## 2. Abbreviations

| | |
|---|---|
| AE | arbuscular and ectomycorrhizal i.e. dual mycorrhizal |
| AM | arbuscular mycorrhiza / arbuscular mycorrhizal |
| C | control (without heavy metals, or without mycorrhizal fungi) |
| CAT | catalase |
| DMF | dimethylformamide |
| DNA | deoxyribonucleic acid |
| DUAL | experiment with dual mycorrhizal symbioses |
| DW | dry weight |
| ECM | ectomycorrhiza / ectomycorrhizal |
| FW | fresh weight |
| GCL | *Glomus claroideum* |
| GIN | *Glomus intraradices* |
| HM(s) | heavy metal(s) |
| HME | *Hebeloma mesophaeum* |
| INVIT | *in vitro* experiment |
| K | experimental site Komín (eventually representing Komín) |
| L | experimental site Litavka (eventually representing Litavka) |
| PIN | *Paxillus involutus* |
| POX | peroxidase |
| SOD | superoxide dismutase |

### 3.1. Abstrakt

Kontaminace půd těžkými kovy představuje značné riziko jak pro zdraví lidí, tak pro samotné životní prostředí. Perspektivní metoda, jak tento problém vyřešit, spočívá v remediaci kontaminovaných půd rychle rostoucími dřevinami na plantážích s krátkým obmytím. Jelikož dřeviny využívány tímto spůsobem (hlavně topoly a vrby) , tvoří symbiózu se dvěma hlavními skupinami mykorrhizních hub, je důležité zkoumat interakce mezi rostlinami, houbami a těžkými kovy, a neomezovat se jenom na interakce rostlin a těžkých kovů. Cílem této práce je přispět k dosud nasbíraným informacím, hledáním vhodných mykorhizních partnerů pro topol a vrbu, tolerantních k zátěži, kterou těžké kovy představují. Toto hledání je založeno na posuzování aktivity klíčového antioxidačního enzymu u vybraných hub, a taky na studiu fyziologické odezvy rostlin k přítomnosti mykorhizního partnera na půdách s vyšším obsahem těžkých kovů. První experiment v podmínkách *in vitro* byl zaměřen na morfometrické charakteristiky mykorhizních hub, pěstovaných na růstových médiích s přídavkem „koktejlu“ těžkých kovů. Na základě výsledků bylo vybráno několik tolerantních izolátů pro pozdější inokulaci rychle rostoucích dřevin, s ohledem na fytoextrakční a fytostabilizační strategii. Druhý experiment analyzoval interakce hub a rostlin (vrb a topolů) na kontaminovaném substrátě a zároveň sledoval toleranci rostlin vůči těžkým kovům, stejně tak, jako jejich příjem. Stromečky byly pěstovány v Petriho miskách, které sloužili jako květináče. Z výsledků experimentů, je zřejmá variabilita hub v schopnosti akumulovat těžké kovy, ale i rozdílné antioxidační mechanismy mezi zkoumanými izoláty. Tato práce navíc ukazuje spceifické odezvy rostlin vůči houbovému partnerovi (jak fyziologické nebo morfometrické), vyzdvihujíc důležitost testování interakcí mezi rostlinami a jejich mykorhizními partnery pro dosáhnutí kýžených výsledků.

**Klíčová slova**: antioxidanty, arbuskulární mykorhiza, duální mykorhiza, ektomykorhiza, fytoextrakce, fytoremediace, fytostabilizace, populus, salix, superoxid dismutáza, topol, těžké kovy, vrba

**3.2. Abstract**

Soil contamination by heavy metals represents rather serious environmental problem for both human health and an environment itself. One of the perspective technologies dealing with this threat that only recently has been intensely developed is phytoremediation by means of short rotation coppice plantations. As plants used in this technology (mostly poplars and willows) host two major groups of mycorrhizal fungi substantially influencing plant physiology it is important to study plant-mycobiont-heavy metals interactions rather than just plant-heavy metals interactions. The present thesis aimed to contribute to the growing knowledge of the field by search for suitable mycobionts of poplar or willow tolerant to heavy metals, by evaluating an activity of the key antioxidative enzyme in selected mycobionts and by looking at physiological responses of plant hosts to their mycobionts in a soil polluted by heavy metals. The first experiment *in vitro* focused on screening of morphometric criteria of fungi growing on solid growth media amended with mixture of heavy metals. Based on the results, several tolerant ectomycorrhizal strains were chosen for the next inoculation of fast growing trees serving phytoextraction and phytostabilisation strategies. The second, re-synthetic experiment was conducted in petri dishes serving as reservoir for plant roots with mycorrhiza with willows and poplars to study their plant –fungal interactions under the effect of heavy metals focused towards plant metal tolerance and uptake. Performed analyses showed that strains differed in the ability do accumulate heavy metals and partly also in their defence against oxidative stress. The present thesis also demonstrates mycobiont-specific physiological and morphometrical responses of the examined plant hosts. Thus, the present thesis underlines neccessity of detailed testing of host-mycobiont combinations in order to achive demanded output.

**Key words**: antioxidants, arbuscular mycorrhiza, dual mycorhiza, ectomycorrhiza, heavy metal(s), phytoextraction, phytoremediation, phytostabilisation poplar, populus, salix, superoxide dismutase, willow

## 4. Introduction

Mining and smelter activities have been taking place in the vicinity of Příbram city in Central Bohemia region of Czech republic since middle ages. They resulted not only in growing wealth of the region but also (and considering used technologies inevitably) pollution of nearby environment. Two main sources of contamination were emissions of pollutants (including HMs) from smelters and water stream deposits due to the ore streaming. The first source lead to pollution of area estimated to cover up to thousand hectares. Untill eighties the smokestack of metal-processing factory Kovohutě Příbram released substantial amounts of Pb into atmosphere, due to lack of management of environmental impact od contaminants emitted. Thus today, the area north-west from the Příbram city is highly contaminated by heavy metals such as cadmium, lead or zinc. The concentrations of these elements in soil often exceed allowed limits for agricultural soils (http://www.pribram-city.cz/index.php?vid=153).

Nowadays society realizes neccesity to remove old ecological burdens such as the one mentioned above. The underlying reasons are by far not just environmental but economical and social as well. This is reflected in financial support of research aimed at developing low-cost, environment-friendly and profitable solutions for removal of these burdens. One such solution for areas polluted by HMs has been developed by plant researchers and is called "phytoremediation". Various phytoremediation techniques are described in more detail latter in the Review section. In 2008, a group of scientists from the Institute of Botany ASCR, Czech University of Life Sciences in Prague and Norwegian ecological and agriculture institute Bioforsk joined in an international project "Energy plantations biotechnology on contaminated land" supported by Financial mechanism EEA/Norway. The project aims to test the possibility of establishing plantations of fast growing trees with short silvicultural rotation (often called short rotation coppice) in areas polluted by (mixtures of) heavy metals using means of fertilisation, soil amelioration and mycorrhizal inoculation. Succesfull finalization of the project should provide a biotechnology usable for farmers and/or agricultural corporations and thus offer an alternate utilization of contaminated land for energy-from-biomass purpose and partial land remediation. Two experimental sites were chosen for model plantations: site "Komín" (meaning smokestack) located nearby the Kovohutě Příbram nástupnická a.s. and site "Litavka" located in the Litavka river basin close to the village Trhové Dušníky village.

This thesis was elaborated within the project scope and its general aim was to address a question of suitable ectomycorrhizal strains for inoculation of fast growing trees and elucidation of their impact on physiology of host trees grown in polluted soil.

## 5. Literature review

### 5.1. Heavy metals classification

When referring to heavy metals, authors usually mean chemical elements from a group of transition metals (sometimes even some methalloids, lanthanides and actinides). However, other authors define the term heavy metals (HMs) in a different way, taking as a criteria either density or their deleterious effects which contributes to classification confusion. The precise definition of HMs is rather difficult. The term "heavy metal" has never been defined by any authoritative body such as International Union of Pure and Applied Chemistry (Duffus 2002). Instead, given definition of HMs is usually based either on physical and chemical properties of chosen elements, or on the toxic effect which they have on living organisms, or on the combination of the mentioned (Duffus 2002). With density taken to be the defining factor in most cases, HMs are thus commonly defined as metals having a specific density of more than 5 g/cm$^3$ (Järup 2003, Schützendübel 2002, Zenk 1996). This is true for all metals used in this study; that is cadmium, zinc and lead. Furthermore the interaction of metallic elements with living systems is mainly dominated by their chemical properties as Lewis acids. Lewis acids are elemental species with reactive vacant orbital or available lowest unoccupied molecular orbital acting as an electron acceptor. Thus classification of metallic elements based on their Lewis acidity permits us to predict preferred ligands as well as general trends in metal complex properties. Overall classification of metal ions according to hard and soft acid scheme is given in Table 5.1. The toxic effect of HMs is discussed in following sections.

| Type-A metal cations | Transition metal cations | Type-B metal cations |
|---|---|---|
| Electron configuration of inert gas | 1–9 outer shell electrons | Electron number corresponds to $Ni^0$, $Pd^0$ and $Pt^0$ (10 or 12 outer shell electrons) |
| | Not spherically symmetric | Low electronegativity |
| Low polarizability | | High polarizability |
| 'Hard spheres' | | 'Soft spheres' |
| ($H^+$), $Li^+$, $Na^+$, $K^+$, $Be^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Al^{3+}$, $Sc^{3+}$, $La^{3+}$, $Si^{4+}$, $Ti^{4+}$, $Zr^{4+}$, $Th^{4+}$ | $V^{2+}$, $Cr^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Ti^{3+}$, $V^{3+}$, $Cr^{3+}$, $Mn^{3+}$, $Fe^{3+}$, $Co^{3+}$ | $Cu^+$, $Ag^+$, $Au^+$, $Ga^+$, $Zn^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Pb^{2+}$, $Sn^{2+}$, $Tl^{3+}$, $Au^{3+}$, $In^{3+}$, $Bi^{3+}$ |
| **Hard acids** | **Borderline** | **Soft acids** |
| All type-A metal cations plus $Cr^{3+}$, $Mn^{3+}$, $Fe^{3+}$, $Co^{3+}$, $UO^{2+}$, $VO^{2+}$ | All divalent transition metal cations plus $Zn^{2+}$, $Pb^{2+}$, $Bi^{3+}$ | All type-B metal cations minus $Zn^{2+}$, $Pb^{2+}$, $Bi^{3+}$ |
| In addition species such as $BF_3$, $BCl_3$, $SO_3$, $RSO_2^+$, $RPO_2^+$, $CO_2$, $RCO^+$, $R_3C^+$ | $SO_2$, $NO^+$, $B(CH_3)_3$ | All metal atoms, bulk metals $I_2$, $Br_2$, ICN, $I^+$, $Br^+$ |
| Preference for ligand atom | | |
| $N \gg P$ | | $P \gg N$ |
| $O \gg S$ | | $S \gg O$ |
| $F \gg Cl$ | | $I \gg F$ |
| Stability sequence | | |
| Cations: | Cations | |
| Stability $\propto$ (charge/radius) | Irving-Williams series: $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ | |
| Ligands | | Ligands |
| $F > O > N = Cl > Br > I > S$ | | $S > I > Br > Cl = N > O > F$ |
| $OH^- > RO^- > RCO_2^-$ | | |
| $CO_3^{2-} \gg NO_3^-$ | | |
| $PO_4^{3-} \gg SO_4^{2-} \gg ClO_4^-$ | | |

*Table 5.1. Classification of metal ions based on numer of outer shell electrons (type A, type B and transition metals) as well as the hard and soft acid classification. Adopted from Gadd 1993.*

### 5.1.1. Health risks

Some of the so called HMs are essential micronutrients for plants as well as other living organisms, these include a number of transition metals (Zn, Fe, Cu). Others such as Pb and Cd are believed not to be involved in metabolism and considered toxic. However a recent finding shows that cadmium cation is used as enzymatic cofactor by a marine diatom species *Thalassiosira weissflogii.* This may be a result of specific environmental conditions of this diatom (Lane 2000). The toxic effect of HM usually occurs when the element is present in excess concentrations, but may be observed even at low concentration of non essential metal (Macfarlane 2001, Yang 2005). Numerous studies described toxic effect of HM on human health; for example cadmium is believed to be responsible for kidney damage and to contribute to cancer occurence, lead is descirbed to cause kidney damage as well as

brain several neuropathy syndroms especially in children (Järup 2003, Warnick 1969). The main source of toxic metal exposure is usually inhalation of airborne particles or take up via food chain. Furthermore metal biological half-life can reach easily 20-30 years, as reported for Cd or Pb (Järup 2003). Even though the source of HM is eliminated, the effects of metal may persist due to its accumulation in affected organism. Apart from these studies, there is a vast number of studies describing the effect and toxicity of HM in plants, which I would like to describe in more detail.

### 5.1.2. Effect of HMs on metabolism

The effect of HMs on metabolism varies widely according to species, chosen element, its concetration availability and some other conditions. The spectrum of metals and especially transient metals that has been acquired for use by enzymatic systems in the time of evolution is a result of their chemical properties and abundance in the Earth's crust (Clemens 2006). Characteristics common to all metals comprise the ability to readily loose valent electrons thus forming cations. Thus some HM ions are exploited in multitude of metabolic pathways including redox reactions, electron trasfers, enzymatic reactions were they often dwelve in the catalytic centre of enzyme. They are also often involved in signaling pathways, and structural functions in nucleic acid metabolism (Clemens 2006, Williams 2000, Zenk 1996). The same properties that make these metal ions indispensable for life, however, are also the reason why they and others can easily be toxic when present in excess (Clemens 2000).

The toxic effect of HM on plants can be observed at various scales - body/organ, tissue level and cellular/molecular level. The usual symptoms are decreased growth rate, decreased photosynthesis, disturbed water uptake, chloroses, necrotic lesions on leaves or even leaf abscission (Schützendübel 2001). In case of serious HMs poisoning or due to other factors the toxic effect of HMs may result in death of the plant individual. The molecular mechanisms underlying observed symptoms are not fully understood, however a great effort has been made to elucidate them. Several distinct mechanisms of HMs toxicity have been described, posing a great challenge for plant metabolism to cope with. I will describe them briefly in following sections.

#### 5.1.2.1. Activation of reduced forms of oxygen and associated oxidative damage

A number of different ROS, including the superoxide anion ($O_2$·-), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (·OH), occur transiently in aerobic organisms. These species are normal byproducts of oxidative metabolism and pose a constant threat to all aerobic organisms (Pinto 20003).

Despite the fact that ROS are used by cells to repel invading pathogens or as a signaling molecules that activate defense response (Mithöfer 2004), and despite the fact that there are many ways (either enzymatic or non-enzymatic) involved in their regulation and detoxification, they are a possible menace because of oxidative damage they may cause when deregulated by, for example, presence of HM at high concentrations (Wong 2004).

