMNb (Modified Melin-Norkrans medium + casein after Kottke *et al.*, 1987) blocks from each ectomycorrhizal fungus were added on the surface of the germination medium, in-between the pine seeds, as described in figure 5B. The co-cultures were stored in a green house where 12 h day-night regime with alternating temperature of 23/17°C and 80% relative humidity were constantly assured. At the end of growing period, the pine seedlings were harvested, rinsed with distilled water and stored at 4°C in Petri dishes (145X20 mm).

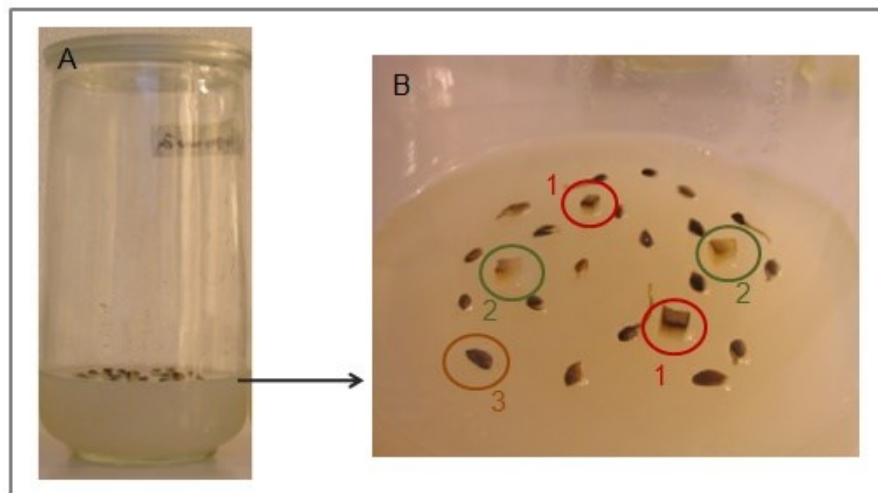


Figure 5, The set up of the co-cultures in the biological experiment. A) the Weck jar in which the co-cultures developed. B) configuration of organisms on the surface of the germination medium: in position 1 are placed the *P. involutus* fungi, in position 2 are placed the *P. tinctorius* fungi, and position 3 indicates one of the *P. sylvestris* seeds

Table 2, Medium growing conditions for the co-cultures

Growing condition	Abbreviation	Metal concentration	Nr. of replicates
Germination medium	C/control	-	5
Germination medium + CuCl₂	minimum Cu	0.3 mM CuCl ₂	5
	medium Cu	1.3 mM CuCl ₂	5
	maximum Cu	7* mM CuCl ₂	5
Germination medium + PbCl₂	minimum Pb	2.6 mM PbCl ₂	5
	medium Pb	9 mM PbCl ₂	5
	maximum Pb	18* mM PbCl ₂	5
Germination medium + ZnCl₂	minimum Zn	1.1 mM ZnCl ₂	5
	medium Zn	5.4 mM ZnCl ₂	5
	maximum ZN	14.3* mM ZnCl ₂	5

* the listed values represent half of the resulted averages, and were modified because would have been too high for a possible growth of the created cultures

2.4 Microscopic investigation of short roots

The short roots were identified using Stemi 2000-C stereomicroscope, individually cut by a scalpel and pretreated at least 48 h in fixative (4% formaldehyde in PME buffer - 50 mM PIPES, 1 mM MgSO₄, 5 mM EGTA) for freeze microtomy sectioning. Then, the short roots were vertically attached to pre-cooled pedestal by applying embedding medium (Leica - Reichert Jung), and allowed to solidify for 15 minutes before cutting. The sectioning was performed with Bench Cryostat Leica CM 1100 equipment (Leica Instruments GmbH, Nussloch) at -19/-20°C. 25 µm thin sections were collected on biological glass slides and observed under fluorescence microscopes for identification of ectomycorrhizal symbiosis features. The Hartig' net identification was conducted with

Olympus BX 50 microscope, and fungal hyphae identification through DAPI nucleic acid staining followed by investigations with Zeiss Axioplan 2 microscope.

2.5 Heavy metal uptake by co-cultures

Four out of five replicates were assessed with respect to metal uptake by co-cultures. Cu, Pb and Zn concentrations were determined by ICP-MS (Inductively Coupled Plasma Mass Spectrometry). For sample preparation the entire biomass (including seedling roots) developed in each selected co-culture was collected, oven dried (60°C) to constant weight, and grinded to less than 2 mm particle size with mortar and pestle. 100 mg of plant material were weighted and digested in 5 mL HNO₃ for 20 minutes in open vessel, heated at 180°C within 15 minutes and maintained for another 15 minutes at the reached temperature by closing the vessel, and cooled down within 30 minutes. The extract was transferred to PMP flask and diluted to 25 mL with deionized water, centrifuged at 3000 rcf in 50 mL centrifuge tubes to separate the undissolved particles (e.g. silicates), and the clear supernatant was measured by ICP-MS (X-Series II, Thermo Scientific). Also approximately 3 g of media samples were collected from each growing condition and the metal concentrations at the end of experiment were determined after the protocol described above. The accumulation potential of the bioremediation system was evaluated in terms of bioconcentration factor (BCF), calculated as ratio between metal concentration found in biomass (C_{biomass}) and initial metal concentration in medium (C_{medium}), determined as sum of final metal concentration in germination medium and metal concentration in biomass.

2.6 Scanning electron microscopy (SEM)

For visualizing the metal distribution into plant tissue, the root, the stem and the needle crown of a mycorrhized seedling were sectioned and separately analyzed at the Center of Electron Microscopy, Jena. To create appropriate cross-sections, the sample preparation required freezing in liquid nitrogen and braking into several pieces of each sectioned part, instead of metal/ceramic cuts which could have brought surface

overlaying with outer metal. To avoid water condensation, the samples were dried in warming vessel (in nitrogen atmosphere). For SEM analyses the sample surfaces needed to become electrically conductive, so they were vertically glued on specimen mounts covered by conductive carbon tabs (“Leit-C”, Plano GmbH, Wetzlar, Germany) and let to air dry overnight. Then, the samples were coated with a thin carbon layer by high-vacuum carbon flash evaporation using a SCD005 (BALTEC, Liechtenstein). The resulted specimens were investigated with FE-SEM LEO-1530 Gemini (Carl Zeiss NTS GmbH, Oberkochen, Germany) at 25 keV using the back-scattered electron detector for localizing the metal particles and the secondary electron detector for tissue morphology images. For characterization of metal accumulations found in the mycorrhized seedling, an elemental microanalysis was performed by SEM-EDX (Energy Dispersive X-ray Spectroscopy), using a LEO-1450 instrument (Carl Zeiss NTS GmbH, Oberkochen, Germany) equipped with EDX system Quantax 200 with XFlash 5030 detector (Bruker AXS, Berlin, Germany). The analyses were conducted on the same specimens used for SEM investigation of metal distribution.

2.7 Transmission electron microscopy (TEM)

To gain crystallographic information of the metal precipitates accumulated into the tissue of the mycorrhized plant, the assemblages were investigated by methods of transmission electron microscopy (TEM) at the Institute of Geoscience, Jena. Platinum was deposited by the gas injection system (GIS) as a protective layer on the outside of the cell wall, which was then cut out by the focused ion beam (FIB) system (FEI Quanta 3D FEG dual FIB-SEM), using Gallium as ion source. Subsequently, the specimen was lifted out and transferred onto an Omniprobe TEM grid by an Omniprobe micromanipulator, and was finally thinned (by cutting out TEM foils as parallel cross sections of the plant cell wall) to electron transparency (~ 200 nm thick) by the FIB technique. The electron transparent foils were characterized by means of bright field imaging, EDX and selected area electron diffraction (SAED) using a kV FEI Tecnai G² TEM.

3 Results

3.1 Environmental risk assessment of the investigated site

Each remediation strategy should be site specific to ensure a proper improvement of the environment, and in this regard, the chemical assessment brought important information on how much the soil quality was affected by the heavy metal contamination. Thus, appreciations on the degree of soil pollution were enabled in order to understand how the local ecosystem can be influenced by the presence of Cu, Pb and Zn elements in soil.

The analyses conducted for total metal content indicated that Zn had the lowest contamination rate in the field, and exceeded the intervention threshold (1500 mg kg^{-1}) at both depths only in S2 and S3 sampling points with concentrations ranging from 4931 mg kg^{-1} to 5626 mg kg^{-1} . The contamination with Cu was increased at S5, S13 and S14 sampling points, where the alert threshold (250 mg kg^{-1}) was exceeded at both depths, and Cu concentrations ranged between $268\text{-}337 \text{ mg kg}^{-1}$. The measurements indicated even higher Cu contamination in S1, S2 and S3 samples ranging between $1157\text{-}1555 \text{ mg kg}^{-1}$, and exceeding the intervention threshold (500 mg kg^{-1}) at both depths. The analyses revealed that Pb had the highest contamination level, exceeding the intervention threshold (1000 mg kg^{-1}) in almost all samples. Exceptions were recorded in S10 and S11 samples, where the alarm threshold (250 mg kg^{-1}) was overpassed only in S11 samples with concentrations ranging between $393\text{-}447 \text{ mg kg}^{-1}$. An alarming soil contamination was indicated in S1, S2, S3, S5, S7, S13 and S14 points due to Pb concentrations which were even ten times higher than the Pb concentration admitted as intervention threshold, ranging between 9733 mg kg^{-1} and 11582 mg kg^{-1} . The results for total metal concentrations are summarized in table 3.

S1, S2 and S3 are found in the area where the former smokestack was demolished (Fig. 4), so the elevated metal contents, including for Cu and Zn, could be a consequence of the soil mixture with demolition waste. S5, S7, S13 and S14 are placed on the W to E wind direction, at approximately 500 m East from the plant. Thus, the increased Cu and Pb contents found in these points could have been generated by plant emissions like other studies in the area have suggested (Damian *et al.*, 2010). The

depth analysis did not indicate significant differences between metal concentrations, so it was assumed that the top layers might have been mixed after periodically landslides generated by ravines that can be observed in the field (Fig. 3). These phenomena could have led to an increased contamination of soil into the depth, beside the atmospheric pollution from the industrial plant. In regard to reference samples, Cu and Zn contents were below the alert threshold, while Pb exceeded the alert level at both depths, indicating that Pb pollution is more spread than it was anticipated.

Table 3, Total concentrations of Cu, Pb and Zn (mg kg^{-1}) in soil samples from the investigation and reference sites, and the corresponding legislative thresholds

Depth (cm)	Cu		Pb		Zn	
	10-30	40-60	10-30	40-60	10-30	40-60
Study area						
Min	29.6	29.2	137	132	113	118
Max	1555	1560	11337	11637	5626	5407
Average	447	470	6156	5920	1036	1130
Reference area						
	70.6	115	536	340	241	141
Legislative thresholds*						
Alert level	250		250		700	
Intervention level	500		1000		1500	

*alert/intervention threshold values for less sensitive soil use according to MO 756/1997

While the average pH of the investigation site was similar to the pH from the reference area, indicating an extremely to moderate acid soil, the TC and the DOC analyses showed significant differences between the two fields (Tab. 4), indicating low activity of soil microorganisms. The vegetative cover in the reference area was abundant in contrast to the one found at the investigation site, reason for which the low pH cannot be the only limiting factor for the lack of vegetation in the investigated field. Thus, it was assumed that the bioavailable metal fractions, whose mobility is directly influenced by the soil pH, induced ecotoxicological effects that inhibited the plant growth and the activity of soil microorganism. Based on the results of the chemical assessment, it was concluded that the soil quality was substantially affected by elevated Cu, Pb and Zn contents, and the site was framed as brownfield contaminated with heavy metals.

Table 4, The pH, total carbon (mg/kg) and dissolved organic carbon (mg/kg) in soil samples

Depth (cm)	pH		TC		DOC	
	10-30	40-60	10-30	40-60	10-30	40-60
Study area						
Min	3.57	3.37	4810	3240	210	127
Max	5.50	5.57	13700	12800	950	750
Average	4.69	4.52	10000	8200	480	400
Reference area						
	4.50	4.38	51900	27200	1940	1550

Assessing the pollution level based only on the total metal content of an element cannot provide relevant information that could explain the nature of pollution and which conditions can pose significant threats for the environment. Therefore, for an appropriate risk assessment, the bioavailable fractions which can be taken up by plants and soil microorganisms need to be determined. Gaining these information provides reliable parameters that need to be considered in regard to bioremediation strategies.

The bioavailability of a metal in soil is determined by the intrinsic solubility and relative dissolution rates of the solid-phase form into a solution phase, considering the uptake of a metal ion by plants (Traina & Laperche, 1991). According to BCR extraction scheme, the contents found in the acid-extractable fraction are mobile, and the metal forms which can be extracted by weak acids may become available for plant uptake because they are directly influenced by the humidity of the soil. For the investigated brownfield, the partitioning of the analyzed metals is presented in table 5. About 20% of the total content of each metal was found in the acid-extractable fraction, and was considered bioavailable for target organisms. About 15% of the total Cu and Zn, and 42% of the total Pb content were found in the reducible fraction, considered to be mobilizable under certain environmental conditions. In this phase, the metals are bound to Fe and Mn oxides, which are considered ideal scavengers of metals, featuring binding abilities based on the redox potential and pH of the soil (Gleyzes *et al.*, 2002). Since the soil pH is ranging between 3.37 and 5.57, the metal contents found in the reducible fraction can be released into the soil and become available for plant uptake.

Table 5, Partitioning of Cu, Pb and Zn (mg kg⁻¹) according to BCR extraction scheme (AE-acid extractable, RED-reducible, OX-oxidisable, RES-residual)

Depth (cm)	Study area		Reference area		
	10-30	40-60	10-30	40-60	
Cu	AE	0.36-1340 (132)*	2.40-532 (81.5)	7.44	5.92
	RED	2.20-576 (76.9)	1.64-362 (55.6)	4.68	4.36
	OX	2.70-466 (72.2)	3.25-520 (77.8)	36.2	18.9
	RES	16.0-1550 (223)	17.1-1690 (221)	30.5	14.3
Pb	AE	2.04-3310 (1662)	40.4-2740 (1184)	41.6	26.5
	RED	20.4-7280 (3017)	35.3-7120 (2202)	254	175
	OX	7.70-14100 (1728)	4.40-11000 (1495)	167	109
	RES	6.43-3920 (651)	4.09-3200 (604)	68.0	47.8
Zn	AE	9.60-1580 (208)	1.40-1500 (325)	75.6	72.0
	RED	5.72-1680 (172)	4.12-1470 (192)	65.6	35.2
	OX	13.3-1440 (211)	5.05-630 (144)	70.0	48.8
	RES	70.4-4600 (476)	65.7-5410 (569)	132	91.6

*Min-max (average)

The oxidizable fraction is considered to be less mobile than the previous one, and about 16% Cu and Zn, and 20% Pb were found to be bound to organic matter and sulphides. This fraction is associated with humic substances which can release small amounts of metals in a slow rate, due to their stable molecular weight, and could become mobile in oxidizing conditions (Filgueiras *et al.*, 2002). In general, metals accumulated in the first three fractions are related to anthropogenic pollution, thus conclude that the non-ferrous metallurgical plant has generated an extensive pollution in the area, especially with Pb, whose proportion was only 15% in the residual fraction. About 50% of Cu and 47% of Zn were found in the residual fraction, which is considered to be immobile. Metals found

in the residual fraction are considered to be incorporated in the crystal structure of primary and secondary minerals, and thus do not pose a risk for the environment.

As the metal fractionation after BCR scheme has indicated, the bioavailability of Cu, Pb and Zn is elevated in the brownfield, and the disturbed soil quality can favor a higher metal uptake by plants, thus generating various ecotoxicological risks that should be predicted in order to develop an efficient bioremediation strategy. However, the soil parameters still indicate appropriate conditions for fungi development which would require an optimum pH 5, usually being able to develop between pH 2 and pH 7 (Smith & Doran, 1996). In this way, the ECM fungi which were chosen for the bioremediation system could enhance the development of *Pinus sylvestris*, which are known for their abilities to grow in heavy metal polluted sites. Through the ectomycorrhizal symbiosis, the tree limitations in nutrient supply would be overpassed by *P. involutus* and *P. tinctorius*, while the missing carbon from the soil and needed for fungal growth would be assured by the trees. Therefore, a self-sustaining ecosystem can be prone as a bioremediation strategy for the investigated brownfield.

3.2 Heavy metal caused effects in microcosm studies

Based on previous research of Bizo *et al.* (2013), non-mycorrhized plants were not able to properly develop in heavy metal stress condition (a good growth being dependent on the fungal interaction), and therefore did not represent a feasible mechanism to be investigated in terms of bioremediation techniques for the present study.

Fungal colonization of root host represents the key achievement for survival of seedlings in metal conditions, therefore a special attention was firstly guided on the fungal growth during the experiment. Both ECM fungi showed three different types of development which can be characterized upon the growth direction of the mycelium. *P. involutus* started to grow from the first week of experiment and developed air mycelia which spread on the surface of the medium (Fig. 6A), and was observed in control, minimum Cu, minimum Pb and minimum Zn. After 2 weeks small signs of air mycelia were observed also in medium Pb, but without further visible development until the 6th

week, when it was noticed that *P. involutus* is slightly growing into the depth of the medium (Fig. 6B). This development was also present in minimum Cu starting with the 4th week of the experiment, after *P. involutus* initially had an air mycelia growing. These growing features are considered inhibiting effects on the spread of fungal air mycelia induced by metal toxicity at 0.3 mM CuCl₂ and 9 mM PbCl₂. By trying to develop into the depth of the contaminated medium, it can be assumed that the fungus was developing a defense mechanism in response to volatile organic compounds (Henke *et al.*, 2014), or was searching for carbohydrate sources since glucose is missing from the germination medium and the ECM symbiosis was not accomplished by that time. At higher metal concentrations, *P. involutus* growth was visible only inside the MMNb blocks (Fig. 6C) through which it was inoculated into the cultures. This development appeared after the 4th week in medium Zn, and between the 6th and 8th week in medium Cu, maximum Cu, maximum Pb and maximum Zn. The metal concentrations related to these growing features are believed to generate high toxicity on the fungal development, and the limitations could not be overpassed since the development of pine trees was also restricted, so the symbioses could not take place.



Figure 6, Growing features of *Paxillus involutus*.

- A. *P. involutus* developing air mycelia which can be observed through the brown mantle which is covering the surface of the germination medium;
- B. *P. involutus* growing into the depth of the germination medium;
- C. *P. involutus* developing inside the MMNb blocks, reason for which there are no visible signs of its growth on the germination medium

P. tinctorius started to grow only from the 6th week of the experiment and the level of development was rather insignificant in terms of mycorrhizal interactions with pine seedlings. Nevertheless, the fungus featured the same growing types as observed in the case of *P. involutus*, most predominantly being the development inside the MMNb blocks (Fig. 7A), and which was present in all growing conditions since the 6th week. After the 8th week of experiment a few air mycelia (Fig. 7B) was observed in control and minimum Cu, and after the 10th week a slight tendency of growing into the depth of the medium was present in minimum Zn (Fig. 7C). Another factor besides metal contamination (e.g. competition between fungi) was assumed to have restricted the development of *P. tinctorius* since the same weak growth occurred also in the control. For this reason, only *P. involutus* was considered to be the symbiont partner involved in the ECM interactions, and all the following results and discussions will be held in regard to this specific fungus.

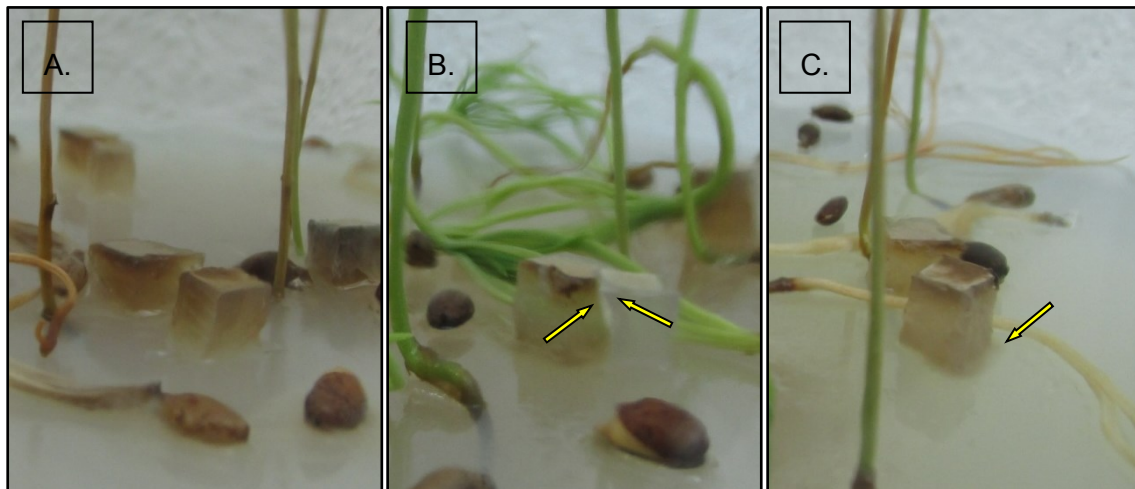


Figure 7, Growing features of *Pisolithus tinctorius*
A. *P. tinctorius* growing inside the MMNb blocks;
B. *P. tinctorius* developing little air mycelia on the edges of the MMNb block (arrows)
C. *P. tinctorius* slightly growing into the depth of the germination medium (arrow indicates the area where the fungal mycelia runs into the medium)

The germination rate of pine seeds was almost complete until the second week in all growing conditions (Fig. 8), indicating that all seeds were properly treated for the

experiment, and all the effects that occurred in their growth can be related to heavy metal stress. Upon the growing behavior of pine seedlings, three different growing types were distinguished: vertical growth, horizontal growth and inverted growth.

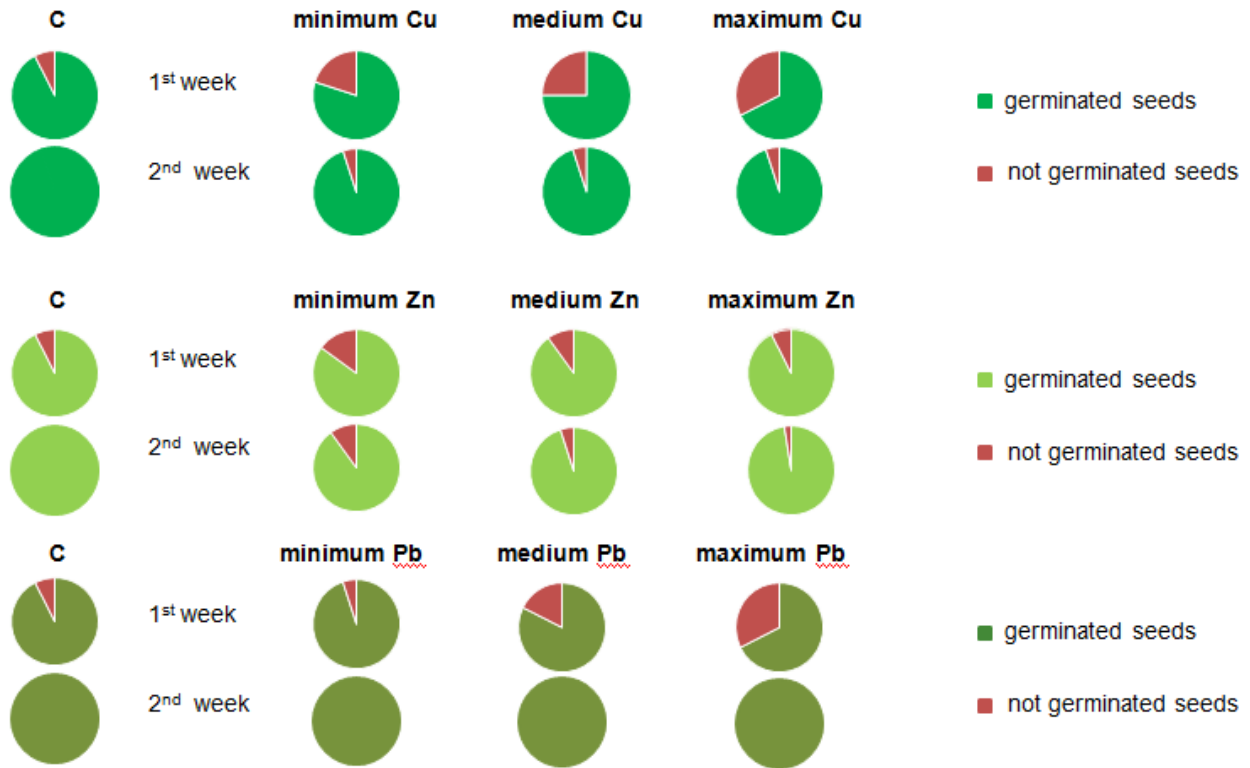


Figure 8, Germination rate of pine seeds after 1st and 2nd week of experiment, where germinated seed is considered the state in which the seed shell was cracked and the seedling is at least 0.1 mm in length

Vertical growth was defined for seedlings which had the roots developed in the medium, and the stem and the needle crown above the medium (Fig. 9). This growth represents the typical development of pine trees found also in nature and was characteristic to cultures grown in control, minimum Pb and minimum Zn. The seedlings looked strong by having a well developed needle crown, and showed a healthy green color. The root length ranged between 3 cm and 5 cm in pure germination medium, while in minimum Zn was decreased to 1-4 cm, and in minimum Pb was even more restricted, the root length ranging from 0.5 cm till 2 cm. The height of the shoots reached approximately 8

cm in control, 6.5 cm in minimum Zn and 6 cm in minimum Pb. An average of 11 seedlings developed in the vertical position in control, while around 7-8 seedlings in minimum Zn and minimum Pb. In comparison to co-cultures in germination medium, the observed differences can be related to 1.1 mM ZnCl₂, respectively to 2.6 mM PbCl₂ metal concentration in growing conditions. The toxic effects exhibited at these concentrations restricted the overall plant development, but did not limited the growth of *P. involutus* which enhanced the resistance mechanisms of the seedlings. *P. involutus* showed the best development in minimum Pb, and since Pb is not known as an essential element, it was believed that metal stress conditions had spurred the ECM interactions, leading to a fast fungal colonization.