According to Haber-Weiss reaction transition metals (such as Fe, Cu, Mn or Co) can catalyze production of highly reactive hydroxyl radicals. The one electron reduction of molecular oxygen to the superoxide radical is thermodynamically unfavorable, but can nevertheless take place by interaction with another paramagnetic center such as HM atom. Transition metals have frequently unpaired electrons, so they are good catalysts of oxygen reduction. The reaction can be described as follows:

$M + O_2 \rightarrow M^{+1} + O_2{\cdot}^-$

where M stands for metal atom. In aqueous solutions at neutral pH $O_2{\cdot}^-$ can generate $H_2O_2$ which can subsequently decompose to produce ·OH by the Haber-Weiss reaction, as follows:

$M^{+1} + O_2{\cdot}^- \rightarrow M + O_2$

$M + H_2O_2 \rightarrow M^{+1} + OH^- + {\cdot}OH$ (often called Fenton reaction)

These reactions are usually summarized as

$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + {\cdot}OH$

Hydroxyl radicals (·OH) produced by the Haber-Weiss reaction can initiate radical chain and thus be responsible for irreversible chemical modifications of various cellular components, leading to severe cellular damage, and ultimately to cell death. When hydroxyl radicals are produced in close proximity to DNA, by adding or removing H atoms to DNA bases or the DNA backbone, respectively, the DNA integrity may be also endangered. Metal ions, through Haber-Weiss reaction, also play an important role in the oxidative modifications of free amino acids and proteins. A major consequence of oxygen free radical damage to proteins is ~~to~~ targeting them for degradation by proteases. Finally, oxygen and transition metals are proved to be involved in lipid peroxidation. Biological membranes rich in polyunsaturated fatty acids are extremely susceptible to these reactions. This may result in oxylipin production, which is substance involved in inter- and intracellular signalling related to multiple defense reactions in response to pathogens (Briat 1998, Howlett 2007, Mithöfer 2004). Nevertheless, accumulation of ROS and successive oxidative stress was reported to increase as a result of exposure of plants to certain HM, which due to their chemical properties, cannot be directly involved in reactions mentioned above. For example cadmium in physiological conditions occurs only in $Cd^{2+}$ state

(Clemens 2006). This increase of oxidative stress is caused by inhibition of antioxidant enzymes and antioxidants such as glutathione, SOD, or others (Clemens 2006, Schützendübel 2002).

#### 5.1.2.2 Inactivation of enzymes

Most heavy metal ions have a strong affinity for ligands containing atoms of oxygen, nitrogen or sulphur, such as phosphates, purines, pteridines, porphyrins, and cysteinyl and histidyl side chains of proteins, which are all abundant in cells. This binding affinity is linked to free enthalpy of the formation of the product of metal and ligand (Schützendübel 2002). Thus, the toxic effects of metals can be attributed partly to the multiplicity of coordination complexes and clusters that they can form (Howlett 2007). The list of enzymes reported to be inhibited by HM resulting in different symptoms of HM toxicity is rather long including: nitrate reductase being inhibited by Cd resulting in decreased absorption of nitrate and its transport from roots to shoots, Cd treatment reduced ATPase activity of the plasma membrane of wheat and sunflower roots (Fodor 1995), which could result in ion leakage from the cell (Hall 2002). Pb and Cd are responsible for inactivation of enzymes with zinc finger motives, which participate in DNA repairs - resulting in increased mutation rate (Clemens 2006). Excessive concetration of Cd, Cu and Zn were reported to decrease photosynthesis either by inhibition of ribulose-1,5-diphosphate carboxylase and enzymes involved in photosynthetic pigment synthesis. Heavy metals have been found to decrease the chlorophyll content and the chlorophyll a/b ratio, and decrease the chlorophyll/carotenoid ratio in terrestrial plants. Heavy metals inhibit the biosynthesis of chlorophyll pigments and enzymes involved in these processes. (Monni 2001, Ralph 1998). Furthermore HM were reported to interfere directly with photosystem II (Tanyolac 2007). As mentioned above, inactivation of enzymes involved in ROS scavenging results in increased oxidative stress (Clemens 2006).

#### 5.1.2.3 Competition of metals

HM either essential or non-essential, when present in high concentrations can compete with essential metals in acquisition by plant roots. For example the uptake of Cd ions is in competition for the same transmembrane carrier with nutrients such as K, Ca, Mg, Mn, Fe, Cu or Zn (Sanità di Toppi 1999) . This is in accordance with findings that Fe limitation leads to increase in Cd accumulation, which can be explained by overexpression of Fe-transport proteins which consequently allow increased Cd influx (Clemens 2006). Another example of HM competition with essential metals results in

decrease in photosynthetic rate that is caused by Zn or Cu replacing magnesium ions functioning as cofactor in various enzymes (Macfarlane 2001). Furthermore Mg ions from chlorophyll were shown to be replaced by HM in vivo in aquatic submersed plants (Küpper 1996). Pb cation is also reported to compete with Ca cation, entering cell via Ca permeable channels and binding with high affinity to Ca binding sites such as calmodulin (Arazi 2000).

**5.2. Phytoremediation**

To avoid these deleterious effects of HMs on organisms, various techniques have been developed, aiming at removal of HMs from the soil, water and sediments. These include HMs precipitation, use of ion exchange, chemical leaching of soil or complete removal and storage of contaminated soil. The latter however have a substantial impact on the environment and are quite costly (Khan 2000, Nedelkoska 2000). In response to this, low cost, more environmental-friendly methods are being developed such as phytoremediation. Phytoremediation is a technique using vascular plants to ameliorate soils or water contaminated with various substances including HM, pesticides, petroleum hydrocarbons, chlorinated solvents, radionuclides or excess nutrients (Khan 2000, Muller 2000,). Phytoremediation covers a range of methods such as phytodegradation, phytostabilisation or phytoextraction (Khan 2000). In the case of HM the two latter can only be used, as HM can not be biodegraded. Phytoextraction (see figure 5.1.) is a process where the ability of plants to extract metals from soils and concentrate them in their harvestable parts is exploited (Muller 2000). Plant species favoured for phytoextraction are often referred to as hyperaccumulators, this means that the concentration in harvestable tissue is higher compared to that in soil (Lunáčková 2003). However high biomass could compensate lower metal concentration in harvestable parts of plants used for phytoextraction, adding to economical feasibility (Vassilev 2002). Phytostabilisation (see figure 5.2.) in contrast does not aim to extract metals from the soil. Phytostabilisation aims to reduce HM mobility, thus decreasing further environmental degradation by leaching into the groundwater or by airborne spread (Muller 2000). Phytostabilisation can be achieved by plant and fungus HM uptake, and storage in below-ground biomass, as was reported for example grass *Cynodon dactylon* (Leung 2007). In both phytoextraction and phytostabilisation, HM tolerant plants need to be used. Plants suitable for phytoremediation can be found within various plant families covering herbaceous species (such as *Sinapis*, *Cannabis* or *Thlaspi*), or woody species such as willows and poplars from the *Salicaceae* family (Kos 2003, Vandecasteele 2006, Vysloužilová 2003). Willows and poplars are especially popular

in phytoremediation research due to observed accumulation of several HM such as zinc or cadmium (Laureysens 2004, Vandecasteele 2006). Another factor favoring the use of poplars and willows for phytoremediation is their great biomass production. Such biomass production is achieved by ability of some of these trees species to resprout after cutting the aboveground part at the base of plants trunk, referred to as coppicing (Al Afas 2004, Laureysens 2004). Coppicing used at short rotation coppice plantations (SRC) is widely used technology often relied with phytoremediation (figure 5.3.). The benefits are that after harvest, plants do not have to be replanted, and at resprouting, can benefit from already established root system. The intervals between 2 harvests usually range between 3-5 years. The effective duration of such plantation is reported to be about 20 years 25 which could lead to considerable amount of HM removed from the soil (Dickinson 2006). However despite the focus on plants as phytoremediation tool, they are not the only organisms involved in the process. Vast majority of vascular plants including the poplars and willows mentioned above form mycorrhizal symbioses (Smith 2009, Tederesoo 2010). This mycorrhizal omnipresence and importance can not be neglected, and it is essential to study and describe plant-fungal-HM-site interactions in order to precisely prescribe “a cure” for degraded environment.

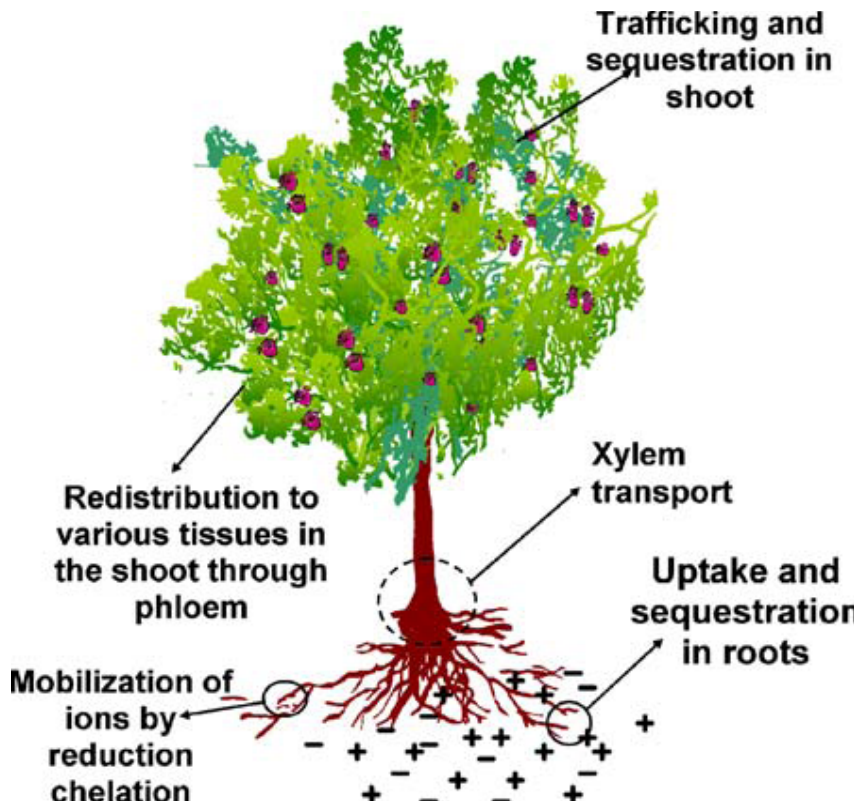


Figure 5.1. Schematic mechanism of phytoextraction (adopted from Padmavathiamma 2007).

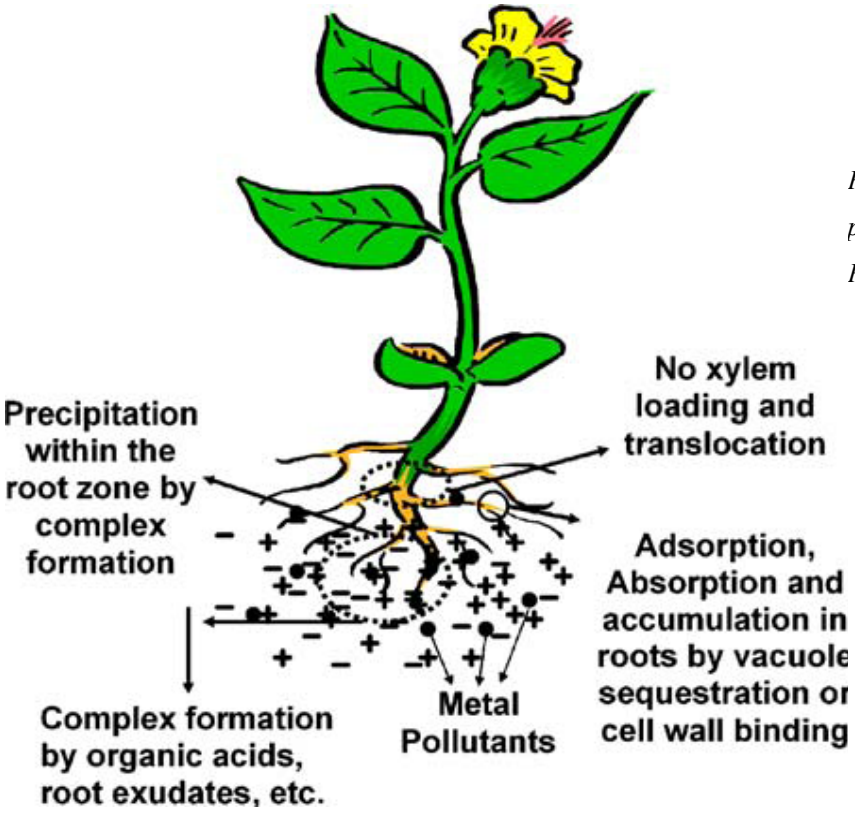


*Figure 5.2. Schematic mechanism of phytostabilisation (adopted from Padmavathiamma 2007)*

*Figure 5.3*. Short coppice poplar plantation source: http://www.energie21.cz/files/image/energie21.cz

### 5.3. Mycorrhizal symbioses

Mycorrhizae are mutualistic associations between fungi and plant roots. Nearly all plant families form root symbiotic organs, termed mycorrhizas, with soil fungi belonging to all the main phyla (Basidiomycetes, Ascomycetes, Zygomycetes). Several types of mycorrhhizal symbioses have been described. The criteria for separating these types has evolved since A.B Frank first described the essential structure and functioning of a symbiotic relationship between trees and fungi that he termed "mykorhiza" (from the Greek: mykés – mushroom, and rhíza – root) (Gryndler 2004). Many attempts were made to classify the variety of mycorrhizal structures considering morphological or ecological factors. I shall introduce the classification published by Gryndler et al. 2004. The endomycorrhizal fungi penetrate host root cortical cell walls whereas the ectomycorrhizas are distinct as fungus is present only in intercellular spaces. The endomycorrhizal types comprise arbuscular mycorrhizal

symbiosis, ericoid mycorrhizal symbiosis, and orchidoeid mycorrhizal symbiosis. There are plants able to form the so called dual mycorrhiza which commonly consists from root system colonised by ectomycorrhizal and arbuscular mycorrhizal fungi. This phenomena has been observed in several genera of some (mainly tree species) families, including *Salicaceae* with genera *Salix* and *Populus* (van der Heijden 2000).

### 5.3.1. Arbuscular mycorrhiza

Arbuscular mycorrhiza (AM) is probably the most widespread and the most general (the least specific as regards to host preference) type of mycorrhizal symbiosis. More than 80% of vascular plants species are believed to form arbuscular mycorrhiza, that is to say circa 11000 genera including 225000 species. (Brundrett 2009). The arbuscular mycorrhiza was first observed in genera Iris by Nägeli. In the 1950's an important breaktrough was made in research by prof. Mosse, who for the first time isolated and cultivated arbuscular mycorrhizal fungi with the host plant in pot culture (Gryndler 2004).

#### 5.3.1.1. Evolution and taxonomy

Arbuscular mycorrhizal symbiosis seems to be phylogenetically the oldest type of mycorrhizal symbiosis (it has probably evolved 400 mil.years ago). These associations are believed to be the essential step in transition of multicellular plants to life on land (Smith 2009). Furthermore fungi involved are considered to be primitve due to their relatively simple spores (azygospores and chlamydospores) and lacking of sexual reproduction.

Nowadays AM fungi are placed in the order Glomeromycota and it is suggested that all the representatives of this order are arbuscular mycorrhizal fungi. However our acquaintance of biological features of this order may be misrepresented due to lack of species with which cultivation experiments were made (Gryndler 2004). Ribosomal genome diversity which is used to distinguish species and evolutionary relations is quite consistent with the absence of sexual reproduction and makes it difficult to define species and individuals. The functional diversity of these fungi is likely to be much greater than is suggested by the number of currently recognized species (Brundrett 2002). Current estimates decipher number of species to some 160 (van der Heijden 2000).