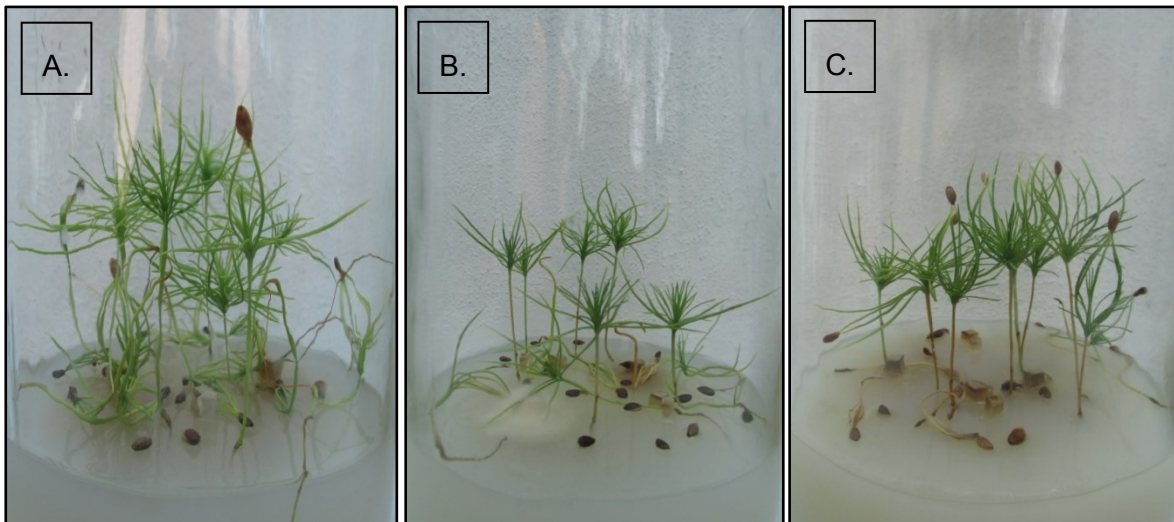


Figure 9, Vertical growth. A. Pine seedlings in C (12 weeks), B. Pine seedlings in minimum Zn (12 weeks), C. Pine seedlings in minimum Pb (12 weeks)

Horizontal growth was associated to seedlings whose roots developed on the surface of the medium (without penetrating into the medium) alike the stem and the needle crown (Fig. 10), and was predominant in medium Zn, while in other growing conditions was representative for a small number of seedlings. The seedlings which had their stem along the media were characterized by an unhealthy tissue, which showed severe discoloration linked to decreased contents of chlorophyll. The length of the roots was less than 0.5 cm and the needle crowns did not complete their development. By the end

of the growing period, most of the seedlings became dried and no fungal interactions were assumed to have occurred. These seedlings seemed to be the most affected by the metals present in the growing media, and the pronounced toxicological effects were related to possible metal absorption through the stem, which was in direct contact with the contaminated media. Not passing through the root, where they could have been subjected to different detoxification mechanisms, the metal ions might have been directed to accumulate in other parts of plant tissue where they exhibit their toxicity.

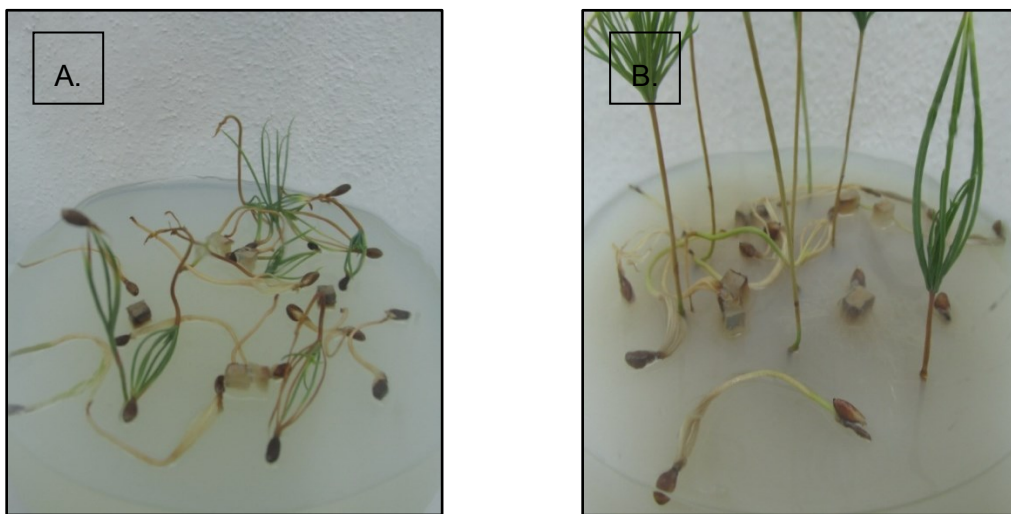


Figure 10, Horizontal growth. A. Pine seedlings in medium Zn (12 weeks),
B. Pine seedlings in minimum Pb (12 weeks)

The inverted growth was characterized by the development of roots in the air (above the medium), stem above the medium and needle crown on the surface of the medium (Fig. 11). This type of growth was representative for seedlings in minimum Cu, and in a lesser rate for minimum Zn and medium Zn. The length of the air roots ranged between 0.1 cm and 2.5 cm, but the fungal colonization could not take place due to the above medium position of the roots, and which was considered a defense strategy of pine seedlings against Cu toxicity. 0.3 mM CuCl_2 metal concentration was put in correlation to reddish colored stems, while 1.1 mM ZnCl_2 and 5.4 mM ZnCl_2 induced reddish colored roots. Starting with the 8th week of the experiment many seedlings started to dry, and by the end of the experiment only the seedlings which developed the needle

crown and kept the stem above the medium were able to survive the metal induced stresses.

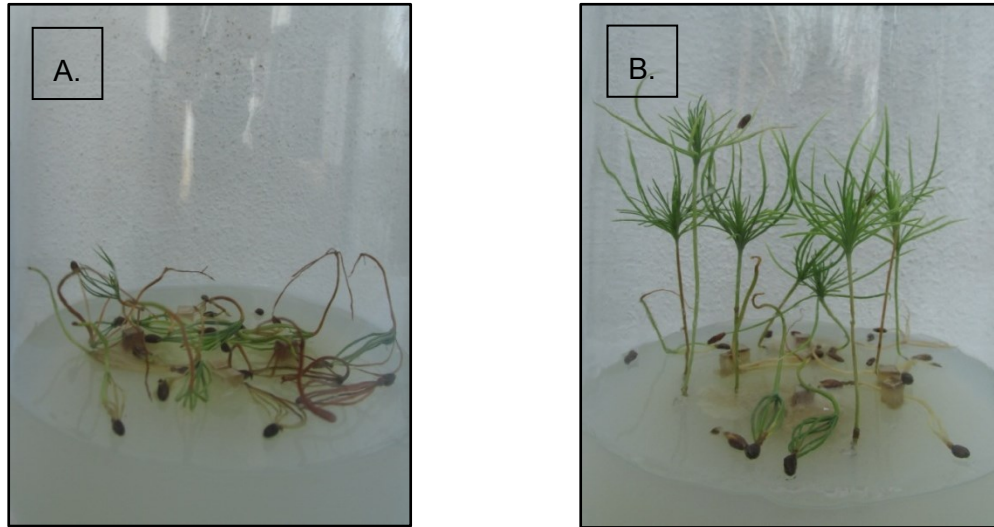


Figure 11, Inverted growth. A. Pine seedlings in minimum Cu (12 weeks), B. Pine seedlings in minimum Zn (12 weeks)

To evaluate the efficiency of the co-cultures in regard to metal uptake, a chemical balance was created (Fig. 12). Because the metal concentrations were too high for organisms to properly develop, the co-cultures from maximum Cu, maximum Pb and maximum Zn were not included in these calculations.

$$\begin{array}{c}
 \text{metal in soil} \\
 \hline
 \text{initial C in medium} \times \text{initial mass of medium} \\
 \hline
 \\
 \text{metal in biomass} \\
 \hline
 \text{C in biomass} \times \text{dried mass of biomass} \\
 \hline
 \\
 \text{metal in medium+fungi in/on medium} \\
 \hline
 \text{final C in medium} \times \text{final mass of medium} \\
 \hline
 \\
 \text{C - metal concentration}
 \end{array}
 =
 \begin{array}{c}
 \text{metal in biomass} \\
 \hline
 \text{C in biomass} \times \text{dried mass of biomass} \\
 \hline
 \\
 \text{metal in medium+fungi in/on medium} \\
 \hline
 \text{final C in medium} \times \text{final mass of medium} \\
 \hline
 \\
 \text{C - metal concentration}
 \end{array}
 +
 \begin{array}{c}
 \text{metal in medium+fungi in/on medium} \\
 \hline
 \text{final C in medium} \times \text{final mass of medium} \\
 \hline
 \\
 \text{C - metal concentration}
 \end{array}$$

Figure 12, The chemical balance formula, where "biomass" includes the mycorrhized seedlings, and "medium+fungi in/on medium" is a variable directly influenced by the development features of the fungus

In the case of co-cultures grown at low levels of metal contamination, 67% of the chemical balance was closed in minimum Cu, 75% was closed in minimum Zn and 99% was closed in minimum Pb. The lacking percentages for Cu and Zn were considered to be a consequence of the reduced spread of fungus mycelia. It was assumed that metal up-take can be influenced by two pathways: either by the mycorrhized seedling, either by the fungal mycelia which did not interact with the plant root and spread in/on the germination medium. Regardless if the metals were absorbed into the plant/fungal tissue, it is clear that the metal mobilization can be influenced by these organisms. Since the final concentration in the medium ("final C in medium") is a parameter of the chemical balance determined by ICP-MS from a piece of the germination medium (collected near the glass wall of the Weck jar), the metal distribution in this medium piece was influenced by the fungal mycelia spread, and therefore its metal concentration was also influenced. As *P. involutus* had an unitary spread all over the germination medium in minimum Pb (Fig. 13c), and thus achieving a representative Pb determination for the final concentration in the medium piece, the restricted spread of *P. involutus* in minimum Cu (Fig. 13a) and minimum Zn (Fig. 13b) generated lower metal contents in the corresponding medium pieces, which decreased their final concentration. As it can be seen in figure 13, the fungus spread is increasing from minimum Cu to minimum Zn, and then to minimum Pb, spreading which influenced the percentages for closing the chemical balances in a rising tendency.

The spreading of *P. involutus* influenced also the percentages at medium level of metal concentrations, where 89% of the chemical balance was closed in medium Cu and medium Zn, and 74% in medium Pb. Since the fungus activity took place only in the MMNb blocks and the seedlings developed only on the surface of the medium (Fig. 13d, e), the dispersal of metals into the medium was expected to remain homogenous, so the final concentration of the medium piece was representative for medium Cu and medium Zn. As soon as *P. involutus* started its development into the depth of the medium (Fig. 13f), the metals could have been mobilized in the direction of the spreading mycelia, thus providing an explanation for the decreased percentage in closing the chemical balance for medium Pb. Based on these observations it was stated that *P. involutus* plays an important role in the mobilization of metals in the medium.

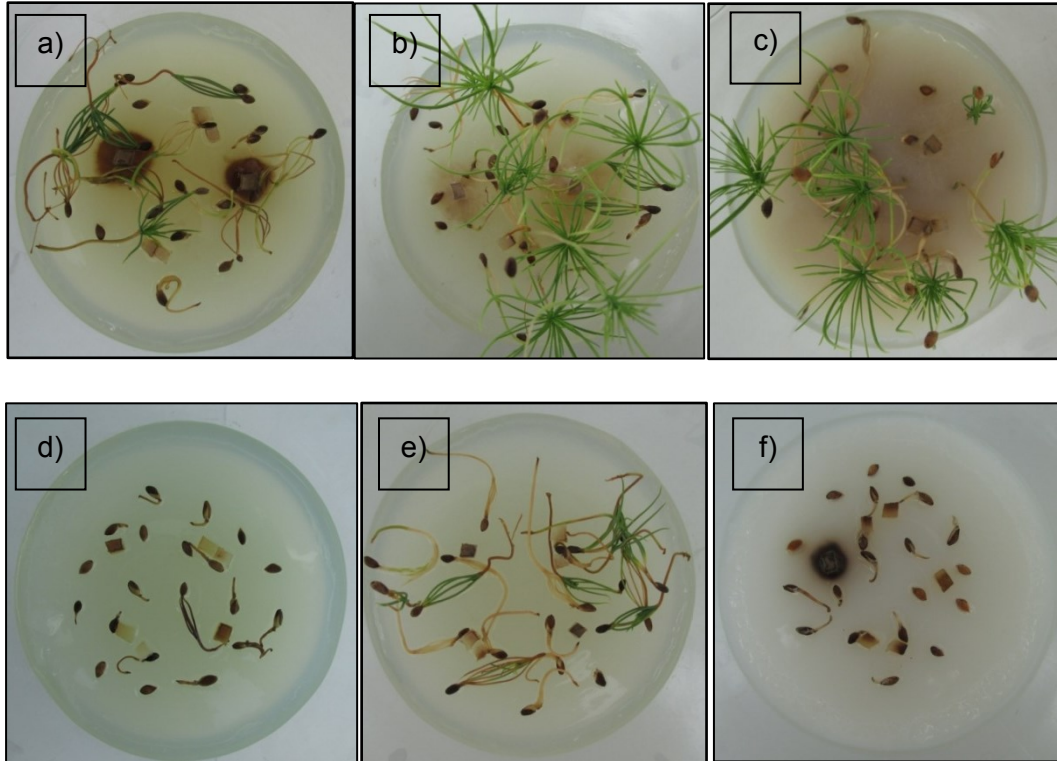


Figure 13, *P. involutus* spreading in a) minimum Cu; b) minimum Zn; c) minimum Pb; d) medium Cu; e) medium Zn; f) medium Pb

3.3 Investigation of the bioremediation system

As bioremediation system were considered the pine seedlings which were characterized by vertical growth in the presence of *P. involutus* found in a developing stage. Therefore, only seedlings from control, minimum Pb and minimum Zn (Fig. 14) corresponded to the required conditions and were subjected to further investigations.



Figure 14, Pine samples from 1-minimum Pb, 2-minimum Zn, 3-control at the end of experiment

3.3.1 Investigation of short roots

For determining the mycorrhization stage, the roots of the seedlings were investigated in regard to short roots formation, a unique feature of ectomycorrhizal symbiosis. Due to development in laboratory medium, which has the texture of a gel with extremely low porosity, the morphology of the root system was strongly influenced and the short roots were lacking the emanating hyphae and the rhizomorph (Fig. 15). Because the experiment was held for a period of 12 weeks, the short roots were found in an early stage of formation, reason for which their shape presented undefined features. While in growing conditions without metal treatment the root did not present obvious signs of fungal colonization (Fig. 15-1), in Pb and Zn treatments (Fig. 15-2,15-3) the root of pine seedlings seemed to be enclosed by the fungal sheath (indicated through a darker tissue layer), and associated to *P. involutus* hyphae which increased the thickness of root. These observations guided to the supposition that the heavy metal stresses promoted the ECM symbiosis to take place so that the organisms can cope with ecotoxicological effects. The present supposition comes to argue the assumption that a fast fungal colonization had led to the best growth of *P. involutus* in minimum Pb.



Figure 15, Colonization of pine root by *P. involutus* and short root formation in 1-control, 2-minimum Pb and 3-minimum Zn

Another specific feature of ECM symbiosis is the formation of Hartig' net in the root of the colonized seedling, which is of important interest because it represents the nutrient exchange area. Fungal nuclei were identified in short root cross-sections (Fig. 16), and indicated the presence of internal fungal hyphae especially in-between epidermis cells.

The decreased identification of nuclei in the cortex was related to an early stage of mycorrhization, and which is in favor to ectomycorrhizal symbiosis occurrence in the bioremediation system.

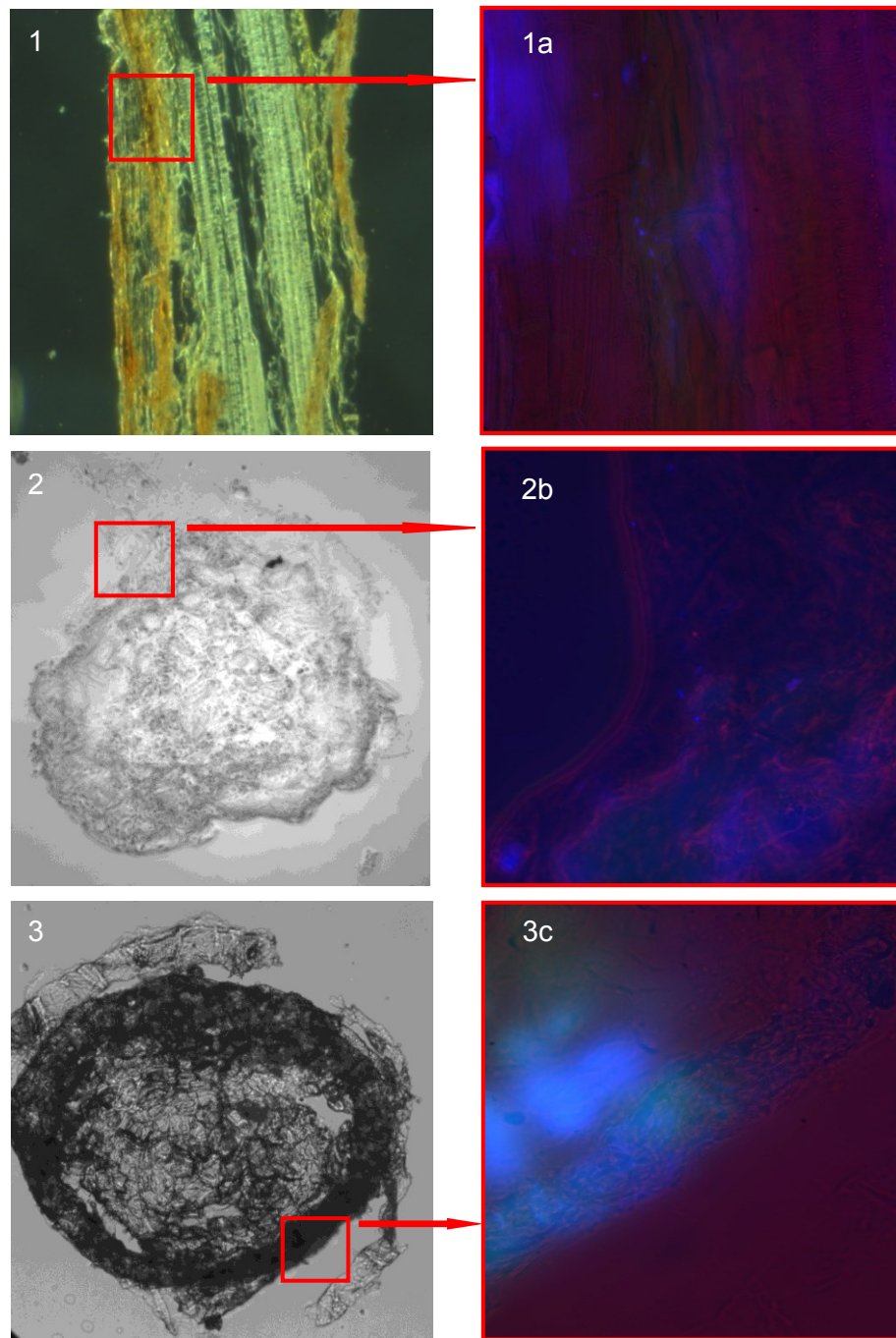


Figure 16, *P. involutus* nuclei stained in root tissue of pine seedlings from 1-1a control, 2-2b minimum Zn, 3-3c minimum Pb

3.3.2 Metal accumulation in the bioremediation system

The bioconcentration factor (BCF) was determined for co-cultures which included mycorrhized seedlings, therefore only the biomass from minimum Pb and minimum Zn was examined (Tab. 6). Being an essential element for plant growth, Zn was expected to present a higher accumulation potential by pine seedlings than Pb which is known only as environmental pollutant, but the results indicated more exclusion processes.

Table 6, Bioconcentration factor (BCF) of bioremediation system

Growing medium	Replicate no.	C _{biomass} ($\mu\text{g/g}$)	C _{medium} ($\mu\text{g/g}$)	BCF
minimum Pb	I	8599	51976	0.165
	II	10308	53685	0.192
	III	11709	55086	0.213
	IV	11909	55286	0.215
minimum Zn	I	2981	7266	0.410
	II	5692	9977	0.571
	III	3749	8034	0.467
	IV	4249	8534	0.498

As the BCF was below 1 unit for both metals, the bioremediation system cannot be characterized by phytoextraction capacity, and this implies the use of other tolerance and detoxification mechanisms that might be specific to phytostabilisation. In regard to plant tissue accumulation, phytostabilisation is correlated to metal ions absorption by the root, followed by adsorption in/on the root cells, and limiting the metal translocation to shoot. By restricting the metal ions transport to other parts of the plant (e.g. stem or needle crown), rhizosphere is the area in which important phytostabilisation processes are assumed to take place, and metal precipitation is expected to occur.

3.3.3 Metal distribution in the bioremediation system

Determining the sample composition in SEM analysis is directly influenced by the atomic number of elements in question, reason for which Zn ($Z=30$) indicated a weak distribution along the outer part of the root. Due to these limitations in the detection technique, the results were considered partly in accordance with the real localization of Zn in the specimen, so the investigation mainly focused on Pb ($Z=82$) localization.

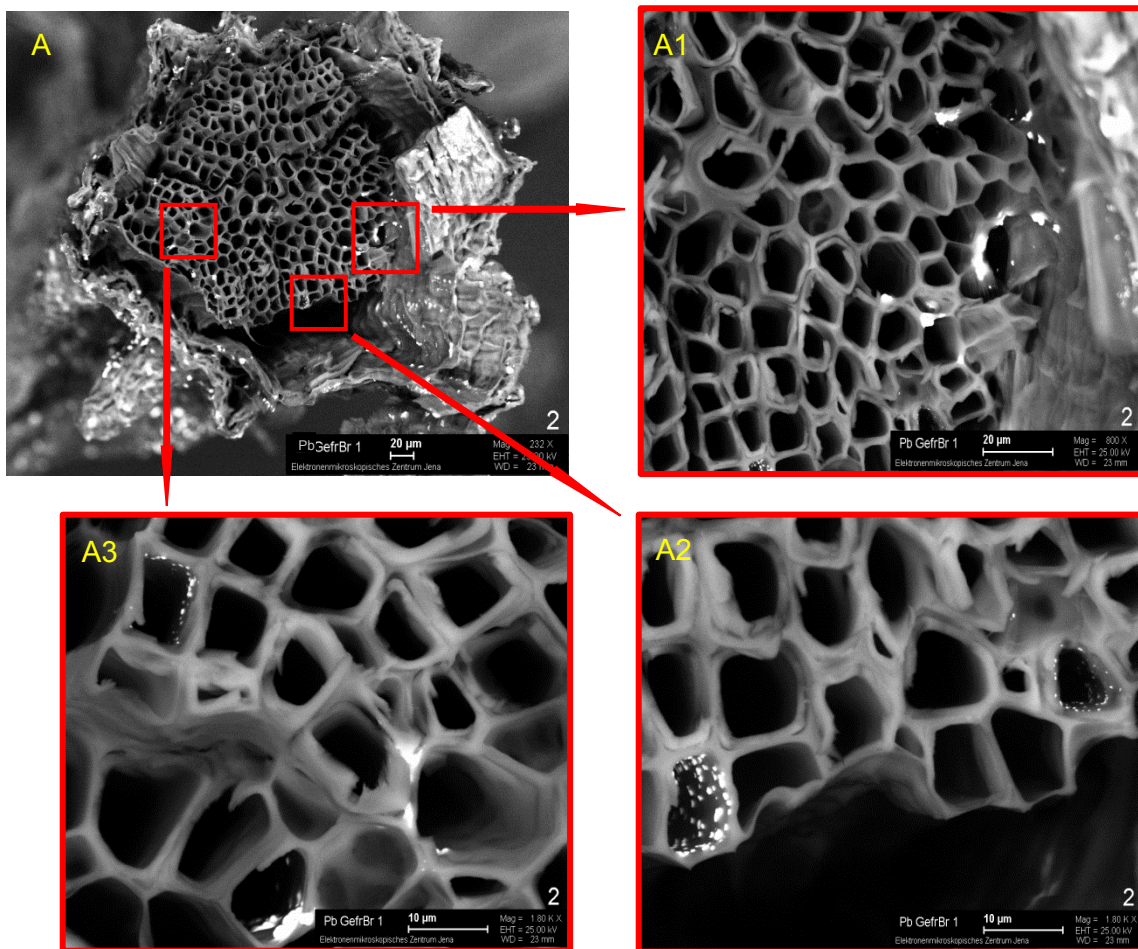


Figure 17, Distribution of Pb particles in the root of the mycorrhized seedling.
A. localization of Pb in root cross-section; A1, A2, A3 close-up with specific Pb accumulations inside the root cell structure

Bright spots were found to be distributed on the surface of the outer part of the root, and inside the root without passing the endodermis (Fig. 17). The SEM-EDX (Fig. 19a) confirmed that Pb is present in the composition of these accumulations, and the bright spots were considered to represent Pb particles (deposits). The accumulations containing the Pb particles were different in size and localization inside the root structure, reason for which the investigations were continued to other parts of the mycorrhized seedling. Only in the needle crown of the plant were found other metal accumulations (Fig. 18), while similar deposits were lacking in the stem. The accumulations were reduced in size compared to the ones found in the root, and were distributed only inside the needle structure (not on the surface of the needle), indicating that their occurrence in this upper part of the plant is due to transport inside the plant tissue and not to absorption from outer Pb sources.

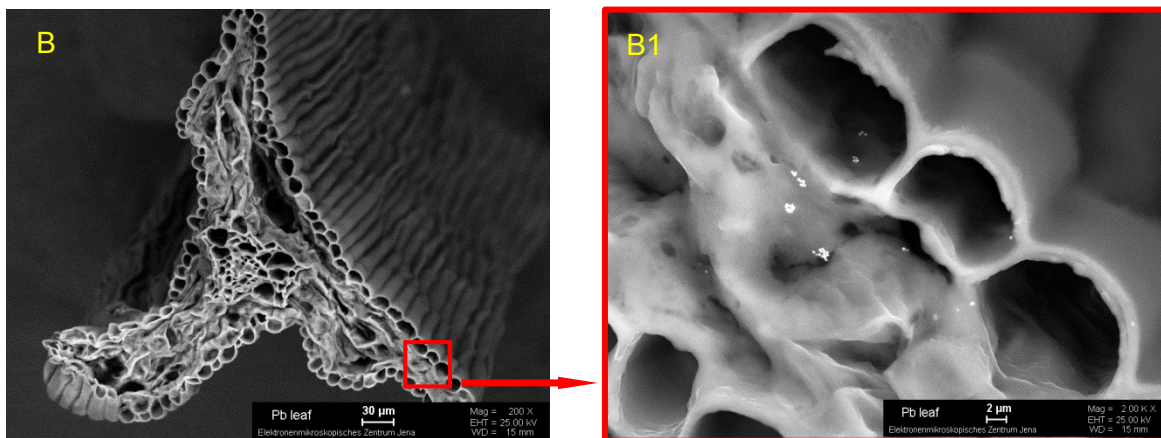


Figure 18, Distribution of Pb particles in the needle crown of the mycorrhized seedling.
B. localization of Pb in needle cross-section; B1 close-up with specific
Pb accumulations inside the needle cell structure

Through SEM-EDX (Fig. 19b) analysis it was confirmed that Pb is present also in the needle, so the overall elemental composition of the identified metal accumulations were compared, both in root and needle. The SEM-EDX investigation was conducted on two different areas of the specimens, one related to pure plant tissue and the other related to metal accumulation. Pb peaks (Fig. 19, yellow arrows) were recorded in the composition of metal accumulations, while in the plant tissues they were absent. The

presence of P and Cl elements in the composition of metal accumulations guided to assumption that Pb precipitates would have been formed inside the mycorrhized seedling, and they were related to tolerance mechanisms of the bioremediation system.