### 5.3.1.2. Ecophysiology and life cycle

In the soil the arbuscular mycorrrhizal fungi are present as spores or as a coenocytic (aseptate) mycelium which is actually one huge branched tubullar cell that has countless amount of nuclei in its flowing cytoplasm. The spore germinates with germination hypha which establishes mycelium that is able to colonize the root. After the mycelium reaches the root, apresorium (morphologiaclly well defined structure) is formed. The apresorium attaches to the root and one or more thick hyphae growing from the apresorium penetrate through the rhizodermis until they reach the cortex. In the cortex the hypha penetrates the cell wall but it never penetrates the plasmatic membrane of the host cell. Cytoplasm of the host cell remains intact. After numerous dichotomic branching of hypha the plasmatic membrane of the host cell intussuscepts and thus gives rise to a furcate follicle (figure 5.4.). This structure (called arbuscule) is place of exquisite counterchange of nutrients, other substances and information (Gryndler 2004). AM fungi also form vesicles in plant cells, which seems to be, at least in some cases sufficient characteristic of functional AM (Brundrett 2009). AM fungi are attributed mainly to supply plant with phosphates (Smith 2009). Phosphorus is often limiting nutrient for plants in the environment. AM fungi can scavenge phosphates with extraradical mycelium, which can grow up to 100 times longer than root hairs, in exchange for assimilated carbon (Javot 2007). Another AM function is to increase plant stress (biotic and abiotic) resistance, (Borowicz 2001, Javot 2007, Tian 2010). Furthermore nitrogen transport has also been reported to be mediated by AM to host plant. AM can take up $NO_3^-$ and $NH_4^+$ (Tian 2010). Recently it has been shown that AM fungi can increase access to organic P and N sources (Javot 2007, Tian 2010).

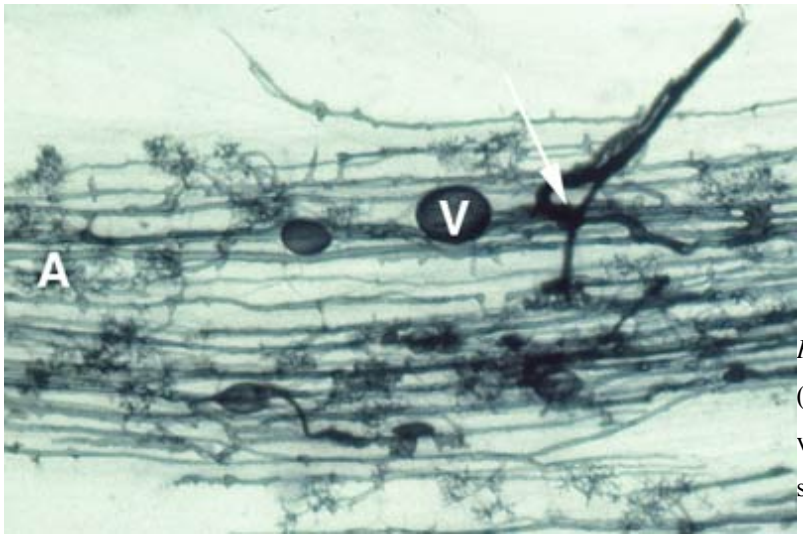


*Figure 5.4.* Part of a colony of a AM fungus (*Glomus* sp.) with hyphae, arbuscules (A) and vesicles (V) growing from an entry point (arrow). source: http://mycorrhizas.info/vam.html

### 5.3.2. Ectomycorrhiza

Ectomycorrhizal (ECM) association dominates in forests ecosystems in Northern hemisphere (Nehls 2010). At least 6000 ectomycorrhiza forming fungal species were described (primarily of basidiomycetes with some ascomycetes and zygomycetes). Yet still their diversity in tropical and southern regions is poorly known (Tedersoo 2010). Although ectomycorhhiza was first observed in the year 1841, it was considered to be a parasitic fungus. The symbiotic essence of this structure was elucidated 45 years later by A.B. Frank. First successful attempt to cultivate ectomycorrhiza was performed by Melin in 1922 on larches grown in aseptic environment. (Gryndler 2004)

#### 5.3.2.1. Evolution

Modern plant phylogenetics suggest that the ECM has arisen independently over the course of evolution in *Pinaceae* and several disparate lineages of angiosperms. They have probably evolved in the past 130 -180 mil.years more than one time, on the score of the polyphyletic origin of ectomycorrhizal fungi. Ectomycorrhizae are thus younger than the ancient arbuscular mycorrhizal symbioses. Futhermore ribosomal genetic analyses suggest that ectomycorrhizal fungi belonging to basidiomycetes evolved on several independent occasions from saprophytic fungi, but what is also interesting, some ectomycorrhizal fungi very likely returned to saprophytic ingestion (Tedersoo 2010).

#### 5.3.3.2. Ecophysiology and life cycle

Considering the polyphyletic status of ectomycorrhizal fungi, their life cycle can not be described precisely, but I shall briefly introduce features typical of Basidiomycetes (eventually Ascomycetes). In the soil ectomycorrhizal fungi are present as spores or as mycelium (aseptate, septate, monokaryotic or dikaryotic). After spore germination, the hyphae merge and a dikaryotic mycelium develops and is able to colonize the root of host plant (Ascomycetes colonize root with monokaryotic mycelium, the dikaryotic hyphae are established in process of fruiting body forming). The colonisation generally begins by aggregation of hyphae around the short secondary roots. The root is then surrounded with compact hyphae sheath. The hyphae grow in the junction between epidermal cells, thus forming the so called Hartig net. Hartig net is a complex organ comprising cortical or epidermal cells of plant roots and fungal hyphae that have ramified intercellularly between the individual root cells to maximize the contact area for reciprocal nutrient transfer (Tedersoo 2010). The fungal cells of Hartig net communicate with the outer environment via the cells of the sheath (mantle) (see figure

5.5.). Under the influence of exogenous (apropriate temperature, humidity, host photosyntetic activity...) and endogenous (sufficient fungal biomass) conditions the fructification takes place. The karyogamy occurs in the fruiting body, followed by the meiosis and formation of the spores. Nevertheless identification of ectomycorrhizal fungi by morphology, lipid profiles or DNA-based methods have shown that the mycorrhizae are often formed by fungi that are not linked to epigeous fruiting bodies. These cryptic fungi may produce hypogeous fruiting bodies or may be asexual, or fruit very infrequently. (Gryndler 2004). Similarly to AM, the biological function of ECM symbiosis is among others exchange of fungus-derived mineral nutrients for plant-derived carbohydrates. In natural forests, major nutrients (N, P) are fixed in organic layer thus one of major ECM function is fungal contribution to tree nutrition by mineral weathering and organic matter decomposition (Nehls 2010). Together with roots, ECM fungus constitute a large carbohydrate sink through increased monosaccharide uptake capacity, glycolysis and intermediate carbohydrate storage pools (trehalose and/or mannitol). As a consequence of high carbohydrate demand, trees may increase photosynthetic capacity (Nehls 2010).

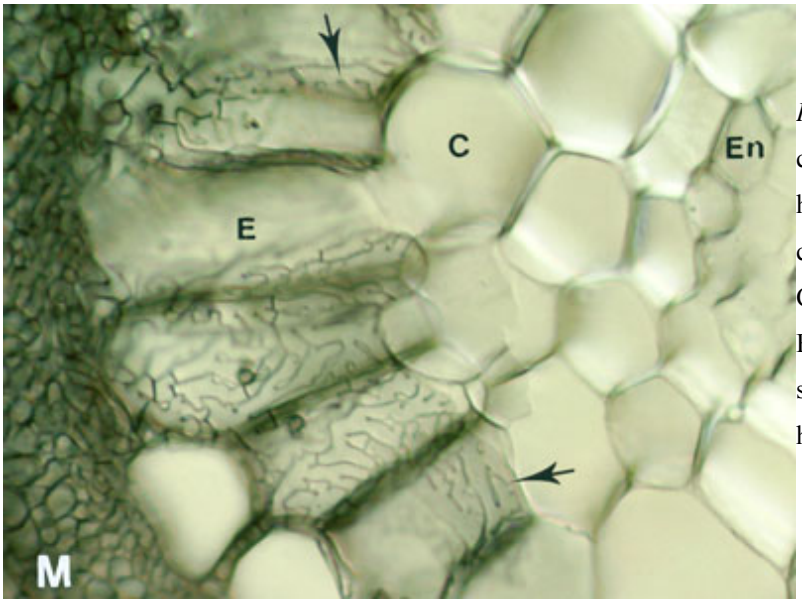


*Figure 5.5. Populus tremuloides* ECM root cross section showing labyrinthine Hartig net hyphae (arrows) around elongated epidermal cells.
C = cortex cell, E = epidermal cell, M = mantle, En = endodermis
source: http://mycorrhizas.info/ECM.html#poplarhartig

### 5.3.3. Dual mycorrhiza

Plants able to form mycorrhizas both with AM and ECM fungi are called dual mycorrhizal species. This phenomena has been observed in some genera of families *Salicaceae* (*Salix*, *Populus*, *Choosenia*), *Betulaceae* (*Alnus*), *Myrtaceae* (*Eucalyptus, Leptospermum*) and recently even in genera of families considered to be solely ectomycorrhizal such as *Fagaceae* (*Quercus*) or *Pinaceae* (*Pinus*, *Tsuga*, *Abies*) or some species from genera *Caesalpiniaceae* and *Uapacaceae* (Moyersoen 1999, van der Heiden 2000, Weijtmans 2007).

Although dual mycorrhizal associations are not well studied in terms of functionality, based on available data dual plant hosts seem to benefit from functional differences between the two mycorrhizal fungal groups (Aguillon 1990, van der Heijden 2001). It has been repeatedly observed that both genotype and environmental factors influence extent of root colonisation by the two mycorrhizal fungal groups (Gehring 2006, Khasa 2002). Out of environmental factors soil moisture, soil fertility, herbivory stress and pollution were reported to significantly influence the arbuscular/ectomycorrhizal ratio (Aguillon 1990, Lodge 1990). Dual mycorrhizal colonisation might be also explained by decreased plant control over colonisation (lower benefit) or by niche differentiation (increased plant benefit). AM were reported to be readily displaced by ECM in root system of willows or eucalyptus, which could be explained by ECM fungi competitive superiority over AM fungi with decreased plant control over colonisation (Gange 2005, van der Heijden 2000). However niche differentiation explanation would be in accordance with fact that ECM do not always prevail in dual plants root system as observed for poplars, or even in willows under some conditions (Beauchamp 2005, van der heijden 2000). Interspecific variations in preference for mycorrhizal partner (or vice versa - variations in fungal preference for host plant) may play a role in the AM/ECM colonisation dynamics (Püttsepp 2004, Tagu 2005). Though the occurrence of AM and ECM fungi was reported to be negatively correlated within one root system, they may nevertheless be functionally complementary (Aguillon 1990). It was hypothesized that the ability of certain plant species to form dual mycorrhizal might have a selective advantage in flood plains with ever changing soil conditions (Aguillon 1990, Lodge 1990).

### 5.4. Plant response to HMs

When plant is exposed to HMs in soil, its response depends on several factors including plant species or even plant genotype, HM involved, its concentration, availability (mobility), soil pH, redox potential, concetration and availability of other elements and other stress factors. Generally plant tolerance to HM is given by its ability to avoid HM and to resist it. The avoidance is realized by hindering HMs from entering apoplast and the resistance by several mechanisms - regulation of HMs transport to various organs, HMs chelation and/or compartmentation of HMs or oxidative protection. These mechanisms will be briefly described in the following sections. Yet as mentioned in the section discussing mycorrhizae most plants are mycorrhizal and thus rather than response of plant itself, response of symbiotic plant-fungus should be considered.

#### 5.4.1 Extracellular detoxification

In a polluted soil, plant can ameliorate its root environment by exudates decreasing pH and by chelating HMs. The acidification has been atributed to plasma membrane-localized proton pumps and to low-molecular weight organic acids. Metal chelating is likely to be mediated by low-molecular weight compounds such as phytosiderophores, citric acid, malate, succinate, oxalate or others (Clemens 2006).

#### 5.4.2. Regulation of HMs transport

After plant has acquired HMs from soil solution, the HMs can be immobilized on cell wall which was reported for several plant and fungal species, for example *Thlaspi caerulescens* accumulated 60-70% of total Ni and Zn in apoplast (Boominathan 2003). This biosorption of HMs on cell wall is possible due pectins, histidyl residues and maybe others ready to acquire metal ions (Sanità di Toppi 1999). Transfer of HMs to symplasm is mediated by various transporters or channels situated on plasma membrane. Proteins involved in HMs transportation across plasma membrane can be divided in several groups according to their structure, position and function.

CPx-ATPases - are transmembrane proteins from a larger family of P-ATPases found in wide range of organisms including bacteria, archea and eukarya. Abbreviation CPx in the name stands for conserved motif in one of transmembrane helixes composed of cystein-prolin and cysteine/serine/histidine respectively. These proteins are responsible for transportation of essenital metals, as well as potentially toxic metals such as Zn, Cu, Cd, Pb (Williams 2000).

CDF - cation diffussion facilliator - is a family of channel proteins also found in wide range of organisms including bacteria archea and eukarya. CDF are implicated in the transport of Zn, Co, Cd. Localized on intracellular as well as plasma membrane, they are thought to be involved in HMs uptake, efflux or sequestration. (Williams1999).

ZIP transporters (ZRT1/IRT1 like proteins) are family of proteins responsible for $Zn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Mn^{2+}$ translocation. Some of them however are permeable for $Cd^{2+}$ ions. CNGC - cyclic nucleotid gated channels, usually responsible for ion ($K^{+}$, $Ca^{2+}$) are reported to be permeable also for Ni, Pb (Eng 1998).

Regulation of proteins involved in HMs transportation could take place on transcriptional as well as post-transcriptional level (Williams 2000). Several experiments with genetically modified plants have demonstrated altered plant/plant cell susceptibility to HMs, as well altered transport of HMs due to altered expression of transporters (Gisbert 2003).

### 5.4.3 Intracellular detoxification, compartmentalization, oxidative protection

Once the HM has entered cytosol the cell can chelate it with various compounds and/or sequester it in vacuole. Chelating compounds differ with species and HM involved - usually plants use several compounds with similar characteristics including amino acids, chaperones, heat shock proteins, small organic acids, phytochelatins, metalothioneins or others. Apart from direct chelating and decreasing the toxic effect of HMs, some of these compounds are also involved in oxidative stress protection and some of them after chelating HMs are readily transported to compartments were they are stored (e.g. vacuoles) (Clemens 2006).

Of the principal importance from the compounds listed above is glutathione (GSH). Glutathione is small oligopeptide composed of γ-glutamyl-cysteinyl-glycine. Glutathione plays a major role in oxidative protection due to its unique structural properties, broad redox potential and abundance (May 1998, Noctor 1998). GSH is also able to directly chelate some HMs such as cadmium (Clemens 2006). Futhermore GSH is essential for phytochelatins (PCs) synthesis.

Phytochelatins are enzymatically synthesized thiol-rich oligopeptides with general structure $(\gamma\text{-glutamyl-cysteinyl})_n$glycine, where n = 2 -11. PCs synthesis is catalyzed by enzyme phytochelatin synthase, which is activated in the presence of HM cation such as $Cd^{2+}$(De Vos 1991). PCs form complexes with HMs and are transported and sequestred in vacuole. (Noctor 1999). As the name suggest they were first described in plant species, however they have also been found in several fungal

species such as Sacharomyces pombe or Boletus edulis (Collin-Hansen 2007). PCs however are believed not to be synthesized in *Salix vinimalis* species (Landberg 2004).

Metallothioneins are also thiol-rich metal-binding polypeptides. However contrary to PCs they are gene-encoded (Hall 2002). Apart from HMs detoxification they were reported to alleviate as oxidative stress in *Quercus suber* (Mir 2004) and ectomycorrhizal fungus *Paxillus involutus* (Courbot 2004).