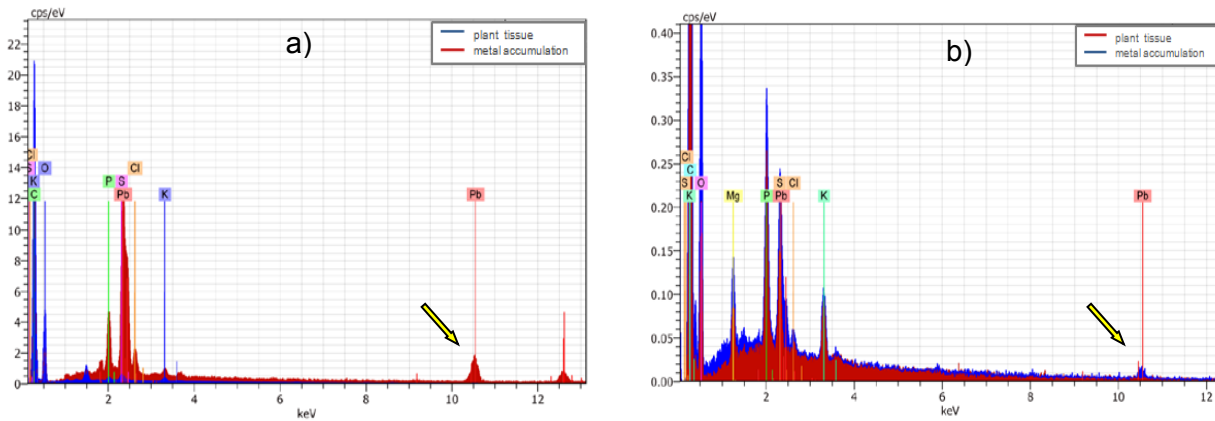


Fig. 19, Spectrum of elemental composition for metal precipitate in a) root, b) needle of the mycorrhized seedling

3.3.4 Identification of lead precipitates

Based on the crystallographic structure of the analyzed Pb precipitates it was proved through electron diffraction (Fig. 20) that the metal accumulations found in the mycorrhized seedling are pyromorphite minerals in the form of chloropyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$).

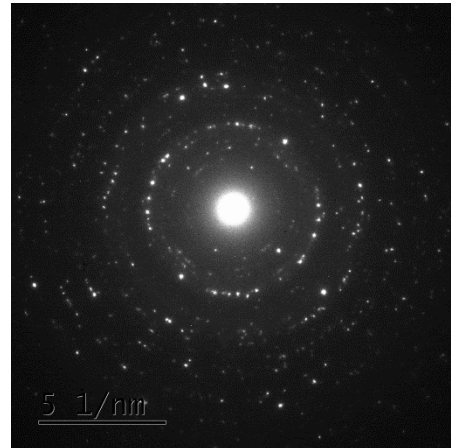
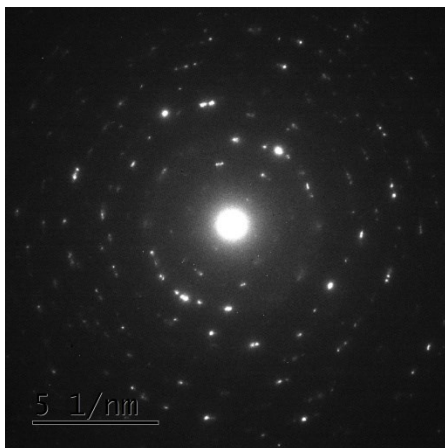


Figure 20, Electron diffraction pattern of Pb particle from a) root and b) needle

TEM investigations revealed that the mineral in the root is characterized by a different morphology than the one present in the needle, thus indicating two different phases of chloropyromorphite (Fig. 21). The presence of Pb minerals in the tissue of the mycorrhized plant are in favor of biomineralization potential of the proposed bioremediation system.

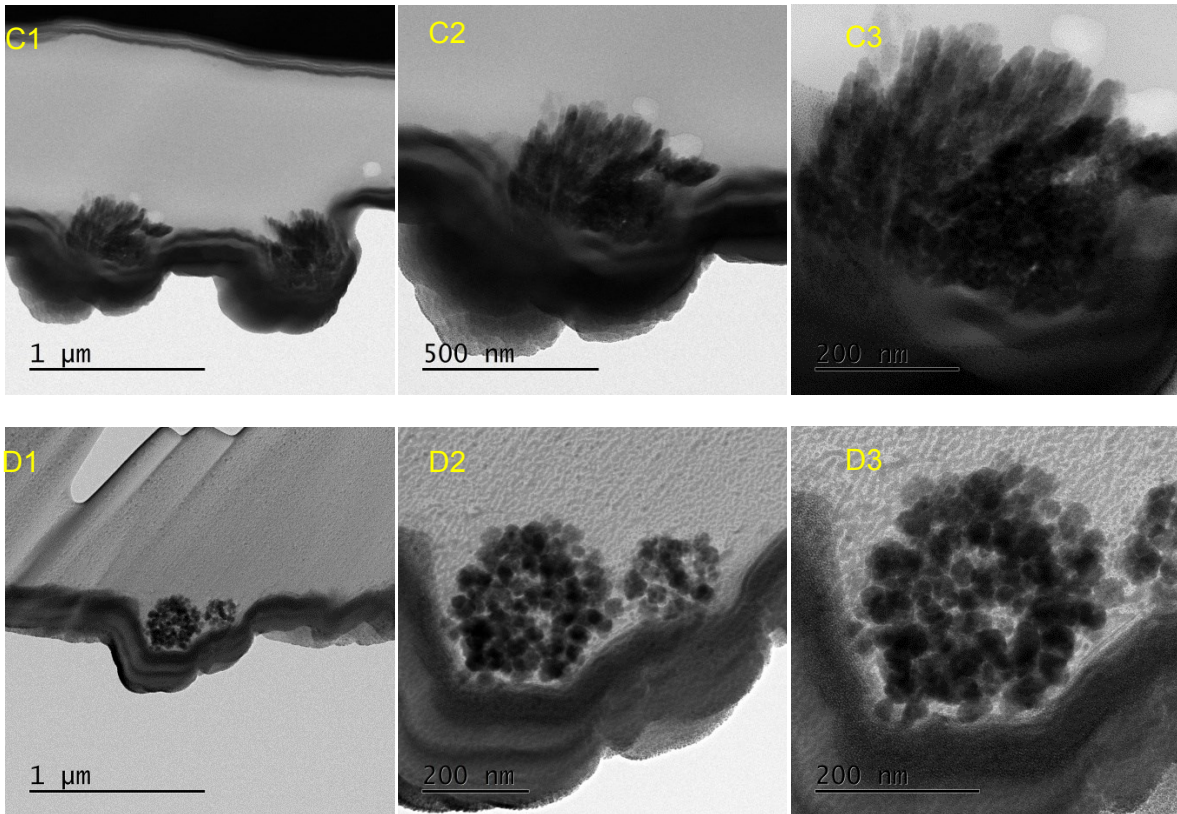


Figure 21, TEM micrographs of chloropyromorphite.

C1 chloropyromorphite phase in root; C2,C3 close-up of chloropyromorphite phase;
D1 chloropyromorphite phase in needle; D2, D3 close-up of chloropyromorphite phase

4 Discussion

4.1 Biomineralization potential

One of the strategies developed by metal resistant organisms implies altering the chemical nature of a toxic metal, resulting in the formation of nanoparticles which no longer cause toxicity of the concerned metal, and can be considered the "by-products" of the involved mechanisms against a specific metal (Pantidos & Horsfall, 2014). The process through which these metallic nanoparticles are being formed is explained as a synthesis that may occur either biogenic, either through chemical and physical methods. Bio-mediated processes are of important interest in the present discussion, and they can be divided into bioreduction, biosorption and bioaccumulation. Bioreduction refers to reduction of a metal ion by the oxidation of an enzyme which results in stable nanoparticles (Deplanche *et al.*, 2010), while biosorption implies the binding of a metal ion to the cell wall of certain organisms (bacteria, fungi or plants), being the first and fast step of the much slower and complex bioaccumulation process (Fomina & Gadd, 2014). The biologically guided synthesis of highly stable and well-characterized nanoparticles is known as biomineralization (Mann, 1993). The present study achieved the identification of Pb particles isolated from Pb deposits found in root and needle tissue of mycorrhized pine seedlings as chloropyromorphite minerals. The presence of these minerals within plant tissue represents the proof that higher plants can immobilize bioavailable metal fractions up-taken from growing substrates through internal biomineralization processes, and might use the biomineralization potential as a defense mechanism against metal toxicity. The chloropyromorphite mineral ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$) is characterized by an extremely low solubility ($K_{\text{sp}}=10^{-84.4}$) (Traina & Laperche, 1999), reason for which its biogenic formation is important in rendering bioavailable Pb in an insoluble form.

Biosorption is defined as a physico-chemical and metabolically-independent process based on a variety of mechanisms including absorption, adsorption, ion exchange, surface complexation and precipitation (Fomina & Gadd, 2014), and is mostly emphasized to removal of metals in terms of biotechnology for pollution treatment (Gadd, 2009). While biosorption is linked as a property of both living and dead

organisms, bioaccumulation is defined as a function of living organisms dependent on a variety of physical, chemical and biological mechanisms including both intra- and extracellular processes where passive uptake plays only a limited role (Fomina & Gadd, 2014). Therefore, the mycorrhized seedlings must have involved a bioaccumulation mechanism since Pb deposits were identified in plant tissue, and this process could occur only when the organisms were alive.

Pb deposits were localized in the free spaces between cells and inside the cells, mainly attached to the cell walls (Fig. 17-18). Due to the regions where metal accumulations were found within plant tissue, biosorption is the process believed to be firstly involved in the biomineralization process. Based on the present definition and significant information provided by elaborated studies (Fomina & Gadd, 2014; Gadd, 2009), the following assumptions can be made in regard to bio-mediated process involved within the investigated organisms: being part of the natural process of nutrient sorption from the growing surface, biosorption implied the inadvertently absorption of toxic Pb ions as passive uptake by living cells, followed by metabolically-independent adsorption of metal ions through surface complexation onto cell walls, and resulting in an accumulation at the ion-cell wall interface; since the formation of new three-dimensional surface species took place (visualized as Pb accumulations/deposits with SEM), and which can be defined as surface precipitates, different systems are believed to be involved in the continuum from adsorption to precipitation.

Reports on metal nanoparticle biosynthesis are relatively new, most of them focusing on recovery of noble metals (e.g. platinum, gold, silver) using different combinations of plant extracts (proteins, enzymes, vitamins, amino acids, organic acids, or polysaccharides) as reducing and/or stabilizing agents (Castro *et al.*, 2014). The great advantage of biosynthesis is that the nanoparticles are being formed under ambient conditions, while the industrial synthesis uses harsh reducing agents, capping agents, organic solvents and high temperatures and pressures for obtaining the same nanoparticles (Crookes-Goodson *et al.*, 2008). From material science perspective, the occurrence of biomineralization in plants can be classified as "green synthesis", and understanding the formation mechanisms of these biomineral composites is considered an inspiration model for the development of new materials (Gilbert *et al.*, 2005)

characterized by such an optimal combination of features as the one found in biogenic nanoparticles: unique physical, chemical, thermal, optical, magnetic and electrical properties (Pantidos & Horsfall, 2014).

4.2 Chloropyromorphite formation

Biom mineralization processes are linked with sorption of metals to cells, followed by interactions with specific groups of the cell wall (Barkay & Schaefer, 2001), and finally leading to biomineral formation if precipitation and nucleation occurs (Burford *et al.*, 2003). Biominerals are biologically-formed minerals by living organisms, whose formation mechanisms are not fully understood at the molecular level (Mount *et al.*, 2004), and which are composed of an organic matrix and nano- or micro-scale crystalline or amorphous components (Gilbert *et al.*, 2005). It is important to distinguish between the biologically induced mineralization, which is a process associated to extracellularly or on the cell surface activity of prokaryotes, and the organic-matrix mediated (controlled) mineralization attributed to eukaryote cells (Gilbert *et al.*, 2005; Lowenstam, 1981). The terminology used in this field is not always relying on these definitions, so the terms can have different associations in other scientific discussions.

In the case of chloropyromorphite, which is characterized by a crystalline structure, the biomineralization mechanisms inside the plant tissue are hard to explain. In regard to organic-mineral interface, starting from hypothesis based on mollusk shell or other models (Gilbert *et al.*, 2005), it can be assumed that the formation of a specific crystal phase, size and orientation is generated by a particular matrix of organic molecules produced by the plant, and which acts as a template for the crystal growth. Not seldom biomineralization has been attributed to various biomolecules like reducing sugars attached to cell walls, polyphosphates, fatty acids, hydrogenases and thiolated peptides (e.g. glutathione) (Crookes-Goodson *et al.*, 2008). It is believed that the organic components are formed first, followed by ion binding on this organic matrix, which can be seen as a nucleation site for the crystal growth (Lowenstam, 1981). Based on SEM micrographs, it can be assumed that the cell wall of pine provided suitable binding sites for Pb ions. The basic structure of the cell wall is composed of pectines, hemicellulose

and cellulose which are cross-linked and interconnected through Ca^{2+} and Mg^{2+} , thus forming an organic matrix in which Ca or Mg ions can be exchanged by Pb^{2+} (Bovenkamp *et al.*, 2013). This process could explain the ion exchange mechanism believed to take place in biosorption. Pb was reported to be bound in carbonate or phosphate forms inside the plant tissue (Peralta-Videa *et al.*, 2009), so another binding site for Pb ions could be the phosphate ones. The formation of pyromorphite requires the interaction between Pb^{2+} , phosphate and chlorine (Rhee *et al.*, 2012), thus the phosphate binding site represents the ideal source for the formation of Pb-phosphate minerals, and could explaining the Pb precipitation claimed to take place in the biosorption process. Because surface complexation of Pb cations is expected to occur, this can be described if a chemical bond is formed between the metal and the electron donating oxygen ion, or if the cation is found in the very close proximity of the surface negative group, but at least one water molecule separates the cation from the base (Fomina & Gadd, 2014). The dentate feature of the particles, noticed in the morphology of chloropyromorphite (Fig. 21), can be associated with these premises of surface complex formation (Gadd, 2009). Other potential binding sites could be represented by the negatively charged carboxyl groups (COO^-) (Clemens *et al.*, 2002) located in amino acids or polysaccharide chains along their proteins, which can mediate (through electrostatic interactions) the attraction of positive ions found in liquid phase and generate the crystal nucleation and growth (Weiner & Dove, 2003). The complexation of Pb by COOH/OH -containing molecules was reported by Schreck *et al.* (2014) in studies using rye-grass as test organism. Based on TEM micrographs (Fig. 21), where chloropyromorphite can be seen in a close association with the cell wall, it can be assumed that the Pb binding sites represent the nucleation sites for pyromorphite minerals, therefore involving a heterogeneous nucleation process which leads to intracellular biogenic nanoparticles in the pine seedlings, and thus explaining the final step of the biomineralization process. Following Mandal *et al.* (2006) studies on algal biosynthesis, formation of nuclei is attributed to enzymatic reduction of metal ions (bioreduction), which subsequently grow through further reduction of metal ions and accumulation of these nuclei, resulting in an epitaxial growth. This growth seems to be more characteristic to the minerals formed in the root, while the chloropyromorphite

formation in the needle presents the features of a cluster development or an aggregate. The growth of the crystals can be directed by a protein template, and in this way the physical dimensions and the features of the template are being preserved in the size, crystallinity and shape of the final nanoparticle (Crookes-Goodson *et al.*, 2008).

Chloropyromorphite ($\text{Pb}_5(\text{PO}_4)_3\text{Cl}$) is considered the most stable mineral from the pyromorphite family ($\text{Pb}_5(\text{PO}_4)_3\text{X}$ where $\text{X} = \text{Br}, \text{F}, \text{OH}$), and its formation is accomplished by the reaction of Pb with different phosphorus sources (Miretzky & Fernandez-Cirelli, 2008). Apatites ($\text{Ca}_5(\text{PO}_4)_3\text{X}$) are phosphate minerals considered geochemically stable forms of P in the environment (Lindsay, 1979), where chlorapatite ($\text{Ca}_5(\text{PO}_4)_3\text{Cl}$), fluorapatite ($\text{Ca}_5(\text{PO}_4)_3\text{F}$), and hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) are among the most used ones in the remediation techniques for Pb polluted surfaces (Traina & Laparche, 1999). Calcium, phosphorus and chlorine are macronutrients for higher plants that may lead to the formation of apatites due to chemical interactions within the aqueous phase of plant uptake, while Pb^{2+} is the principal dissolved form of lead (Sauvé *et al.*, 2000) without any biological significance. Therefore, it is questioned how chloropyromorphite is being formed inside the plant tissue? Prior formation of this mineral outside the plant, followed by plant uptake and storage into tissue is out of the question because plant cells do not perform endocytosis. Possible mechanisms for soil and water induced formation of pyromorphite have been often discussed (Miretzky & Fernandez-Cirelli, 2008; Traina & Laperche, 1999), but the plant internal environment is influenced by various mechanisms that can be quite different than the once involved in nature. Therefore, due to the controlled environment which was created in the conducted experiments, it is assumed that biologically mediated mineralization has led to chloropyromorphite formation in mycorrhized pine seedlings as a consequence of biomineralization potential. Since the specific morphology, size and crystallinity of the nanoparticles are a result of the organisms' genetic control (Weaver & Morse, 2003), the presence of different chloropyromorphite phases between the root and the leaf of the same plant strengthens the hypothesis that various metal resistance mechanisms are being involved by the same organism.

4.3 Mineralogy of lead in biological systems

A large number of studies focused on Pb uptake and provided quantitative data on toxic ions, while the metal distribution into plant tissue benefits from a lesser documentation. Identification of elemental constituents of metal deposits to determine the chemical forms in which Pb could be accumulated in plant tissue receives increased attention lately, but the evidence on biogenic chloropyromorphite are rather limited. The majority of these investigations were conducted on plant roots belonging to hyperaccumulators (mainly represented by grass species), reason for which the present study brings valuable evidence on chloropyromorphite formation inside the roots and leaves of a tree species framed as metal resistant plant. Laperche *et al.* (1997) claimed the presence of chloropyromorphite on the surface of *Sorghum bicolor* (sudax) roots, but XRD evidence was missing from the study and the determination of chloropyromorphite was related to EDS measurements combined with solubility products and physical features of the particles. This technique is insufficient for an adequate mineral identification, and may be expected to rely on subjective assumptions. Additionally, the examination was conducted on possibly unwashed roots which grew in soil treated with apatites, so the formation of pyromorphite minerals would be rather a consequence of apatite amendments than a by-product of the defense mechanisms involved by the plant. Cotter-Howells *et al.* (1999) reported the presence of Pb-P grains with a pyromorphite-like structure in the outer wall of the epidermis of *Agrostis capillaris* roots, but the observations tried to distinguish between pyromorphite (in the form of chloropyromorphite) and Pb-hydroxyapatite (also known as hydroxyapatite) phases. The diffraction analyses were conducted on ashed (at 400⁰C for 8 h) samples of roots, conditions which could have interfered with the crystalline structure of the Pb minerals, a drawback mentioned also by Kopittke *et al.* (2008). At the same time, Kopittke *et al.* (2008) reported the presence of chloropyromorphite in vacuoles within signal grass (*Brachiaria decumbens*) and Rhodes grass (*Chloris gayana*), and attributed the pyromorphite minerals to apoplastic sequestration only in signal grass, which is a Pb resistant plant. However, the identification of chloropyromorphite is not supported by diffraction patterns because the electron beam damaged the Pb-deposits as the authors have reported, so the speciation of Pb compounds was based on multiple EDS spectra

which were averaged and scaled relative to the Pb M α 1 peak. This cannot be accepted as a reliable speciation technique, because the mineral determination relies again on estimations and assumptions. When discussing the localization of Pb deposits, another possible drawback of Kopittke *et al.* (2008) can be represented by the fixing step in the sample preparation, which might have changed the distribution of Pb deposits within the root as Laperche *et al.* (1997) reported for their study, or by the cutting process of ultrathin sections (performed without embedding), which could have brought overlapping of inner particles with particles from the outer part of the roots. In another study, Meyers *et al.* (2009) reported the presence of chloropyromorphite in the apoplast of *Brassica juncea* roots, but the evidence is based on averaged EDS spectra following the Kopittke *et al.* (2008) modeling pattern, which was already discussed as an insufficient proof. Moreover, the EDS spectra from Meyers *et al.* (2009) are lacking the Cl peak, meaning that chloropyromorphite identification is highly doubted since the presence of chlorine in the Pb deposits is unsure. Schreck *et al.* (2014) discussed about pyromorphite presence in rye-grass (*Lolium perenne*) leaves, but the results were partially confirmed by X-ray absorption spectroscopy (XAS). Also, the investigation was conducted on plant powder resulted from unwashed leaves, reason for which the real source of the assumed to be pyromorphite minerals could be doubted. The surface of the leaves might have been contaminated by atmospheric deposition of Pb particles due to the reduced growing sizes of the plant that developed in the proximity of contaminated soil. To date no other studies have reported the presence of pyromorphite minerals within plant tissues.

Therefore, because the mentioned investigations did not provide precise and reliable evidence on biogenic pyromorphite in plant tissue as a consequence of root exposure to Pb, the present study is considered to bring the first proof of chloropyromorphite formation in *Pinus sylvestris* roots and leaves. Additionally, the extraction of metal nanoparticles from the biological samples is a real success of the study and for the researches held in this scientific field, representing the key step in the identification of metal particles. Researchers' attempts to extract nanoparticles from cells usually involved physico-chemical methods that might have interfered with the morphology and structure of nanoparticles, or have used enzymatic lysis of plant cells which has the

drawback of being a destructive technique for the samples. Since the sample preparation for the present study relied on freezing and braking (not cutting) different plant parts, the origin of the identified minerals was precisely localized, and the SEM micrographs are expected to provide a real metal distribution within mycorrhized pine tissue, where Pb particles were identified in epidermis and cortex of the colonized root, and in mesophyll, hypodermis and epidermis of the needle.

In regard to Pb distribution, various studies focused on the metal distribution inside different plant species, but the investigations were mostly conducted on seedlings generated in lab conditions or pot experiments. In this way, the assessment of metal distribution can be related only to the first stages of plant development, leaving an open question on how the accumulation process can be influenced in the following phases. The advantage in using these seedlings is provided by their reduced growth sizes which allow visualization of metal distribution among the complete cellular composition of different structures (root, stem or leaf) when cross-sections are being used for electron microscopy. The sample sizes are limited by the specimen mounts used in microanalytical methods, which usually have a ranging diameter between 5 mm and 25 mm, so only restricted plant tissue can be subjected to metal localization for plants found in more advanced developing stages. The micrographs resulted from seedling investigations can provide pathways for a general modeling of metal distribution at cellular level, but special care has to be given for images captured from samples which have undergone special tissue preparation. Redistribution of ions was reported to occur for tissue fixation methods (Jentschke & Godbold, 2000; Laperche *et al.*, 1997), or during dehydration through ethanol series, which could also induce artifacts formation (Hartley *et al.*, 1997; Orlovich & Ashford, 1993). The cutting of thin sections could bring modifications or disruptions of cellular structures if the biological tissue is not embedded in resins (process which however requires ethanol dehydratation), or can create surface overlaying with outer metal. Also chances to lose metal particles in the embedding process have been mentioned in different studies (Antosiewicz & Wierzbicka, 1999; Meyers *et al.*, 2008). These observations can rise critical remarks since many studies for metal distribution have relied on electron microscopy evidence provided by samples which were subjected to preparation processes as the ones mentioned above.

Therefore, the creation of a protocol which could overcome all these weaknesses in the preparation step of biological tissues would bring a great step ahead for the scientific work involved in this research field.

4.4 Mechanisms proposed to enable ECM associations in bioremediation

Many publications have claimed to present metal toxicity reduction by ectomycorrhizas, but only few studies bring evidence for such an effect. The improved development of plants exposed to toxic metal concentrations was considered an ameliorating effect provided by specialized fungal species only when the fungal tolerance exceeded the tolerance of the host plant (Jentschke & Godbold, 2000). Altering the metal sensitivity of the plant by EMC fungi can be accomplished directly by reducing the metal availability to the host root, or indirectly by modifying the physiological processes of the plant. However, the improved tolerance enhanced by these fungi is conditioned also by the metal concentration and its speciation (Jentschke & Godbold, 2000).

Little is known about the defense mechanisms of metal resistant plants, most of the studies being conducted on Ni, Cd, or Zn hyperaccumulating species (Krämer, 2010). Involved mechanisms for Pb tolerance and detoxification are relatively unknown, and the limited researches performed upon this topic have mainly reported the restriction of Pb to roots, with reduced amounts of metal translocation to shoots (Tian *et al.*, 2010). The distinct mineral structures of chloropyromorphite found in the mycorrhized seedling guided to the hypothesis that the root mechanisms differ from the needle crown ones, involving transport of Pb^{2+} inside the vascular tissue of the plant through the liquid phase. Due to the presence of Pb precipitates on the root surface and inside the root tissue (Fig. 17), it can be assumed that the absorbed metal ions are partially restricted in the root area based on the principles of phytostabilization, while another fraction is translocated to shoot, where other mechanisms are involved in Pb tolerance. These assumptions assert that the proposed bioremediation system benefits from various detoxification and tolerance mechanisms, both at cellular and whole mycorrhized plant level, and these mechanisms can be simultaneously activated.

4.4.1 Lead transport at root level

In regard to absorption and transport of metal ions, Pb^{2+} were taken up by the colonized root along with water absorption, process that could have been enhanced by the *P. involutus* hyphae, known to facilitate the transport of water and certain nutrients to the roots. Based on SEM investigations that revealed the presence of Pb accumulations on the outer surface of the root (Fig. 17), it is believed that extracellular chelation and cell wall binding was promoted by the fungal mantle and root exudates. Rhee *et al.* (2012) reported outer pyromorphite formation by fungi, and suggested that fungal metabolites, particularly organic acids, play an important role in mediating extracellular precipitation of secondary minerals. Extracellular precipitation as lead oxalate (Fomina *et al.*, 2005c) and Pb accumulation within the fungal biomass (Fomina *et al.*, 2005b) have been indicated as consequences of fungal excreted chelators, while the accumulation of Pb was related to phosphate coordination in *P. involutus* tissue (Fomina *et al.*, 2007). Other studies on *P. involutus* exposure to Cd have shown the same involvement of oxalic acid (among other organic acids) in reducing the uptake of Cd into fungal cells, together with Cd cell wall binding (Blaudez *et al.*, 2000). However, acid exudation is considered to be metal and species dependent (Bellion *et al.*, 2006), and more studies are required for a better understanding of the molecular mechanisms involved in the synthesis and release of these organic compounds. Binding to the cell wall is related to metal precipitation, and the free amino, carboxyl, hydroxyl, phosphate and mercapto groups found in the composition of the fungal cell wall represent potential binding sites (Bellion *et al.*, 2006). Pb sorption on the mycelium of *P. involutus* was found to occur by Marschner *et al.* (1998), and binding to cell wall was assumed to take place at cation exchange sites, possibly followed by metal precipitation. In this way, the fungal mantle, which is a complex matrix composed of external hyphae, acts like a barrier using different processes that restrict the metal movement to the pine root.