Membrane transporters or channels also contribute to intracellular detoxification of HMs; Nramp (natural resistance-associated macrophage protein) are transporters conferred to mediate divalent cations of Mn, Co and Cd. In plants localized in the vacuolar membrane, they are likely to be involved in metal sequestration or mobilization from the vacuole (Williams 2000). CPx ATPases coould also be responsible for compartmentalization of HMs eg sequestration in vacuole (Williams 2000).

As mentioned above - HMs can induce several oxidative stress, thus HMs detoxification usually involves oxidative stress protection. Plants are endowed by several antioxidative mechanisms involving induction of antioxidative enzymes (such as superoxid dismutase (SOD), catalase (CAT), ascorbate peroxidase, glutathione reductase, glutathione transferase etc.) and antioxidants scavenging ROS (GSH, ascorbic acid, various compounds of secondary metabolism etc.) (Dixit 2001).

### 5.4.4. Plant-mycorrhizal interactions

The mechanisms of fungal tolerance are also similar to those involved in plant metal tolerance, including extracellular chelation, cell wall binding (chitin-Cd complexes), regulation of HMs transportation, intracellular detoxification and/or sequestration and antioxidative mechanisms (SOD, CAT, GSH) (Bellion 2006, Leyval 1997). As true for plants, mycorrhizal fungi also show great variability in HMs tolerance, either interspecific or intraspecific (Bladuez 2000). It has been repeatedly observed that mycorrhizal fungi increase tolerance of their hosts to HMs (Ahmed 2006, Hartley1997, Leung 2007, Sell 2005, Schützendübel 2002). However fungi can increase the tolerance of their hosts only when their own tolerance exceeds that of the host plant (Jentschke 2000). Nevertheless some papers showed that mycorrhizal colonisation may lead to increase in HMs uptake resulting in toxic concentrations in host plants (Khan 2000). Jentschke and Godbold summarised the findings in the table comparing shoot metal content from different plant species inoculated or non-inoculated with ectomycorrhizal fungi; inoculated plants contained from less than 20 to more than 150% metals in

shoots compared to non-inoculated plants (Jentschke 2000). Fungal symbiosis may increase overall metal content even if HM concentration remains the same, by increasing plant growth rate (Jentschke 2000, Sell 2005, Baum 2006). Mycorrhizal fungi may also affect plant HM translocation rom roots to stems and from stems to leaves. Although mechanisms behind this are not clear, one of proposed way is alteration of plant hormonal status (Jentschke 2000).Yet whatever is the plant tissue HMs concentration, mycorrhizal fungi can increase plant resistance to HMs present in its tissues (Rabie 2005, Sell 2005). Possible mechanisms may include better plant nutrition, influence of plant physiological repsonse to HM stress, restricted translocation from roots (mycorrhizae) to shoots or just hindered acces of HM to root surface in case of ectomycorrhizas (Schützendübel 2002). It has been reported that AM colonisation induced and activated antioxidative systems in host plants on soils contaminated by HMs, resulting in increased plant tolerance towards HM (Rabie 2005). Several studies point out the importance of intraspecific variation of studied mycorrhizal species. For example inoculation of *Pinus sylvestris* seedlings with 2 strains of ECM fungi *Suillus bovinus,* resulted in different tolerance to Zn treatment and fungal performance significantly affected plant nutrient status (Adriaensen 2003). Examples showing different AM susceptibility to HM has also been shown, with widely ranging results: Depending on growth conditions, fungus, and metal, the effect of maize root colonisation by AM fungi either reduced metal content of the plants or increased its uptake (Hall 2002). Thus further research is needed to elucidate impact of mycorrhizal symbiosis on plant tolerance to HMs on one hand and on soil dynamics of HMs on the other hand.

## 6. Aims

As mentioned in the Introduction this work is a part of the project aimed at developing phytoremediation biotechnology at heavy metals polluted land. This work traced out three objectives:

1) to find ectomycorrhizal strains tolerant to heavy metals and suitable for inoculation of fast growing trees
2) to compare superoxid dismutase activity of the selected strains as a key antioxidative process
3) to evaluate the influence of selected mycorrhizal fungi (both AM end ECM) on performance, few physiological parameters and heavy metals accumulation of the plant hosts when these were grown in soil contaminated by HM

To elucidate the above mentioned problems the following questions were asked:

Ad 1)

Do screened ectomycorrhizal isolates differ in their ability to grow on agar media amended with HMs *in vitro*?

Is it possible to find HMs-tolerant ectomycorrhizal strains differing in the ability to accumulate several HMs and thus to select strains putatively usable for inoculation of fast growing trees serving phytoextraction/phytostabilisation strategies?

Ad 2)

Do selected strains with different HMs accumulation differ also in superoxid dismutase (SOD) activity?

Does SOD activity simply reflect the amount of particular HM in mycelium?

Does SOD activity differentially respond to examined HMs?

Ad 3)

Providing it will be possible to find strains according to the demands specified in objective 1:

Do the strains modulate the accumulation of HMs by their plant hosts in a way expected based on the results of *in vitro* screening?

Does the response depend on particular host tree?

Is the symbiosis reflected on plant biomass acquisition and/or other morphometric parameters?

Do mycorrhizal fungi modulate the effect of HMs on host plant physiology (photosynthesic rate, photosynthetic pigment concentration and leave surface chemistry)?

## 7. Materials and Methods

### 7.1. Experiment INVIT

The aim of the experiment was to find strains of ECM fungi tolerant to the mixtures of heavy metals with different ability to accumulate HMs in their mycelia. The purpose of this screening was to select strains that would successively be used for inoculation of fast growing trees in field experiment (short rotation coppice plantation) and in pot experiments with HMs-contaminated soil. Tolerant and high HMs-accumulating EcM strains were hypothesised to be suitable for enhancing the uptake of HMs by plants (phytoextraction strategy) while tolerant but low accumulating EcM strains were assumed to be suitable for immobilization of HMs in the soil (phytostabilisation strategy). The experiment also addressed the question of the superoxid dismutase activity of selected EcM strains to the heavy metals of interest (Cd, Pb, Zn).

#### 7.1.1. Experiment INVIT part A - screening

##### 7.1.1.1. Experimental time frame

The experiment was conducted at the end of year 2007 (precultivation of fungal strains) and the beginning of year 2008 (experimental cultivation, harvest and evaluation). Experimental procedures took place as depicted below (figure 7.1):

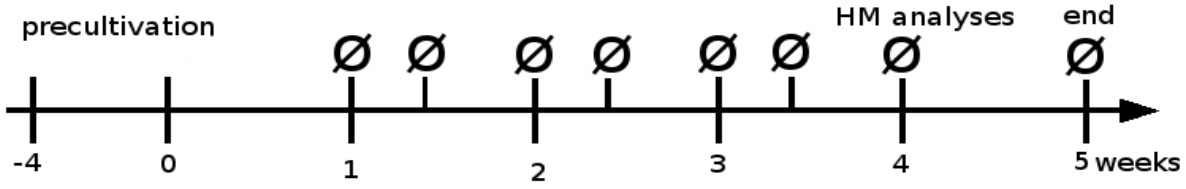


*Figure 7.1. Time frame for experiment INVIT part A, precultivation of fungal isolates took place prior to start of the experiment (0 weeks), symbol ø represents measurement of radial colony growth.*

##### 7.1.1.2. Fungal species and strains selection

20 strains of ECM fungi were chosen (table 7.1), among which, there were strains obtained in the vicinity of experimental fields near Příbram city. Fungi obtained "*in situ*" were isolated from fruitbodies or poplar and willow mycorrhizal tips. All of the selected species are known to form mycorrhizal symbioses with plants from *Salicaceae* family (Baum 2006, Baum 2009, Cripps 2003, DeBellis 2006, Gunderson 2007, Sarmento 2006/2007, Selosse 2003, van der Heijden 2003, Visser

1998).

| Isolate | Species | Isolate origin |
|---|---|---|
| AMU-1 | Amanita muscaria (L.) Lam. | Šumava CR |
| ABY-1 | Amphinema byssoides (Pers.) J. Erikss. | Šumava CR |
| CFI | Cadophora finlandica (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew. | Příbram CR |
| CGE-4 | Cenococcum geophilum Fr. | Finland |
| HBR-1 | Hebeloma bryogenes Vesterh. | Šumava CR |
| HFA-1 | Hebeloma fastibile (Pers.) P. Kumm. | Germany |
| HLE-1 | Hebeloma leucosarx P.D. Orton | Germany |
| HME-1 | Hebeloma mesophaeum (Pers.) Quél. | Slovinsko |
| HSE | Hebeloma senescens (Batsch) Berk. & Broome | Mělník CR |
| LAU-1 | Leccinum aurantiacus (Pers.) Gray | Czech Republic |
| LDU-1 | Leccinum duriusculum (Schulzer ex Kalchbr.) Singer | Příbram CR |
| LLA-3 | Laccaria laccata (Scop.) Cooke | Czech Republic |
| Lsp | Lactarius sp. | Šumava CR |
| PIN Maj | Paxillus involutus (Batsch) Fr. | Germany |
| PIN-8 | Paxillus involutus (Batsch) Fr. | Příbram CR |
| PTI-1 | Pisolithus tinctorius (Mont.) E. Fisch. | Portugal |
| TCI-1 | Tricholoma cingulatum (Almfelt) Jacobashch | Germany |
| TSC-1 | Tricholoma scalpturatum (Fr.) Quél. | Příbram CR |
| TSC-2 | Tricholoma scalpturatum (Fr.) Quél. | Příbram CR |
| Tsp | Thelephora sp. Ehrh. ex Willd. | Příbram CR |

*Table 7.1. Fungal strains used in the experiment INVIT.*

### 7.1.1.3. Culture media and growth conditions

All fungal strains were grown on modified Melin–Nokrans medium (mMNM), containing in 1 l of distilled water: 0.5 g malt extract, 0.3 g $KH_2PO_4$, 0.25 g ammonium tartrate (dibasic), 0.15 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2$, 0.025 g NaCl, 5.4 mg $FeCl_3.6H_2O$, 100 μg thiamine-HCl, 24 μg biotin, 24 μg pyridoxine, 4.5 g glucose and 15 g agar. pH of the media was set to 7.0 prior to autoclaving. Control variant was left free of heavy metals, while variants "K" and "L" were amended with HMs (as shown in table 7.2) corresponding roughly to bioavailable pool of soil HMs at sites "Komín" and "Litávka" respectively. Sites "Komín" and "Litávka" depict experimental plantation sites near Příbram city in Central Bohemia in the Czech Republic (49.706595°N 13.975886°E ; 49.718608°N 14.013335°E for "Komín" and "Litávka" respectively). The average soil characteristics together with HMs concentrations at the experimental sites are summarized in table 7.3.

| Treatment | | Medium | amendment in µM | | | |
|---|---|---|---|---|---|---|
| | | | $Na_2HAsO_4.7H_2O$ | $Cd(NO_3)_2.4H_2O$ | $Pb(NO_3)_2$ | $ZnSO_4.7H_2O$ |
| **C** | control | mMN | 0 | 0 | 0 | 0 |
| **K** | Komín | mMN | 9 | 1,4 | 170 | 20 |
| **L** | Litávka | mMN | 15 | 7,4 | 62 | 680 |

*Table 7.2. Concentration of HMs used in the growth media in experiment INVIT "A"*

| site | Concentration mg/kg | | | | | | | Concentration % | | pH | | Bioavailable concentration mg/kg | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cd | Pb | Zn | K | Ca | Mg | Cu | N | C | pH(H2O) | pH(KCl) | Cd | Pb | Zn |
| **Komín** | 10,0 | 2172,3 | 318,3 | 9576,3 | 17727,1 | 354,4 | 36,3 | 0,29 | 2,39 | 6,46 | 5,74 | 3,5 | 733,6 | 27,9 |
| **Litávka** | 36,1 | 2940,2 | 3861,9 | 8996,8 | 15676,0 | 327,5 | 75,8 | 0,43 | 3,74 | 7,00 | 6,58 | 18,1 | 278,7 | 974,0 |

*Table 7.3. Soil characteristics from sites "Komín" and "Litávka". Bioavailable concetrations - concentrations extracted with weak acid (0.11M acetate).*

Each fungal strain was cultivated on 3-compartment Petri dish as depicted in figure 7.2. Three replicates per variant were used to monitor radial growth of mycelia. For the measurement of heavy metals accumulation in fungal mycelia, three more replicates were prepared with a disc of sterilized cellophane placed on the surface of agar media.

Each Petri dish contained all of the 3 types of medium, one in each compartement. All compartments were inoculated by a 5mm plug from 1-2 months old colony MMN. Fungal cultures were incubated for 34 days in the dark at 21° C.

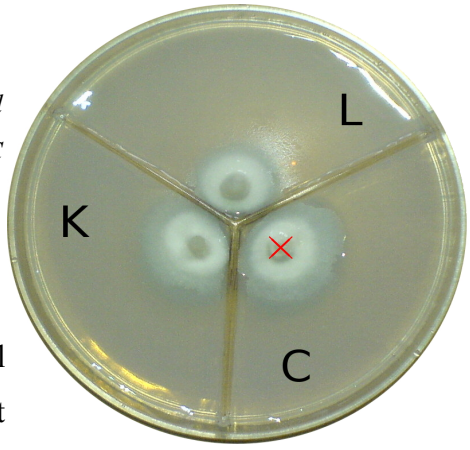


*Figure 7.2. 3-compartment Petri dish with 3 heavy metal treatments: C- control treatment, K - Komín, L- Litávka. The red cross points to original fungal plug in C treatment .*

### 7.1.1.4. Biometric measurements and harvest of the experiment

Radial colony growth (RCG) was measured as a mycelial colony radius on the axis bisecting the colony centre (a mean of at least two values was calculated). It was monitored every 3 to 4 days for 34 days. Fungal colony growth was described by non-linear parametric function fitted to colony diameters observed in time. Fitted equation (Weibull):

$estRCG = RCGmax*(1-e^{(-a*t)^b})$

where “estRCG” is estimated colony diameter in time “t”, based on “RCGmax” - maximal colony diameter, and “a” and “b” parameters altering curve shape. The parameters describing fungal growth are maximal colony size (RCGmax) and time when colony reaches half of its maximal size (T1/2 = $(LN(2)/a)^{(1/b)}$) (López 2004).

At the end of the experiment, mycelia were scrapped from the cellophane and the fresh and dry (oven-dried at 65°C to constant weight) weights of mycelia assessed. The original inoculated plugs were omitted from the analyses.

#### 7.1.1.5. Evaluation of mycelial content of heavy metals

Preparation of samples and the following analyses were done at the Czech University of Life Sciences Prague. An aliquot part of the fungal samples was decomposed using the dry ashing procedure in a mixture of oxidizing gases ($O_2$ $O_3$ $NO_x$) at 400 °C for 10 h in Dry Mode Mineralizer Apion. The ash was dissolved in 1.5% HNO3 (Analytika, Czech Republic). Metal concentrations in digests were determined using ICP-OES (VARIAN VistaPro). The standard reference material DC73350 Leaves of Poplar (China National Analysis Centre for Iron and Steel, China) was used for evaluating the measurement precision.