Once the metal ions enter into the fungal cells, intracellular mechanisms are involved in the metal detoxification processes. Several studies on *P. involutus* (Howe *et al.*, 1997; Ott *et al.*, 2002) have indicated the role of metallothiones (MT) and glutathiones (GSH) as metal chelators, where MT are responsible for the sequestration of environmentally toxic metals (e.g. Cd, Hg) and keeping the homeostasis of essential trace metals (e.g.

Cu, Zn), while GSH prevent the cell injuries by chelating and sequestering the metal ions (Bellion *et al.*, 2006). The presence of GSHs and MTs in *P. involutus* was asserted for Cd exposure (Courbot *et al.*, 2004) and Cd sequestration was proposed through vacuolar compartmentation (Blaudez *et al.*, 2000), while no evidence has been found for Pb exposure. Lead being known as an environmental pollutant as cadmium, may have been subjected to the same intracellular detoxification processes, which could have been followed either by enhanced efflux of Pb^{2+} from the cytosol, or either by subcellular compartmentation in the vacuoles as Pb-conjugated glutathione. Also vacuolar compartmentation by polyphosphate granules has been proposed by several studies, but the evidence were considered unconvincing, especially the ones based on TEM micrographs showing metal depositions into vacuoles, because metal redistribution during the specimen preparation might have occurred (Hartley *et al.*, 1997). These detoxification mechanisms may have been supported by metal transport proteins as they were indicated by Williams *et al.* (2000), but more research is required for the special case of Pb since most of the putative mechanisms involved in fungal metal tolerance and detoxification rely on limited studies. Generalization should be made with care because it is known that toxicity is specific to each heavy metal, and the mechanisms involved against metal stresses are particular to each fungus.

The external absorptive hyphae, responsible for nutrient uptake, may also enhance the Pb^{2+} uptake and transport them inside the root through the developing internal hyphae, but a part of the metal ions get transformed to less soluble Pb compounds due to fungal biomineralization potential (Rhee *et al.*, 2012). It is relatively hard to predict the source for Pb precipitates in the area where the internal hyphae are penetrating in-between the root cells due to the close associations of the ECM symbionts. Still, some assumptions can be made based on organic acids which may be involved in Pb complexation. Increased oxalic acid exudation was reported for pine exposed to elevated concentrations of toxic metals (Ahonen-Jonnarth *et al.*, 2000), while lead oxalate was found as an extracellular precipitate of ECM fungi in other studies (Fomina *et al.*, 2005c; Johansson *et al.*, 2008). First, it may be hypothesized that *P. involutus* is inducing oxalate formation inside the root tissue which acts as a ligand for Pb^{2+} , resulting in precipitated Pb-oxalate, without posing any toxicological threats to the plant. Second, it

may be assumed that some of the Pb^{2+} which are transported through apoplastic route can be subjected to chelation by oxalic acid excreted by the root cells as a metal exclusion mechanisms. At the same time, the movement to vascular tissue of Pb^{2+} found in the apoplast can be restricted by the hydrophobicity of the internal fungal hyphae, which are branching in-between the cortex cells for the development of the Hartig' net (Fig. 22). In this way, the fungal mantel can be seen as a biofilter (Krupa & Kozdrój, 2007) against metals, and evidence for Pb and Zn exclusions from root tissue by certain mycorrhizal fungi was discussed by Jentschke and Godbold (2000). In this phase, a part of the metal ions can be bound on the fungal cell wall (outer surface of the internal hyphae) or on the pine root cell wall (epidermal and cortical cells). Investigations conducted for distinguishing between fungal and plant metal uptake have indicated that the metal adsorption on fungal cell walls is not stronger than that on host tissue (Jentschke & Godbold, 2000).

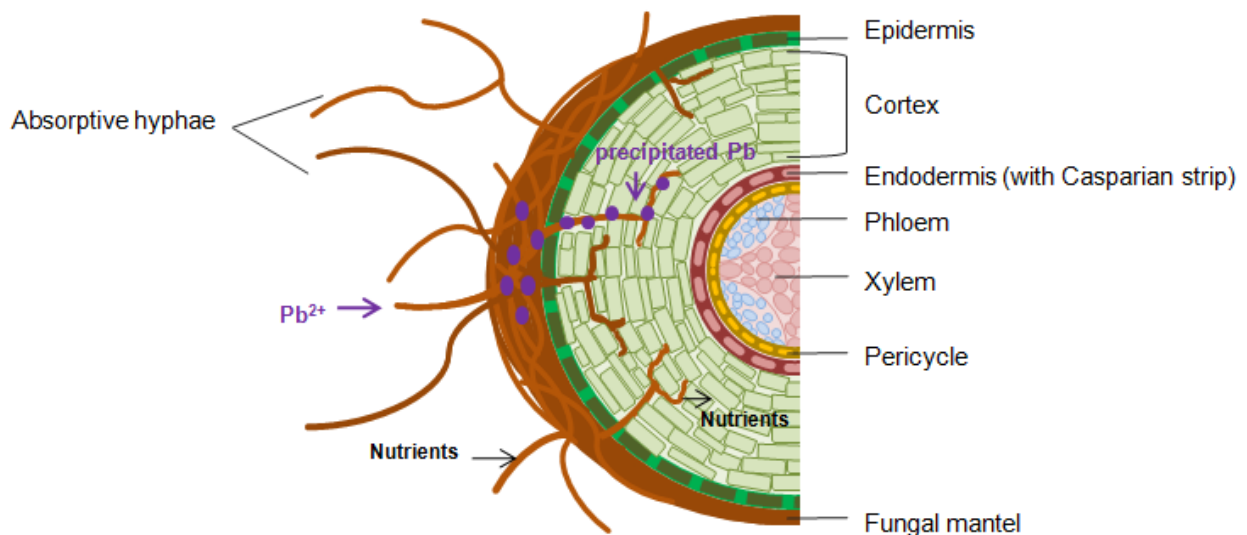


Figure 22, Schematic representation of lead transport by *Paxillus involutus* hyphae inside the mycorrhizized root of *Pinus sylvestris* (cross-section)

Uptake into roots is believed to be mediated by membrane transport proteins for essential metal ions, but no specific transporter has been proved to accept Pb^{2+} as substrate (Fischer *et al.*, 2014). The cell wall represents a low affinity and selectivity ion

exchanger involved in the first part of the metal uptake process, followed by uptake across the plasma membrane mediated by specialized transporters such as channel proteins or H⁺-coupled carrier proteins (Clemens *et al.*, 2002). Cell wall capturing by means of proteins, polysaccharides as pectines, cellulose and ligno-cellulose matrices is being seen as a common strategy for metal disposal in the plant structures (Marmioli *et al.*, 2005). Also the plasma membrane, which is characterized by negative potential and intracellular high-affinity binding sites (Clemens *et al.*, 2002), could have favored the deposition of Pb²⁺. SEM investigations indicated the presence of Pb accumulations between and inside the root cells, attached to the cell walls (Fig. 17), but the plasma membrane cannot be visualized in SEM micrographs because the sample preparation for microscopy required dried samples. Differentiating between fungal and plant extracellular/intracellular mechanisms for metal defense would be rather hard in the root area due to root colonization by *P. involutus*.

Furthermore in the transport of metal ions, before reaching to endodermis, the water containing Pb²⁺ is blocked by the Casparian strip, which is an inner cell layer that surrounds the vascular strand of the root, and is mainly composed out of suberin, an inert impermeable waxy substance (Kolattukudy, 1984). Meyers *et al.* (2008) provided microscopic evidence of Pb deposits found in the extracellular space of cortical cells localized to the adjacent endodermal cells, suggesting the exclusion role of the Casparian strip. In this way, water is forced to shift from the apoplastic route to the symplastic route, a process through which the plant is selecting the solutes which may pass further into the plant tissue (Kolattukudy, 1984). Before reaching into the vascular cylinder, the metal ions are directed to travel through cortical and endodermal cells, where Pb²⁺ can be subjected to intracellular mechanisms for metal tolerance and detoxification specific to higher plants. Tolerance in higher plants is often associated with maintenance of membrane integrity, and is consider essential for intracellular detoxification processes to take place (Meharg, 1993). Cytosol chelation and vacuolar sequestration are expected to occur as intracellular metal detoxification processes (Fischer *et al.*, 2014).

These mechanisms might have been involved in metal defense also previous to pine mycorrhization, because it was assumed that the symbiosis could have started after

approximately two months from the inoculation of pine seeds. *P. sylvestris* being known as a metal tolerant plant is expected to rather use exclusion mechanisms like reduced influx across plasma membrane and increased efflux outside the cytosol (Fig. 2) of Pb^{2+} in order to assure metal homeostasis (Hall, 2002). These hypotheses rely on researches conducted on bacteria, and in which efflux pumping systems were identified for Cu, Cd, Zn, Co and Ni; the metal resistance systems were based on efflux of toxic ions, involving transporters such as P-type ATPases or cation/ H^+ antiporters (Silver, 1996). In other studies, Schlunk *et al.* (2015) have shown that *mte1*, a member of the MATE family, is responsible for detoxifying Cu^{2+} either by enhanced efflux, or by vacuolar compartmentation in ECM fungi, and linked the high expression of *mte1* in symbiotic tissue (Krause & Kothe, 2006) with the role of transporting cations with different oxidation states, probably by glutathions. Other MATE proteins identified in plants proved to chelate Al^{3+} in the rhizosphere through efflux of citrate (Durrett *et al.*, 2007). In tobacco plants was described a plasma membrane transporter associated to Ni tolerance and hypersensitivity to Pb (Arazi *et al.*, 1999), reason for which particular studies on *P. sylvestris* are recommended in order to bring more evidence for these specific transporters. Even though the evidence is limited, several classes of metal transporters have been proposed to be involved in metal uptake and homeostasis in plant cells, among which transporters from heavy metal CPx-ATPases, the Nramps and CDF family (Williams *et al.*, 2000) and ZIP family (Guerinot, 2000) were associated to different tolerance mechanisms in regard to Cd, Fe, or Zn uptake (Hall, 2002).

Intracellular chelation in higher plants is related mostly to phytochelatins (PCs) which are inducible by the presence of the metal itself (Marmiroli *et al.*, 2005). To date, PCs are specific only for plant detoxification mechanisms (Bellion *et al.*, 2006), and are a synthesized product of glutathione (Rauser, 1995) assumed to be involved in transporting the resulted metal complexes into the vacuoles (Clemens *et al.*, 2002). Most of the studies were conducted on defense mechanisms of *Arabidopsis*, where PCs indicated an important role in the detoxification of different compounds of Cd and As, but showed no detoxification effects on Zn and Ni (Hall, 2002). Fischer *et al.* (2014) provided evidence that PC synthesis has an important role for Pb detoxification in *Arabidopsis thaliana* seedlings, so it can be assumed that Pb^{2+} could be bound and

transported by pine PCs. Sharma *et al.* (2004) reported the involvement of sulfur ligands in Pb detoxification and labeled them as indicative for phytochelatins and glutathione, both being considered to have a role in Pb²⁺ complexation, while studies on rice plants (Verma & Dubey, 2003) have indicated the role of peptides on metal-binding and considered this process a specialized mechanism for Pb detoxification. Still, other studies have suggested that the role of PCs would be rather related to metal homeostasis, sulphur metabolism or anti-oxidants (Rauser, 1999) than to detoxification of increased metal concentrations (Hall, 2002). Metal detoxification in plants can be also related to organic acids and amino acids, which could act as potential ligands for heavy metals (Rauser, 1999). Chelation mediated by citrate, histidine and nicotianamine seemed to influence the metal transport to the xylem, while chelation with PCs or MTs was related to root sequestration (Clemens *et al.*, 2002). However, the evidence are little and more particular studies would be needed in order to assert a specificity of metal ligands in detoxification metal pathways. Similar to fungi, plants have been reported to benefit of vacuolar compartmentation as intracellular sequestration mechanism. Some investigations on Ni and Zn have brought evidence of their accumulation into the vacuoles (Kupper *et al.*, 1999; Persans *et al.*, 1999), while other studies on Ni suggested that the vacuoles do not represent an accumulation site for the metal in question (Gries & Wagner, 1998). In regard to Pb vacuolar sequestration, Meyers *et al.* (2008) provided TEM micrographs for intracellular deposition of Pb in vacuoles of a hyperaccumulating plant, but these evidence would be recommended to be asserted also by genetic proofs.

4.4.2 Lead transport in shoots

After passing the endodermis, the Pb²⁺ found in the liquid phase are transported to the pine needles through the vascular tissue as xylem sap. SEM investigations did not show any presence of Pb accumulations in the stem of the mycorrhized seedling, reason for which it is believed that the pine stem is responsible only for supporting the transporting vessels, without considerable involvement in metal accumulation. Furthermore, Pb²⁺ have reached the needles of the seedling, where Pb particles were localized in the

mesophyll, hypodermis and epidermis tissue, and the SEM analyses have indicated that these Pb accumulates are smaller in size and fewer in abundance (Fig. 18) compared to the ones found in the root (Fig. 17). Therefore, it is believed that only a small fraction of Pb^{2+} was taken up to the needle crown, while the rest of the fraction got immobilized at root level. Once the Pb^{2+} got translocated into the needle tissue through apoplast or symplast distribution, the formation of minerals either in the vacuole or outside the cytosol is possible. The TEM investigation could show that the plant cell wall has responded to the mineralization process, which implies that the formation of chloropyromorphite has occurred after contact of metal ions with living tissue, and formation of new indented cell wall material is attributed to the mineral growing phases. Assumptions in regard to transport of Pb particles from root to needle can be excluded based on SEM investigations where no Pb particles were found in the xylem area. TEM investigations determined a different chloropyromorphite phase in the needle than the one in root, suggesting that distinct mechanisms may be involved in the overall metal defense beside the plant representative ones, already discussed. The presence of chloroplasts in the needle cell structure is the main difference from the root cells, while the tissue presents distinguishing anatomy, e.g. resin canals, stomas, cuticles etc.. All these differences could be related to the different phases of chloropyromorphite, and furthermore to various biomineralization mechanisms, but more specific research needs to be conducted on the particular cases of *P. sylvestris* needles.