### 7.1.2. Experiment INVIT part B -mechanisms of HM tolerance

#### 7.1.2.1. Experimental time frame

The experiment was started in at the late June of 2009. Experimental procedures took place as depicted below (figure 7.3):

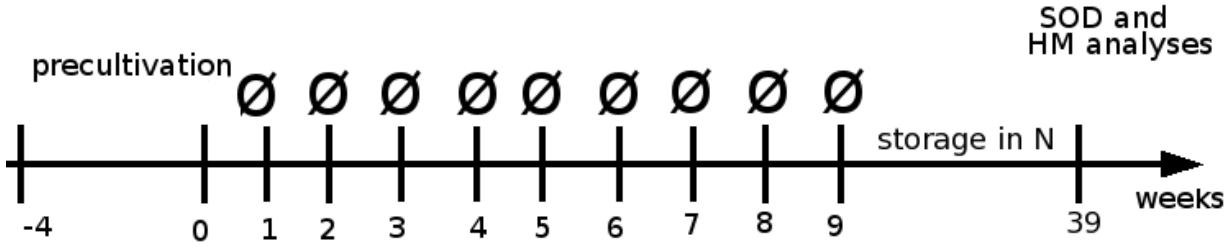


*Figure 7.3. Time frame for experiment INVIT part B, precultivation of fungal isolates took place prior to start of the experiment (0 weeks), symbol ø represents measurement of radial colony growth.*

#### 7.1.2.2. Fungal species and strains selection

4 fungal isolates from previous part (part A) of the experiment were chosen, according to their HM tolerance and accumulation. High accumulating fungi *Paxillus involutus* (Batsch) Fr. strain PIN Maj and *Pisolithus tinctorius* (Mont.) E. Fisch strain PTI-1 and low accumulating fungi *Hebeloma mesophaeum* (Pers.) Quél. strain HME-1 and Tricholoma scalpturatum (Fr.) Quél. srain TSC-1 were used in part B of the experiment.

#### 7.1.2.3. Culture media and growth conditions

All fungal strains were grown on modified Melin–Nokrans medium (mMNM), containing in 1 l of distilled water: 0.5 g malt extract, 0.272 g $KH_2PO_4$, 0.92 g ammonium tartrate (dibasic), 0.11 g $MgSO_4 \cdot 7H_2O$, 0.033 g $CaCl_2$, 0.018 g NaCl, 2.7 mg $FeCl_3.6H_2O$, 1.12 mg $MnSO_4.4H_2O$, 1.15 mg $ZnSO_4.7H_2O$, 100 µg thiamine-HCl, 24 µg biotin, 102 µg pyridoxine, 5.1 g glucose and 20 g agar. pH of the media was set to 5.5 prior to autoclaving. Control variant was left free of heavy metals, while variants Cd Pb and Zn were amended in 1 l of media with: 4 mg $CdSO_4$, 9 mg $PbSO_4$ and 264 mg $ZnSO_4.7H_2O$ respectively.

Each fungal strain was cultivated on square Petri dish. 4 replicates per isolate and given treatment were used to assess biomass production and HM content. To assess SOD activity, 4 more replicates per isolate per treatment were used

Each Petri dish contained one type of medium, with a disc of sterilized cellophane placed on the surface of agar media. Each petri dish was inoculated by five 5mm plugs from 1-2 months old colony grown on MMN. Fungal cultures were incubated in the dark at 21° C.

#### 7.1.2.4. Biometric measurements and harvest of the experiment

Radial colony growth (RCG) was measured as a mycelial colony radius on the axis bisecting the colony centre (a mean of at least two values was calculated). It was monitored every 7 days until reasonable amount of mycelia was obtained or the mycelium covered the petri dish.

#### 7.1.2.5. Evaluation of mycelial content of heavy metals

Same procedure for HM mycelial content was used as in INVIT part A (see section 7.1.1.4.).

### 7.1.2.6. Evaluation of superoxide dismutase activity

Fungal mycelia were scrapped from the cellophane, and stored in liquid nitrogen until processed for SOD analyses. The SOD activity was estimated in mycelial supernatant obtained by a procedure described by Janknegt (2007). Briefly, for each sample, approximately 200 mg of frozen fungal mycelia were homogenized in 2ml extraction buffer (50mM KH2PO4, 0.1mM EDTA, 1% Triton X-100 in $dH_2O$, pH 7.8) for 1 min by IKA Ultra Turrax T-10 homogenier (IKA® Werke GmbH & Co. KG, Staufen, Germany) in ice bath (maximum speed). The homogenate was then centrifuged at 20000g, 4°C for 20min. Activity of SOD was assessed using Superoxide Dismutase Assay Kit (Biovision Research Products, Mountain View, USA). The enzymatic reaction in the kit uses superoxide anion, which reduces WST-1 resulting in production of water soluble formazan dye. This reaction is linearly related to the xanthine oxidase activity. SOD inhibits WST-1 reduction by superoxide anion, which allowed us to determine SOD activity colorimetrically. The absorbance was read at 450 nm following 20min incubation at 37°C. The SOD activity was then calculated according to user's manual provided with the kit.

## 7.2. Experiment DUAL

This experiment aimed to study the effect of mycorrhizal symbiosis on plant performance and physiology of plant host grown on soil contaminated by HM. It also addressed a question about interactions between different mycorrhizal fungi colonizing the same root system. The experiment consisted of several combinations of two species of the family *Salicaceae*, 2 AM fungi of the genus *Glomus* and 2 ectomycorrhizal fungi chosen based on the results of previous experiments.

### 7.2.1. Experimental time frame

The experiment started in May, year 2009. Experimental procedures took place as depicted below in figure 7.4.:

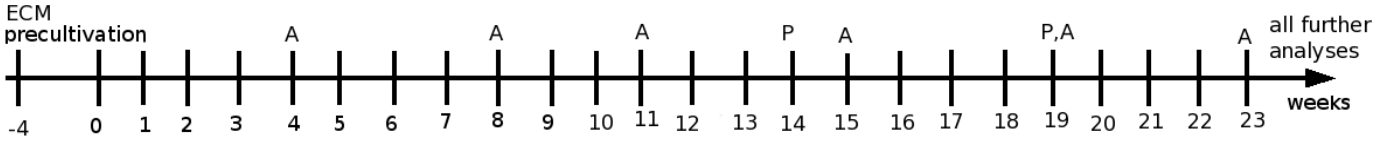


*Figure 7.4. Time frame of experiment DUAL. ECM precultivation took place prior to start of the experiment (0 weeks). A- measurements of plant above-ground architecture (height, stem lenght, branch counting) P- analyses related to photosynthesis (measurement of photosynthetic rate, pigment concentration, and Raman leaves spectra).*

### 7.2.2. Selection of fast-growing trees' clones and mycorrhizal fungi

Two fast growing clones of willow and poplar species used in the experiment "DUAL" were *Salix alba Linnaeus* clone S-117 (Code of Ministry of Agriculture) and *Populus nigra Linnaeus* clone Wolterson (originating from the Netherlands). The selected fast growing clones have high potential to accumulate biomass and to remediate fields contaminated by heavy metals (Deraedt 1998, Vysloužilová 2003). Two ECM fungi were selected for inoculation - *Paxillus involutus* (Batsch) Fr. and *Hebeloma mesophaeum* (Pers.) Quél. The selection was made based on observed formation of mycorrhiza between chosen chosen fungal species with *Salicaceae* family (Cripps 2003, van der Heijden 2003), as well as on the results of both INVIT experiments which are described in Results and Discussion. Two AM fungi were chosen for inoculation - *Glomus intraradices* N.C. Schenck & G.S. Sm. (isolate ph5) and *Glomus claroideum* N.C. Schenck & G.S. Sm.(BEG96 isolate). The selection was based on reported HMs tolerance of both species and the previous work from my colleagues in the Institute of Botany, ASCR (Janoušková 2006, Sudová 2007). Notably, *G. intraradices* ph5 originates from the close vicinity of experimental sites near Příbram city.

Experimental design consisted of 8 experimental fungal treatments for each plant species (see Table 7.4) Each treatment received equal amount of both AM and ECM fungal inoculum either alive or autoclaved; control treatment (K) was supplemented with autoclaved AM and ECM inoculum. 4 treatments were amended with single live fungus with complementary autoclaved inoculum added - PIN treatment with live *P. involutus* inoculum and dead AM inoculum, HME treatment with *H. mesophaeum* inoculum and dead AM inoculum, GCL treatment was inoculated with live *G. claroideum* and dead ECM inoculum was added, GIN treatment alike was inoculated with live *G. intraradices* inoculum and dead ECM inoculum was added. ECMX treatment was amended with both live ECM fungi and dead AM inoculum added to substrate. AMX treatment was amended with both live AM fungi and dead ECM inoculum added to substrate. Finally AE treatment contained all four live fungi. The amount of each inoculum added to treatment is summarized in table 7.4.

| Treatment | Inoculum [ml] | | | | | |
|---|---|---|---|---|---|---|
| | *G. claroideum* | *G. intraradices* | *H. mesophaeum* | *P. involutus* | AM autoclaved | ECM autoclaved |
| **K** - control | - | - | - | - | 10 | 20 |
| **AE** - mixed | 5 | 5 | 10 | 10 | - | - |
| **AM** - arbuscular | 5 | 5 | - | - | - | 20 |
| **ECM** - ectomycorrhizal | - | - | 10 | 10 | 10 | - |
| **GCL** - *G.claroideum* | 10 | - | - | - | - | 20 |
| **GIN** - *G. intraradices* | - | 10 | - | - | - | 20 |
| **HME** - *H. mesophaeum* | - | - | 20 | - | 10 | - |
| **PIN** - *P.involutus* | - | - | - | 20 | 10 | - |

*Table 7.4. Experimental treatments and fungal inoculation of DUAL experiment.*

### 7.2.3. Setup of the experiment

The soil substrate used in pots was obtained from the upper organic soil layer (0-0.25m) at the experimental plantation site "Komín" near Příbram city (soil characteristics are summarized in table 7.3). Soil samples were processed through 5 $cm^2$ sieve and gamma-sterilized. Bacterial eluate was added to soil 2 weeks prior to commencing the experiment; bacteria were obtained from a non-contaminated upper organic layer of kambisol near the Institute of Botany, ASCR (49.995367°N, 14.566755°E). The eluate was prepared as follows: 400ml of soil was dissolved in 3l $dH_20$ and filtered twice through 2-4 layers of filter paper KA 2-M (Papírna Perštejn s.r.o., Perštejn, Czech Republic). The eluate was distributed homogenously to total bulk of soil for experiment.

Tree cuttings were kept in darkened moist room at 4˚C untill the start of the experiment. Three days prior to commencing the experiment, the cuttings were soaked in water at room temperature and exposed to light. AM fungal inoculum was grown in pot cultures with maize as host in greenhouse 4 months prior to experiment (Janoušková 2006). Colonized maize roots fragments, extra-radical mycelium and spores were collected several days before inoculation and stored refridgerated in water. ECM fungal inoculum consisted of a mixture of mycelium grown in liquid PDA medium and perlit-based MMN medium (ratio 1:2). The mixture was prepared 1 day before commmencing the experiment for each fungus separately. The inoculum cultivated on liquid PDA medium was filtred through kitchen sieve (0.5 mm) to get rid of the medium. Subsequently deionized water was added to the mycelium chunks and mixed with the perlit-based inoculum. Half of each inoculum mixture was autoclaved in

order to obtain dead inoculum applicable to control variant to equate inoculum (nutrient) input.

Special containers made from Petri dishes (15 cm in diameter) were prepared and used in the experiment. A hole was cut to place the cutting, as depicted in figure 7.5.

Cuttings were 5 cm long, with both ends newly cut. Only cuttings with at least 2 buds, no roots and no leaves were used. Freshly cutted pieces were surface sterilized in 6% $H_2O_2$ (2 times in $H_2O_2$ for 1min and rinsed in $dH_2O$ after each $H_2O_2$ treatment).

Each Petri dish received additional 5ml of bacterial eluate (obtained from soil samples used for cultivation of GIN, GCL from greenhouse and Salix alba and Populus nigra from plantations (all in equal amounts in doses as described above). Following the containers mounting and placement of the cuttings, the containers were sealed with 3M - Micropore surgical paper tape (3M, St. Paul, Minnesota). Furthermore, the hole in which cutting was placed, was sealed with sterilized gardening wax. Wax was also deposited on top of the cutting to prevent excessive drying. Containers were then wrapped with an aluminium foil, to keep the roots in dark. Petri dishes with plants were weighed and cutting diameter was measured.

### 7.2.4. Growth conditions

The cultivation was conducted in a growth chamber (photon flux ca 500 $\mu mol.m^2.s^{-1}$, daylight/darkness 16h/8h at 24 °C and 18 °C respectively; relative air humidity 50-70%) for 6 months (7th May - 11th October 2009). Pots were randomized and watered with $dH_2O$ once a week. Nevertheless, plants exhibited water stress symptoms 8 weeks after the start of the experiment and thus they were irrigated more frequently, according to their needs (2-3 times a week). Each experimental variant (willows and poplars together) was placed on a one plate where it was watered (figure 7.6.). The position of containers within each plate was randomized every 4 weeks. The order of plates was shifted every 4 weeks as well to eliminate possible boarder effect.

*Figure 7.5. Container made out of 15cm Petri dish with poplar plant.*

*Figure 7.6. Experimental setup - rows of containers on one plate represent one experimental treatment.*

### 7.2.5. Biometric measurements and harvest of the experiment

Lenghts of the plants stems and mortality were monitored monthly. At the end of the experiment, above-ground biomass was processed; fresh and dry (oven-dried at 65°C to constant weight) weights were assessed for leaves and stems separately.

### 7.2.6. Evaluation of photosynthetic activity

Photosynthetic activity was measured twice, 14$^{th}$ week since the start of the experiment and at the end of experiment (19$^{th}$ week). Measurements were carried out by Li 6400 System portable gas exchange measuring system (Li 6400 System, Li Cor , Lincoln, Nebraska). Actual net photosynthetic rate (μmol CO2.m$^{-2}$.s$^{-1}$) and stomatal conductance (mol H2O.m$^{-2}$.s$^{-1}$) were evaluated by the system. Measurements were carried only on poplar plants due to poor willow leaves quality. Measurements were carried out under constant conditions (800 μmol.m$^{2}$.s$^{-1}$, ambient $CO_2$ concentration 370 ppm)

using the 5th unfolded leaf from the shoot apex. The photon flux density of 800 μmol.m$^2$.s$^{-1}$was chosen according to light curves obtained prior to measurements to ensure full light saturation of the photosynthetic systems. Each measurement lasted 20 minutes to allow the leaf to adapt to measurement chamber and to obtain steady values of conductivity and photosynthetic rate. Only values obtained at the 20th minute were compared.

### 7.2.7. Evaluation of photosynthetic pigments' content

The photosynthetic pigments' content was measured twice,14th and 19th week since the start of the experiment. Chlorophyll A, chlorophyll B and total carotenoid concetration were measured spectrophotometricaly (Lichtenthaler, 1987; Porra 1989). Fresh leave material (ca 50 mg from the 5th unfolded leave from shoot apex) was cutted with scissors to small ca 5mm*1mm pieces and extracted in dimethylformamide (DMF). The extraction of photosynthetic pigments in DMF took place at 4˚C and lasted for 2 weeks. Afterwards, absorption at 480, 647, 664 and 750 nm was measured on Hach DR 4000 U spectrophotometer (Hach Company, Loveland, Colorado). The concetration of photosynthetic pigments were calculated according to Lichtenhaler's equations as follow (Lichtenthaler, 1987).

ChA = 11.65 * A664 - 2.69 * A647

ChB = 20.81 * A647 - 4.53 * A664

c = (1000*A480 - 0.89ChA - 52.02 * ChB)/245

Where ChA, ChB and c represent chlorophyll A, chlorophyll B and carotenoid concentration in μg/ml respectively. The absorption at 750nm was used as a purity indicator of the sample. If the absorption exceeded 0.06 the sample was centrifuged to get rid of inpurities.

### 7.2.8. Analyses of Raman spectra

Raman spectra were collected using a Fourier transform near-infrared (FT–NIR) spectrometer Equinox 55/S with FT-Raman module FRA 106/S (Bruker, Germany). One spectrum per plant was collected. The fixed fresh leave (4$^{th}$ unfolded leave from shoot apex) was irradiated by the focused laser beam with a laser power 50 mW of Nd:YAG laser (1064 nm, Coherent). The scattered light was collected in backscattering geometry. A quartz beam-splitter and Ge detector (liquid N2 cooled) were used to obtain inteferograms. 1024 scans were accumulated for an individual spectrum of the given leaf. A standard 4 cm-1 spectral resolution, ‘zero filling’ 8 and Blackmann–Harris cosine apodization function was used for all data accumulation and Fourier transform processing. The spectra acquired using software package OPUS (Bruker,Germany) were exported to JCAMP-DX format for

chemometric evaluation. The PCA analysis of FT Raman spectra was performed on spectral data in the Stokes range (3600 – 100 cm-1) using the Uncrambler 9.2 (Camo,Norway). A full cross validation procedure was applied.

### 7.2.9. Evaluation of heavy metal content

Preparation of samples and the following analyses were done at the Czech University of Life Sciences Prague. An aliquot part of the plant samples was decomposed using the dry ashing procedure in a mixture of oxidizing gases ($O_2$ $O_3$ $NO_x$) at 400 °C for 10 h in Dry Mode Mineralizer Apion. The ash was dissolved in 1.5% HNO3 (Analytika, Czech Republic). Metal concentrations in digests were determined using ICP-OES (VARIAN VistaPro). The standard reference material DC73350 Leaves of Poplar (China National Analysis Centre for Iron and Steel, China) was used for evaluating the measurement precision.

### 7.2.10. Evaluation of fine root colonisation by mycorrhizal fungi

Roots of each plant were gently rinsed under sap water, and stored in 25% ethanol-water solution for succesive observations.

ECM root tips colonisation was assesed under stereomicroscope. On average 250 root tips were examined for each plant. Morphotypes found were described, photographed and stored in 5ml vials in 25% ethanol-water solution for DNA analysis. The relative abundance of each morphotype (number of root tips of each morphotype/total number of fine roots) was calculated for each sample (Aucina 2007).

AM root colonisation was determined after cleaning the roots in KOH for 40 min at 80˚C and subsequent staining with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989) using the grid-line intersect method (Giovannetti and Mosse, 1980).

### 7.2.11. Molecular characterization of mycorrhizal fungi

#### 7.2.11.1. DNA isolation

From each ectomycorrhiza sampled, DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The ectomycorrhizal tips were mechanically disrupted using small plastic mortar with sand, cooled with liguid nitrogene. 400 µl of AP buffer was added to each disrupted sample and incubated at 65°C for 10 min to lyse the cells. After incubation, 130 µl of Buffer AP2 was added to the lysate from each sample, the solution was mixed, and incubated for 5 min on ice, this step ensures proteins, polysaccharides and detergent precipitation. The lysate was then centrifuged for 2 min at 20,000 g. 1.5 volumes of buffer A3/E was added to recovered lysate and mixed immediately. The mixture was transferred to the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min at >6000 g. At this point DNA binds in the DNeasy Mini spin column membrane. It was washed with 500 µl AW buffer, centrifuged for 1 min at >6000 g. Again the mmebrane was dried with 500 µl AW buffer, while centrifuged for 2 min at 20000 g. The DNA was then eluated from the membrane with 100 µl 10mM TRIS/HCl buffer pH 8. After 5 min incubation at room temperature, the buffer was centrifuged twice for 1 min at >6000 g. The eluate was then used for PCR.

#### 7.2.11.2. PCR - polymerase chain reaction

Following extraction, the internal transcribed spacer regions I and II and the nuclear 5.8S rRNA gene were amplified on Eppendorf Master Cycler (Eppendorf AG, Hamburg, Germany) using the primer sets ITS-1F/ITS-4 (White 1990). The PCR mixture for one sample was composed of 14.6 µl of nuclease free water, 2.5 µl of 10x PCR buffer without $MgCl_2$, 2 µl of $MgCl_2$ (2 mM), 0.4 µl of each primer (0.4 mM), 2.5 µl of deoxynucleotide triphosphate (0.2 µM) and 0.12 µl of Taq polymerase (25U/µl) and 2.5 µl non-diluted template (diluted 50 times). All chemicals were obtained from Fermentas (Fermetnas, Ontario, Canada). Thermal cycling conditions were as follows: initial denaturalisation at 94˚C for 30 sec; 35 cycles at 55˚C for 30 sec, and a final elongation at 72˚C for 80 sec. PCR products were assessed by electrophoresis (45 min at 100 W on 1.5% agarose gel) before sequencing. Gels were stained with ethidium bromide and photographed under ultraviolet light.

If DNA stained bands were blurry or weak, the PCR product were used for nested PCR as follows: The nested PCR mixture per one sample consisted of 14.8 µl of nuclease free water, 2.5 µl of 10x PCR buffer without $MgCl_2$, 2 µl of $MgCl_2$ (2 mM), 0.5 µl of each primer (0.5 mM), 2.5 µl of deoxynucleotide triphosphate (0.2 µM) and 0.2 µl of Taq polymerase (40U/µl) and 2 µl of 1250-times

diluted template. All chemicals were obtained from Fermentas (Fermetnas, Ontario, Canada). Thermal cycling conditions (35 cycles in total) were the same as for PCR reaction above. Nested PCR products were assessed in same manner as for PCR reaction above.

#### 7.2.11.3. Sequencing and BLAST-based identification

Sequencing was done by GATC Biotech AG (Konstanz, Germany). The obtained ITS-sequences were used as probes in BLAST searches (GenBank, NCBI) in order to retrieve the most similar sequences for identification. BioEdit software was used for handling the sequences and the alignments (version 7.0.0.; Hall 1999).

### 7.3. Statistical analyses

Data were analysed using STATISTICA 9.0 (Statsoft, USA) statistical package and were subjected to Kolmogorov - Smirnov goodnes of fit test of normality and Cochran's homogenity of variance test. If these preliminary tests weren't violated, data were analysed by one-way ANOVA and statistically significant differences were identified by Fisher least significance difference (LSD) test with $p < 0.05$. If Cochran's homogenity test was violated, data were analyzed by Kruskal-Wallis ANOVA test with $p < 0.05$ as well. Correlations were assessed by non-parametric Spearman correlation test.

## 8. Results

### 8.1. Experiment INVIT

#### 8.1.1. Part A

##### 8.1.1.1. Colony growth and tolerance

Fungal colony growth was measured regularly as desribed in Materials and methods. The obtained growth curves were fitted by non-linear parametric Weibull function to derive curves parameters usable for further statistical analysis. The key parameters obtained were maximal colony size (RCGmax) and time when colony reached half of its maximal size (T1/2). Figure 8.1. depicts both obtained growth data and corresponding Weibull function for isolate PIN Maj on C, K and L treatments.

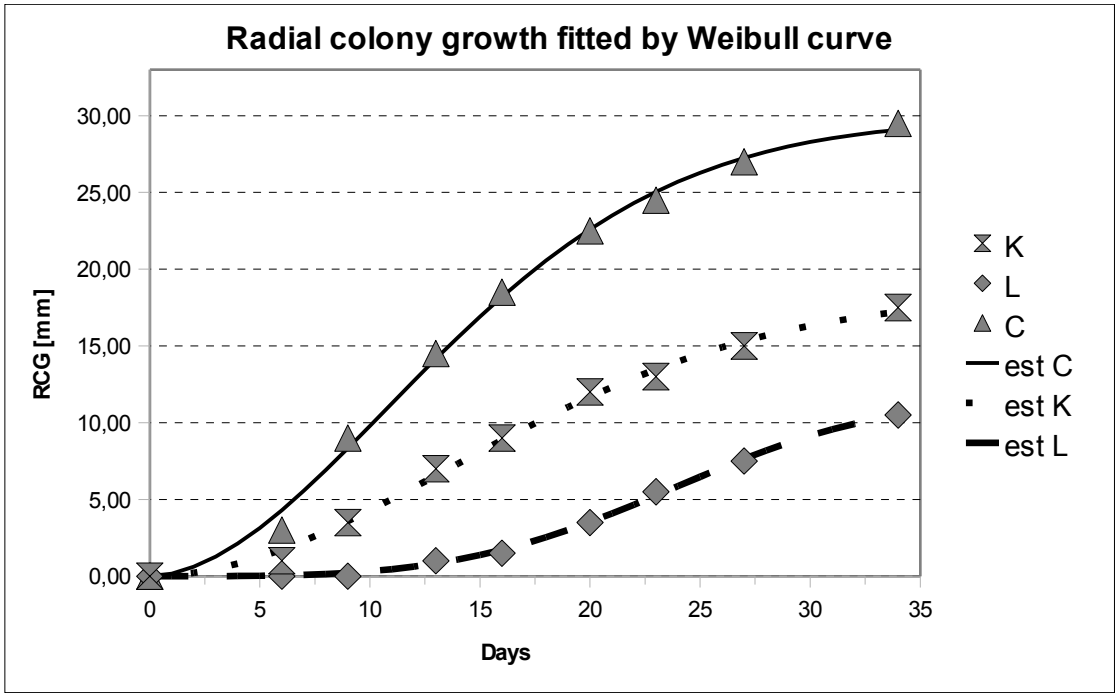


*Figure 8.1. Fungal growth as observed in PIN Maj strain in all three treatments (C - control, K - "Komín", L - "Litavka" as specified in Materials and methods) and fitted curves based on Weibull equation, estimating radial colony growth for each treatment respectively (est C, est K and est L ).*

Intraspecific (among strains) comparisons of derived parameters were done for control treatment to evaluate their ability to grow in axenic culture and thus their suitability for inoculation. Tolerance of fungal strains to heavy metals was evaluated by comparison of RCGmax and T1/2 within each strain between control, K and L treatment. Differences in heavy metal tolerance between isolates

were assessed comparing RCGmax%C and T1/2%C for K and L treatments, respectively. These relative parameters were obtained by dividing values of given parameter (either RCGmax or T1/2) grown on media treated with HMs (K or L treatment) by values of the parameter in control treatment. Fungal strains did not differ in growth parameters on K treatment, but differed in both parameters on L treatment. These results are summarised in table 8.2., where fungal isolates are sorted with relative parameters descending from most tolerant to the least.

Fungal growth could not be fitted in several cases, either due to linear growth (Lsp) or too slow growth of several strains (LDU-1, HFA-1 and HME-1) on L treatment preventing curves fitting. Several significant differences were manifested with LLA-3, PIN Maj, PTI and CFI as the fungi with largest colonies and LLA3, ABY-1 and both *P. involutus* strains as the fastest growing colonies within control treatment. Isolates AMU -1, ABY-1, HFA-1, HLE-1, HME-1, HSE-1, LLA-3, LDU, PIN 8 , TCI, TSC-1 and Tsp showed no significant differences in T1/2 caused by HMs treatment. Strains LAU, TSC-2 were retarded to the same extent by both HM treatments compared to control treatment. PIN Maj, PTI-1, CFI-1, and HBR-1 colony growth was retarded more by L treatment than by K treatment, and out of these only PIN Maj exhibited the T1/2 in K treatment different from control. Only CGE-4 and CFI - 1 isolates displayed T1/2 decrease in L treatment compared to K and CFI -1 even compared to C - thus growing faster, but decrease in RCGmax in both strains in L treatment in fact decreased overall colony growth. When RCGmax was compared within each fungal strain, strains PTI-1, PIN-8, LDU, LLA3, HME-1, HLE-1, HFA -1 and ABY-1 showed no significant differences. Isolates AMU-1, CGE-4,HSE-1, TSC-1 and Tsp were significantly smaller only in L treatment compared to both K and C treatments. Isolates LAU-1, HBR-1and CFI were bigger in control compared to both HM treatments and finally PIN Maj, TCI-1 and TSC-2 showed significant differences in RCGmax in the following manner: C > K > L.

| Strain | Treatment | RCGmax [mm] | | | | | T1/2 [days] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mean | ± | SE | T | Strain | mean | ± | SE | T | Strain |
| AMU-1 | C | 10,8 | ± | 0,9 | a | c,d | 20,9 | ± | 1,6 | NS | h |
| AMU-1 | K | 10,9 | ± | 1,2 | a | | 19,9 | ± | 1,2 | | |
| AMU-1 | L | 2,3 | ± | 0,3 | b | | 19,4 | ± | 1,3 | | |
| ABY-1 | C | 6,4 | ± | 0,8 | NS | b | 12,3 | ± | 0,4 | NS | a |
| ABY-1 | K | 4,5 | ± | 0,7 | | | 14,4 | ± | 0,9 | | |
| ABY-1 | L | 2,6 | ± | 0,3 | | | 15,9 | ± | 0,5 | | |
| CGE-4 | C | 10,8 | ± | 0,8 | a | c,d | 19,4 | ± | 0,4 | a,b | f,g,h |
| CGE-4 | K | 11,4 | ± | 0,4 | a | | 21,3 | ± | 1,3 | a | |
| CGE-4 | L | 6,8 | ± | 0,5 | b | | 17,9 | ± | 0,7 | b | |
| CFI | C | 17,2 | ± | 0,7 | a | g | 20,4 | ± | 0,3 | a | g,h |
| CFI | K | 12,1 | ± | 1,7 | b | | 20,6 | ± | 1,7 | a | |
| CFI | L | 8,7 | ± | 0,4 | b | | 15,1 | ± | 0,7 | b | |
| HBR-1 | C | 11,0 | ± | 0,8 | a | c,d | 16,6 | ± | 0,8 | a,b | b,c,d,e |
| HBR-1 | K | 8,1 | ± | 0,2 | b | | 16,1 | ± | 0,9 | a | |
| HBR-1 | L | 7,1 | ± | 0,3 | b | | 19,8 | ± | 1,4 | b | |
| HFA-1 | C | 3,8 | ± | 0,5 | NS | a | 17,9 | ± | 1,2 | NS | d,e,f,g |
| HFA-1 | K | 3,3 | ± | 0,1 | | | 19,3 | ± | 1,1 | | |
| HFA-1 | L | N.D. | | | | | N.D. | | | | |
| HLE-1 | C | 6,3 | ± | 0,6 | NS | a,b | 19,3 | ± | 0,7 | NS | f,g,h |
| HLE-1 | K | 6,3 | ± | 1,2 | | | 19,1 | ± | 0,9 | | |
| HLE-1 | L | 3,9 | ± | 0,9 | | | 23,6 | ± | 0,9 | | |
| HME-1 | C | 5,5 | ± | 0,5 | NS | a,b | 15,7 | ± | 1,4 | NS | b,c,d |
| HME-1 | K | 5,4 | ± | 1,0 | | | 16,9 | ± | 0,3 | | |
| HME-1 | L | N.D. | | | | | N.D. | | | | |
| HSE-1 | C | 13,2 | ± | 1,2 | a | d,e,f | 15,7 | ± | 0,4 | NS | b,c,d |
| HSE-1 | K | 11,4 | ± | 0,9 | a | | 15,7 | ± | 0,5 | | |
| HSE-1 | L | 3,9 | ± | 0,1 | b | | 15,9 | ± | 0,5 | | |
| LLA-3 | C | 31,1 | ± | 0,8 | NS | i | 12,5 | ± | 0,4 | NS | a |
| LLA-3 | K | 30,9 | ± | 1,1 | | | 13,8 | ± | 0,9 | | |
| LLA-3 | L | 31,2 | ± | 0,9 | | | 14,3 | ± | 0,2 | | |
| LAU-1 | C | 13,7 | ± | 1,0 | a | e,f | 20,0 | ± | 0,3 | a | g,h |
| LAU-1 | K | 8,4 | ± | 1,0 | b | | 25,2 | ± | 1,5 | b | |
| LAU-1 | L | 7,5 | ± | 0,0 | b | | 25,0 | ± | 0,5 | b | |
| LDU-1 | C | 5,5 | ± | 0,4 | NS | a,b | 24,9 | ± | 2,6 | NS | i |
| LDU-1 | K | 6,3 | ± | 0,3 | | | 25,5 | ± | 1,3 | | |
| LDU-1 | L | N.D. | | | | | N.D. | | | | |
| Lsp | C | N.D. | | | | | N.D. | | | | |
| Lsp | K | N.D. | | | | | N.D. | | | | |
| Lsp | L | N.D. | | | | | N.D. | | | | |
| PIN-8 | C | 5,4 | ± | 0,5 | NS | a,b | 14,4 | ± | 1,5 | NS | a,b,c |
| PIN-8 | K | 3,7 | ± | 0,2 | | | 19,3 | ± | 4,0 | | |
| PIN-8 | L | 3,1 | ± | 0,1 | | | 21,5 | ± | 2,2 | | |
| PIN Maj | C | 30,5 | ± | 0,9 | a | i | 14,1 | ± | 0,5 | a | a,b |
| PIN Maj | K | 18,0 | ± | 0,3 | b | | 17,0 | ± | 0,5 | b | |
| PIN Maj | L | 10,5 | ± | 0,5 | c | | 22,6 | ± | 0,5 | c | |
| PTI-1 | C | 25,7 | ± | 2,4 | NS | h | 17,2 | ± | 1,2 | a | d,e,f |
| PTI-1 | K | 23,4 | ± | 1,0 | | | 14,6 | ± | 0,4 | a | |
| PTI-1 | L | 21,2 | ± | 1,0 | | | 22,9 | ± | 0,6 | b | |
| TCI-1 | C | 12,6 | ± | 0,4 | a | d,e | 18,9 | ± | 0,4 | NS | e,f,g,h |
| TCI-1 | K | 8,4 | ± | 1,5 | b | | 20,3 | ± | 2,0 | | |
| TCI-1 | L | 4,9 | ± | 0,4 | c | | 17,3 | ± | 0,9 | | |
| TSC-1 | C | 12,0 | ± | 0,4 | a | c,d,e | 16,9 | ± | 0,2 | NS | c,d,e,f |
| TSC-1 | K | 11,9 | ± | 0,2 | a | | 17,3 | ± | 0,2 | | |
| TSC-1 | L | 5,6 | ± | 0,2 | b | | 17,1 | ± | 0,3 | | |
| TSC-2 | C | 15,5 | ± | 0,2 | a | f,g | 16,3 | ± | 0,1 | a | b,c,d |
| TSC-2 | K | 11,8 | ± | 0,2 | b | | 18,1 | ± | 0,8 | b | |
| TSC-2 | L | 4,9 | ± | 0,6 | c | | 19,3 | ± | 0,3 | b | |
| Tsp | C | 10,0 | ± | 0,1 | a | c | 19,2 | ± | 1,1 | NS | f,g,h |
| Tsp | K | 10,4 | ± | 0,3 | a | | 21,4 | ± | 1,3 | | |
| Tsp | L | 2,6 | ± | 0,4 | b | | 18,7 | ± | 0,5 | | |