Predicting the complete defense system of a metal resistant plant can be challenging because the assumptions need to take in consideration that the plant is a multicellular organism characterized by tissue and cell differences. Complications in comprehension can arise also from intercellular and intracellular transport, and the multiple metal pathways that can be implied by detoxification mechanisms, which depend on the specific toxicological effects of each metal and can differ between the plant species. As in the case of *P. involutus*, assumptions for Pb defense mechanisms of *P. sylvestris* should be generalized with care upon the existent studies, especially differentiating between evidence brought on hyperaccumulators and metal resistant plants. Schützendübel and Polle (2001) showed in their studies that ECM associations between *Pinus sylvestris* and *Paxillus involutus* contained increased concentrations of secondary

metabolites than non-mycorrhizal roots in response to Cd induced stresses, so extended research on other metals framed as environmental pollutants to identify their resistance mechanisms might bring important information in respect bioremediation tools for soil decontamination.

4.5 Ecological role of the ECM symbiosis on bioremediation

4.5.1 Afforestation strategies

Metal polluted soils must be excluded from agricultural production and subjected to remediation procedures in order to assure a responsible land management (Bojarczuk & Kieliszewska-Rokicka, 2010). Afforestation has been often indicated as a proper alternative for reducing the ecological impact generated by elevated concentrations of heavy metals. Understanding the different responses in regard to metal tolerance or sensitivity is important for predicting the potential role of living organisms to be involved in bioremediation strategies. Long-term site restoration might be difficult due to the chemical and physical properties of the soil (Hartley-Whitaker *et al.*, 2000), but the tree's chances to survive are significantly increased by the ECM colonization as it was shown in the conducted experiments.

Pinus sylvestris tree species mycorrhized by *Paxillus involutus* ectomycorrhizal fungi seemed to be in accordance with the biodiversity of the investigation area, and their selection as symbiont partners proved to be efficient for the bioremediation strategy. Pine is a well known forest-forming tree often used in restoration processes due to fundamental habitat requirements of contaminated sites (Chudzinska *et al.*, 2014). Artificial afforestation of brownfields could provide prevention against soil erosion through the root system of pine trees which can aggregate soil particles. Mycorrhized trees have been reported to benefit from a highly branched root system (Smith & Read, 1997), thus increasing the pine potential for soil aggregation. Once the stability of the topsoil is reached, surface stability being one of the main conditions for remediation success, the development of a vegetation cover favors the decrease of metal leaching by enhancing the water-storage capacity of the soil, and evapotranspiration (Brunner *et*

al., 2008). The branched roots of mycorrhized pine provide also an increased surface for metal binding and sequestration, thus enhancing the potential of reducing the metal toxicity in the brownfield. The mobility of the contaminants can be further reduced when soil amendments such as compost, phosphate fertilizers, organic matter or clay minerals are added in the afforestation process (Palmroth *et al.*, 2006). At the same time, compost addition improves the development of the vegetative cover, which is directly involved in the production of organic matter. The latter implies increasing the nutrient cycling, promoting biotic communities and creating a forest micro-climate (Bert *et al.*, 2009). Another advantage of afforestation is brought by the presence of pine trees that form green corridors which act like sinks for dry atmospheric deposition (Alriksson & Eriksson, 2001), thus decreasing the soil contamination through air pollution.

4.5.2 Secondary implications for green industry

Previous studies (Alriksson & Eriksson, 2001; Chappelka *et al.*, 1991) have indicated reduced translocation to shoots in regard to biomass concentrations of Pb. This pattern of metal distribution and accumulation was also observed in the mycorrhized seedlings of the present study. By restricting the metal ions distribution to the root area through phytostabilization mechanisms, the generated vegetative cover of the proposed bioremediation system would not pose a supplementary environmental threat for the above ground living organisms. Even more, the low phytoextraction potential indicated by the subunitary BCF (Tab. 6) promotes the use of the generated biomass in different sectors of industry. The SEM investigations for metal distribution indicated that no metal accumulations are taking place within the stem of *P. sylvestris* seedlings. Therefore, the stem wood of the trees could be further used in timber industry as solid wood for construction, wood-based products in pulp and paper industries, or fuel production, by enzymatic hydrolysis of wood residues into ethanol, as a substitute to gasoline (Mai *et al.*, 2004). For all that, extended studies focused on metal accumulation during longer stages of tree development are required if further utilization is envisaged for the plant species used in the bioremediation process. At the same time, the investigated

organisms can be prone as efficient and cheap biosorbents that can be used in the research fields of element recovery and recycling, beside the great opportunities brought for pollution treatment. Plant materials as leaves, bark and sawdust which are often seen as waste biomass could be further used as biosorbents due to their metal sorbing properties (Ahluwalia & Goyal, 2007).

From an ecological point of view, wood acts as a carbon sink against the greenhouse effect by maintaining a neutral CO₂ balance when cultivation for sustainable development is considered. In terms of biotechnology, wood is a polymeric composite considered a renewable raw material, whose technical properties are influenced by the cell wall composition (Mai *et al.*, 2004). Cellulose, hemicellulose and lignin are the main structural components of wood cell walls, composing a ligno-cellulosic matrix that assures important functions for the plant (Davison *et al.*, 2013). Cellulose is a β -1,4-linked glucose polysaccharide which forms crystalline hydrophobic microfibrils, while hemicellulose are amorphous single-chain polysaccharides that interact with cellulose fibrils, and are made up of galactoglucomannans, which have a backbone of β -1,4 linked D-mannopyranose and D-gpucopyranose units in softwood species, such as pines (Davison *et al.*, 2013). Cellulose can be a suitable metal binding site due to its symmetrical structure in parallel chains, along which glucose monomers linkages may form covalent bonds through the oxygen atoms (Marmioli *et al.*, 2005). Lignin is a three-dimensional polymer, polyphenolic polymer that mediates the adhesion of fibrils by encasing the previous cell wall polysaccharides, providing mechanical and elastic support (Davison *et al.*, 2013). Ligno-cellulosic matrices have been also reported as lead binding sites (Marmioli *et al.* 2005) that might be persistent during long periods of time. In this way, if the bioremediation strategy is based on plants which do not require to be periodically harvested, the above ground biomass can contribute to the overall metal binding potential of the tree, additionally to the root system.

4.5.3 Bioremediation outlook of heavy metal contaminated soils

The European policy encourages the redevelopment of brownfields and their revaluation after removal of hazardous threats associated with ecological risks and risks

for humans. Due to the complexity of threats related to soil pollution, the present remediation technologies are often related to excavation of contaminated soil, *ex situ* treatments, and disposal as landfill, technology which implies high costs. Opposite, bioremediation could represent an eco-friendly and low-cost alternative for *in situ* remediation. Phytostabilization represents one of the branches used in bioremediation strategies, and could be characterized by implying the following endpoints: soil stabilization, erosion control, reduction of metal leaching, reduced phytotoxicity, reduced uptake of pollutants in roots, reduced translocation of pollutants in aboveground parts, and a system with plant litter that does not increase pollutant availability (Bert *et al.*, 2009). The developed bioremediation system, composed of *Pinus sylvestris* and *Paxillus involutus* as symbiont partners, seems to be in agreement with all these requirements based on the investigations that were carried out in the present study, and correlated with specific literature. The final goal for *in situ* phytostabilization is the immobilization of metals into insoluble precipitates or minerals for relatively long periods of time, and decreasing the chances of other mineral phases to be remobilized by the dynamic interactions of biogeochemical parameters within a given environment (Moon *et al.*, 2007). One of the advantages of using specialized plants in phytostabilization approaches is represented by metal precipitation and mineral formation inside the plant tissue. In this way, a part of the soil contaminants get sequestered inside the plant, without being further released into the environment as toxic by-products and without posing any ecotoxicological threats to the plant metabolism. Since mycorrhized seedlings of pine have shown chloropyromorphite formation within plant tissue, the ECM symbiosis has proven to be effective for metal immobilization through biomineralization process, and decreasing the mobility of the contaminant through processes of biosorption and bioaccumulation. It can be assumed that the proposed bioremediation system acts as a combination between passive and active remediation techniques. Passive remediation approaches imply monitored natural attenuation (MNA) which relies on natural physical, chemical and biological processes (US EPA, 1999), while active remediation strategies promote metal-phosphate formation (Traina & Laperche, 1999; Zhang *et al.*, 2010). To date, the majority of metal immobilization

studies focused on bacteria, but the role of ECM symbiosis should be extended to detailed analyses of fungal and plant activities that are involved in metal immobilization.

In conclusion, a self-sustaining ecosystem can be a viable option for the bioremediation of brownfields contaminated with heavy metals. The appropriate selection of metal resistant organisms which could form a strong bioremediation system represents the key success of the remediation strategy. To enhance the restoration process, it is recommended that the plants chosen for afforestation to be previously colonized by ECM fungi, especially the plant species dependent on ECM symbiosis for survival. If a brownfield proposed for bioremediation is characterized by areas where metal contamination is recording alarming concentrations, phytostabilization approaches can be combined with phytoextraction methods, thus involving the use of hyperaccumulator plant species until the metal contents get reduced to acceptable levels for other forms of bioremediation. For improving the landscape, different grass species (e.g. *Avena pratensis*, *Festuca ovina*) and flowering plants (e.g. *Silene vulgaris*, *Ameria maritima*) naturally found in metal contaminated sites can be planted, in this way enhancing the biodiversity of the area.

Nevertheless, *in situ* remediation strategies depend on local geochemical parameters which include geology of the area, concentrations of soluble metal fractions, pH and redox state. In order to test the sustainability of the proposed bioremediation technique, an *in situ* experiment should be further conducted in the investigated site from Baia-Mare, where the same ECM organisms should be tested. In contrast to the axenic conditions which were used for the present experiment and which were able to bring more clear answers in regard to interactions between organisms and metals, the field experiment will increase the hypothesis related to possible organisms and mechanisms involved in metal immobilization. Due to the presence of unknown soil microorganism (including bacteria), the results that may come out of this field experiment would need to be related to a broader scale of possible factors involved in metal immobilization. However, the main goal of the experiment should focus on the surviving abilities of the proposed ecosystem in real contaminated conditions.

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6 Acknowledgements

It is a great pleasure to express my deepest gratitude and acknowledgement to all the great persons who dedicated their time and contributed to the accomplishment of my doctoral thesis. Since the present study was founded on the basis of a cotutelle, the acknowledgements will be presented in a chronological order.

A wonderful collaboration started with my first research program as a master ERASMUS student in 2012, and for this I am expressing my appreciation to Prof. Dr. Erika Kothe and the working group from the Institute of Microbiology - Microbial Communication for introducing me in the wonderful world of science. Following this experience, in 2012 Prof. Dr. Eng. Alexandru Ozunu offered me the great opportunity to start a PhD program on a favored topic, and Prof. Dr. Erika Kothe accepted and intensively supported the joint supervision of the PhD program. I am thanking both my supervisors for providing a proficient coordination throughout the whole PhD program.

I would like to thank Dr. Erika Levei and her colleagues from the Research Institute for Analytical Instrumentation Subsidiary, Cluj-Napoca who helped me with the investigations of soil samples, and brought an important contribution in the chemical assessment of the present study. I am also grateful for the great guidance and advices in the writing of the papers. I am thanking to Dr. Carmen Roba from Faculty of Environmental Science and Engineering who was also involved in the chemical analyzes.

Special thanks to PhD student Steffi Formann for always helping me with the best advices and being a reliable partner in my lab experiments held in the Institute of Microbiology, Jena. Also Dr. Katrin Krause and all the members of Basidiomycetes group are acknowledged for providing their support and encouragements throughout my periods spent in Jena. Thanks to your warm professionalism it was a real pleasure to work together with you and have you as my colleagues.

I am thanking to Dr. Dirk Merten from the Institute of Earth Science, Jena for the ICP-MS measurements of heavy metal contents in co-cultures.

I am expressing my gratitude to Dr. Sándor Nietzsche from Center of Electron Microscopy, Jena for the great involvement in the investigation of metal accumulations in plant tissue, performing the SEM micrographs and SEM-EDX microanalysis. Thank you very much for the time dedicated in teaching me valuable principles of electron microscopy, and for all the proficient discussions held.

I am also thanking to Prof. Dr. Juraj Majzlan from Institute of Geosciences, Jena for the helpful discussions in regard to metal mineralization, and guiding the investigations in the direction of pyromorphite identification in plant tissue.

Many thanks to Prof. Dr. Falko Langenhorst and Dr. Ulrich Mansfeld from Institute of Geosciences, Jena who accepted the challenging task of extracting metal particles from biological samples, and performed the electronic diffraction for determination of metal minerals.

I am thanking my coordination commission from Faculty of Environmental Science and Engineering, Cluj-Napoca, Prof. Dr. Eng. Cristina Roșu, Assistant Professor Kinga Reti and Assistant Professor Eng. Cristina Modoi. Also my colleagues are acknowledged for their friendly support throughout the doctoral program.

7 Declaration of original work

I declare that the dissertation "BIOREMEDIATION FOR HEAVY METAL CONTAMINATED BROWNFIELDS" is the product of my own work which was independently written, and any forms of assistance have been acknowledged. The dissertation has not been submitted in other faculty or university for any other degrees or purposes.

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