*Table 8.1. Growth parameters of selected ectomycorrhizal strains. Explanation of fungal strains abbreviations and treatments is given in Materials and Methods. Parameters (derived from Weibull growth curves): RCGmax, maximal radial colony growth; T1/2 time at RCGmax/2 (data are expressed as mean ± standard error (n=3)); N.D.- not determined; Statistics: RCGmax and T1/2 were assessed separately. Column "T" - significance of the treatment effect within given strain; column "Strain" - significance of strain effect on parametres from control treatment. Different letters denote statistical difference at $p<0.05$. NS not significant.*

| Strain | RCGmax%C ± SE | Statistics |
|---|---|---|
| LLA-3 | 100,32 ± 2,93 | a |
| PTI-1 | 82,62 ± 3,97 | b |
| HBR-1 | 63,75 ± 2,42 | c |
| CGE-4 | 63,16 ± 4,67 | c |
| HLE-1 | 63,03 ± 15,16 | c |
| PIN-8 | 57,06 ± 1,84 | c,d |
| LAU-1 | 55,11 ± 0,36 | c,d |
| CFI | 50,29 ± 2,23 | d,e |
| TSC-1 | 46,11 ± 1,55 | d,e,f |
| ABY-1 | 41,15 ± 5,29 | e,f,g |
| TCI-1 | 38,62 ± 2,95 | f,g,h |
| PIN Maj | 34,24 ± 1,61 | g,h,i |
| TSC-2 | 31,25 ± 3,59 | g,h,i,j |
| HSE-1 | 29,47 ± 0,76 | h,i,j |
| Tsp | 26,33 ± 4,1 | i,j |
| AMU-1 | 21,74 ± 2,96 | j |
| HME-1 | ND | ND |
| HFA-1 | ND | ND |
| LDU-1 | ND | ND |
| Lsp | ND | ND |

| Strain | T1/2%C ± SE | Statistics |
|---|---|---|
| CFI | 73,78 ± 3,47 | a |
| TCI-1 | 91,53 ± 5 | b |
| AMU-1 | 92,52 ± 6,3 | b |
| CGE-4 | 92,76 ± 3,61 | b |
| Tsp | 97,05 ± 2,44 | b |
| TSC-1 | 100,79 ± 1,72 | b,c |
| HSE-1 | 101,28 ± 3,32 | b,c |
| LLA-3 | 114,1 ± 2,01 | c,d |
| TSC-2 | 118,65 ± 2,13 | d,e |
| HBR-1 | 119,24 ± 8,36 | d,e |
| HLE-1 | 122,23 ± 4,41 | d,e,f |
| LAU-1 | 124,96 ± 2,25 | d,e,f |
| ABY-1 | 128,92 ± 4,16 | e,f |
| PTI-1 | 133,08 ± 3,58 | f |
| PIN-8 | 149,31 ± 14,9 | g |
| PIN Maj | 159,81 ± 3,78 | g |
| HME-1 | ND | ND |
| HFA-1 | ND | ND |
| LDU-1 | ND | ND |
| Lsp | ND | ND |

*Table 8.2. Tolerance of fungal strains within treatment L described by RCGmax%C and T1/2%C (RCGmax and T1/2 relative to parameters of control treatment respectively). Explanation of fungal strains abbreviations and treatments is given in Materials and Methods. Parameters (derived from Weibull growth curves): RCGmax, maximal radial colony growth; T1/2 time at RCGmax/2 (data are expressed as mean ± standard error (n=3)); N.D.- not determined. Fungal isolates are sorted from the most tolerant to the least tolerant isolate in given parameter.*

### 8.1.1.2. HMs uptake

Examined fungal isolates significantly differed in the ability to accumulate HMs in their tissues. Results showing accumulation of individual HMs by strains of given treatment are shown in figures 8.2.-8.7.

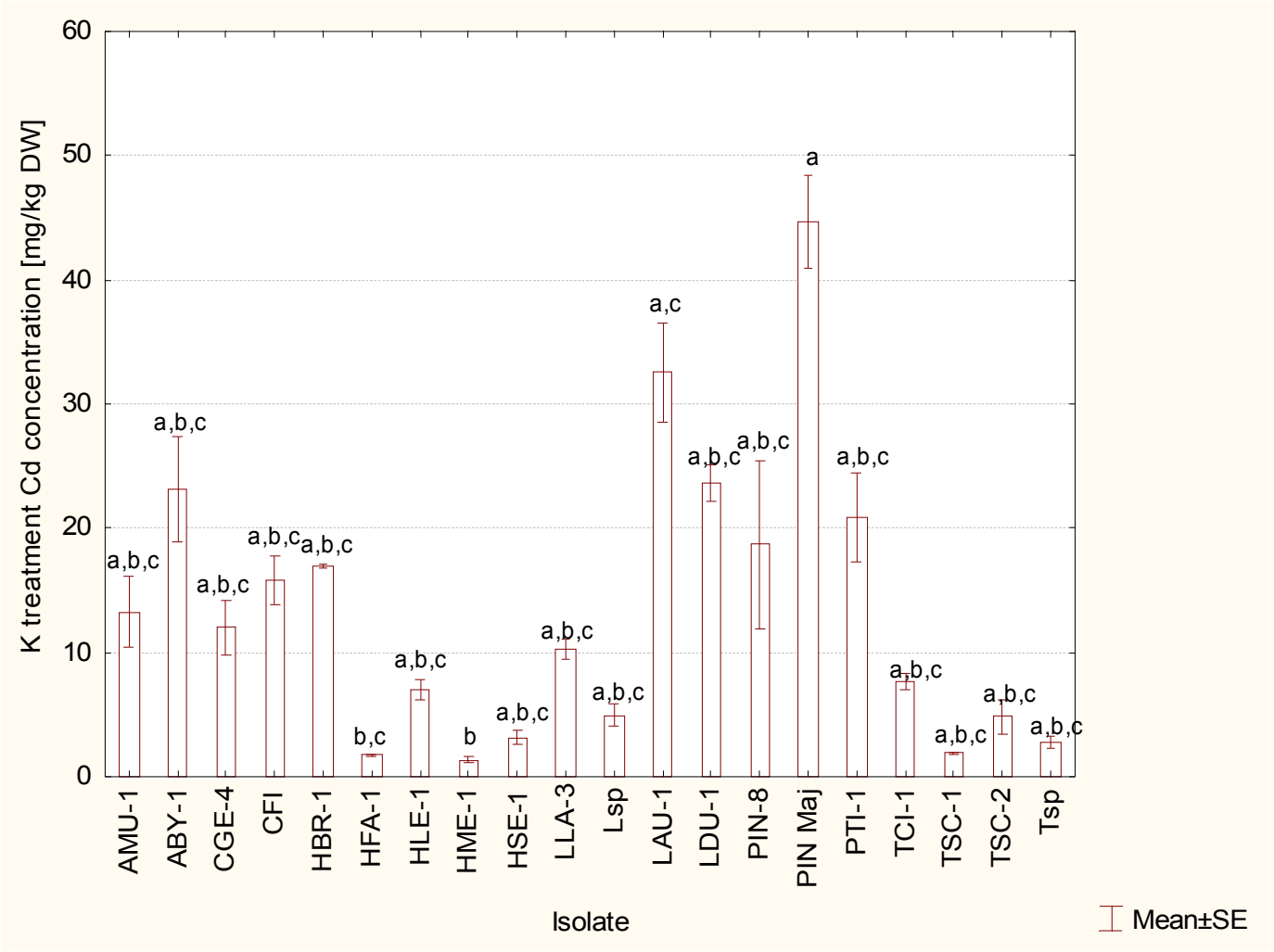


*Table 8.2.Accumulation of Cd [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment K. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at $p<0.05$.*

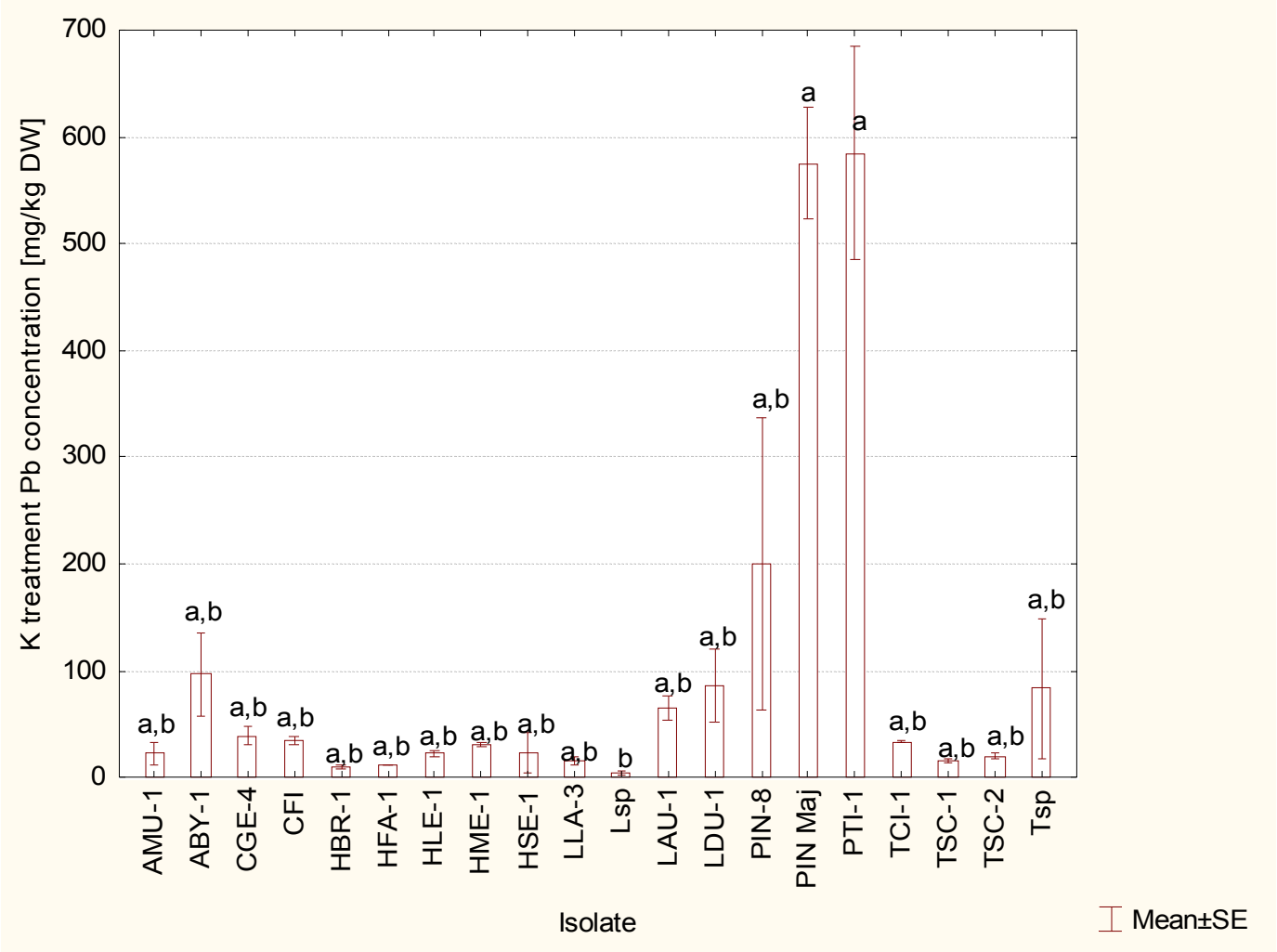


*Table 8.3.Accumulation of Pb [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment K. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at $p<0.05$.*

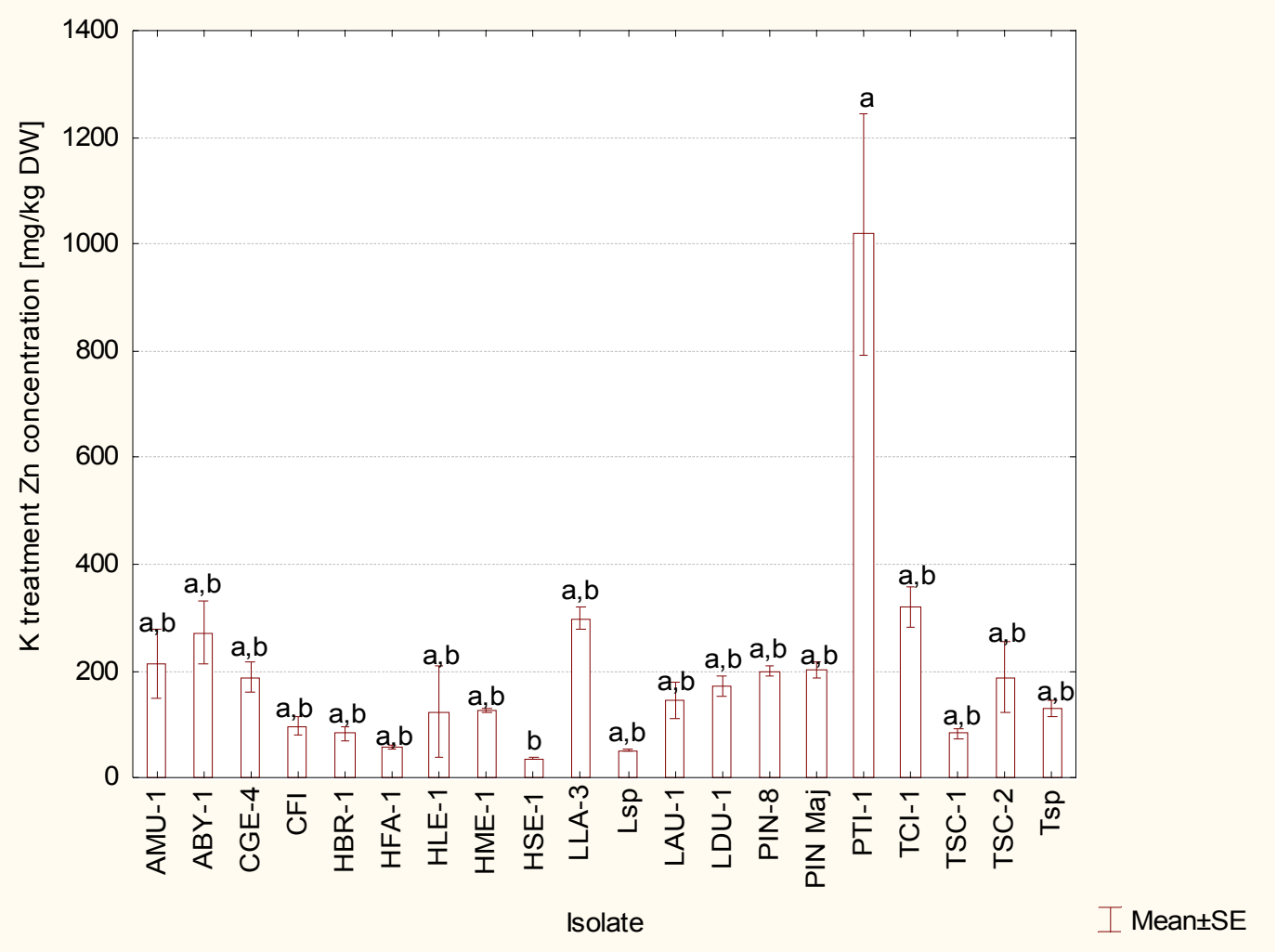


*Table 8.4.Accumulation of Zn [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment K. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at $p<0.05$.*

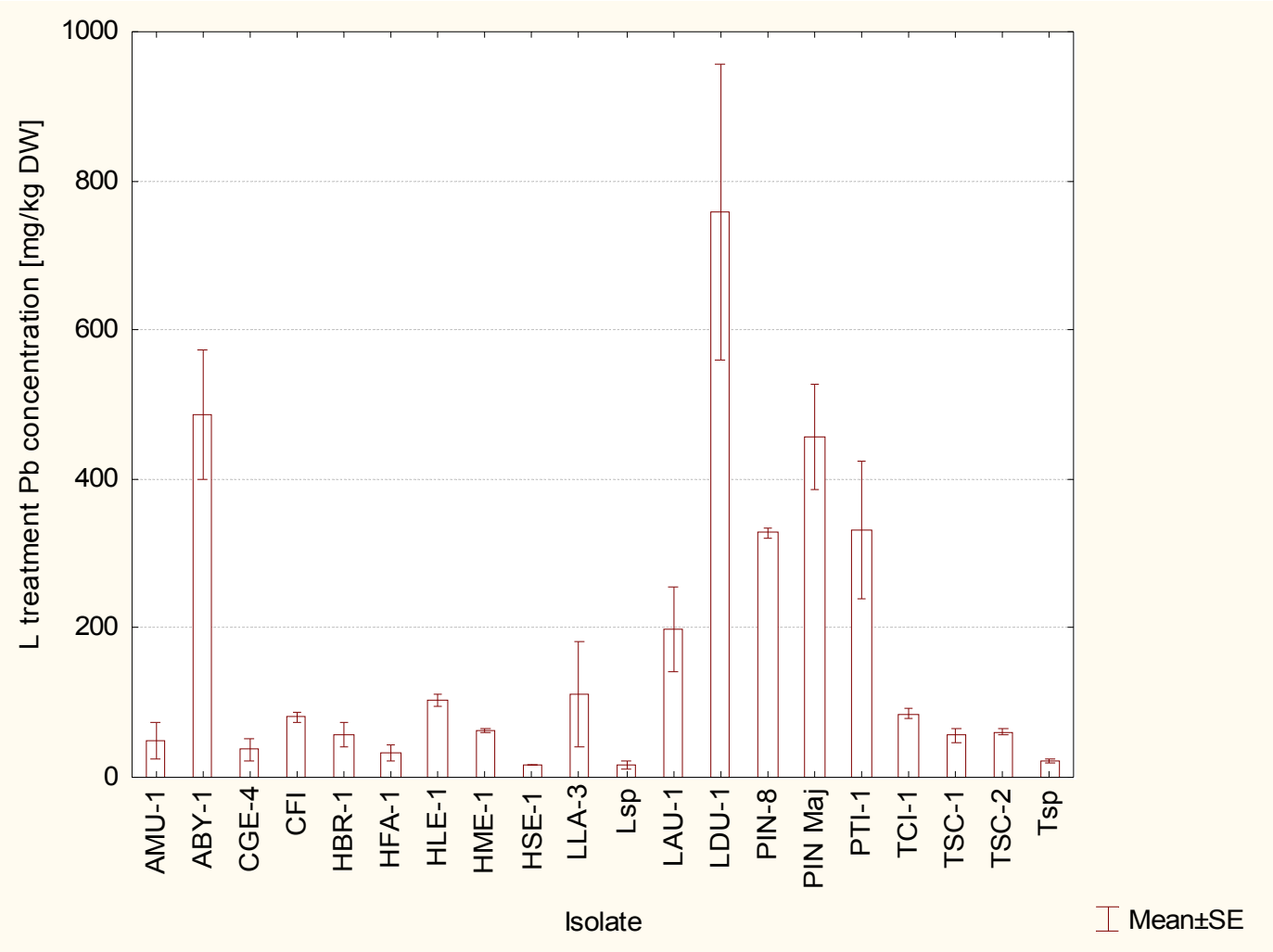


*Table 8.5.Accumulation of Pb [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment L. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). No significant differences were found at p<0.05.*

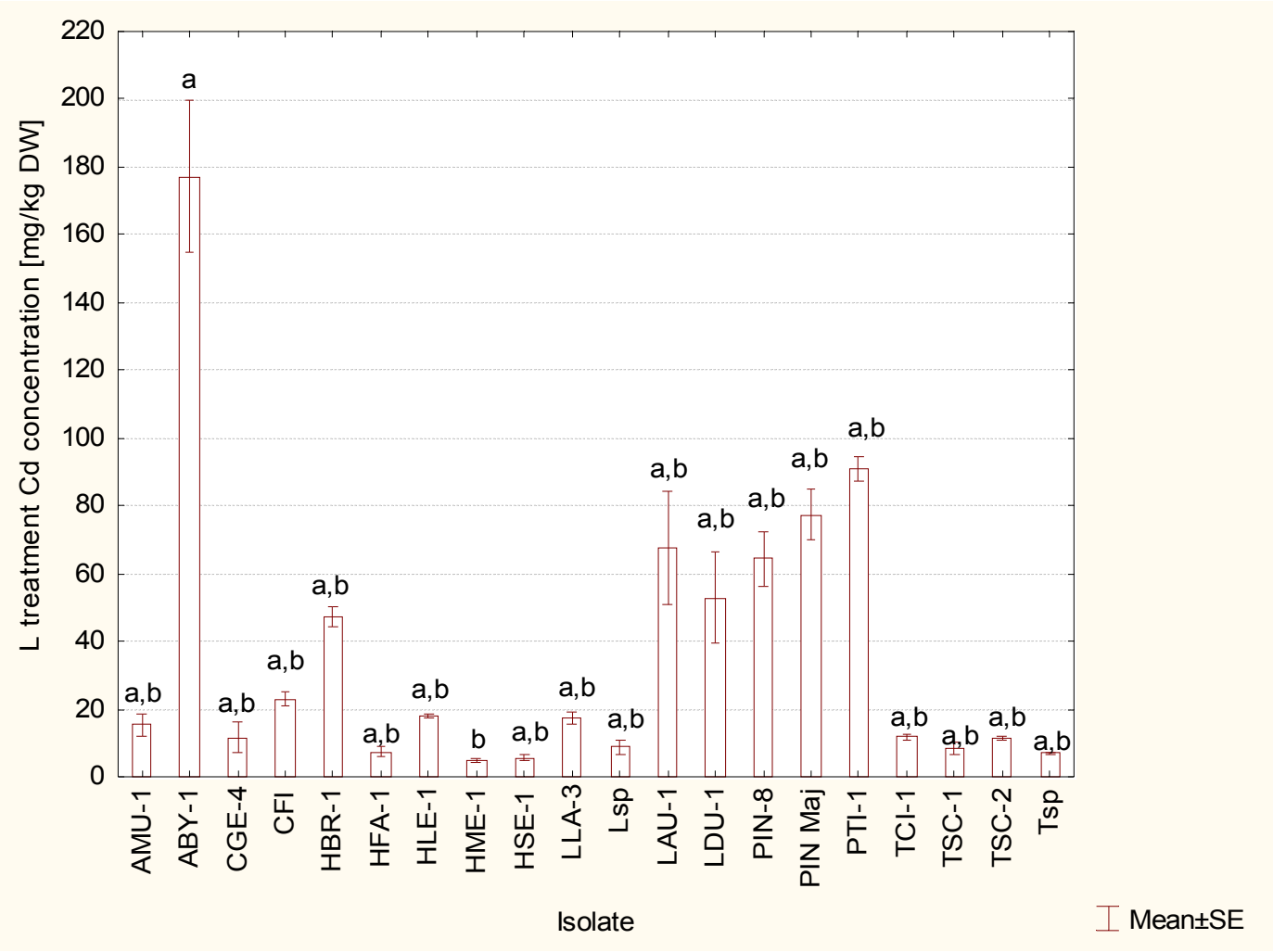


*Table 8.6.Accumulation of Cd [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment L. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at $p<0.05$.*

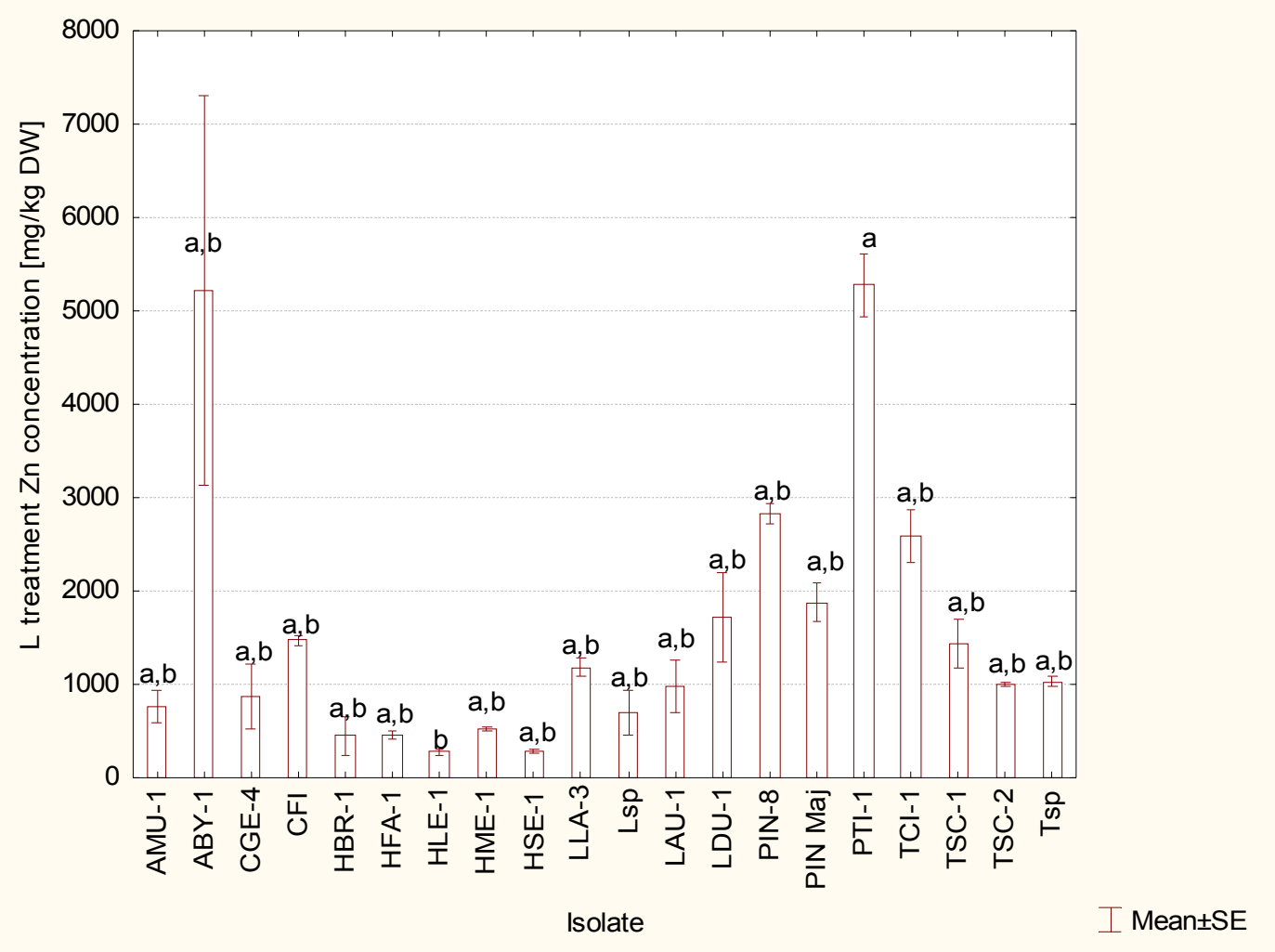


*Table 8.7.Accumulation of Zn [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment L. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at $p<0.05$.*

### 8.1.2. Experiment INVIT Part B

#### 8.1.2.1. Colony growth

Colony growth of the four chosen ECM strains was assessed and is depicted in figure 8.8. The fungi were let to grow untill reasonable amount of mycelia was achieved for use in further analyses or the mycelium covered the petri dish. All strains were retarded in growth on media amended with individual HMs (Cd, Pb, Zn), except for PIN Maj on Zn treatment, PTI-1 on Cd treatment and both mentioned isolates on Pb treatment. In the case of Pb treatment the concentration of Pb corresponded to the highest concentration that could be achieved in the used agar medium. Use of higher concentration of Pb would lead to Pb salt precipitation.

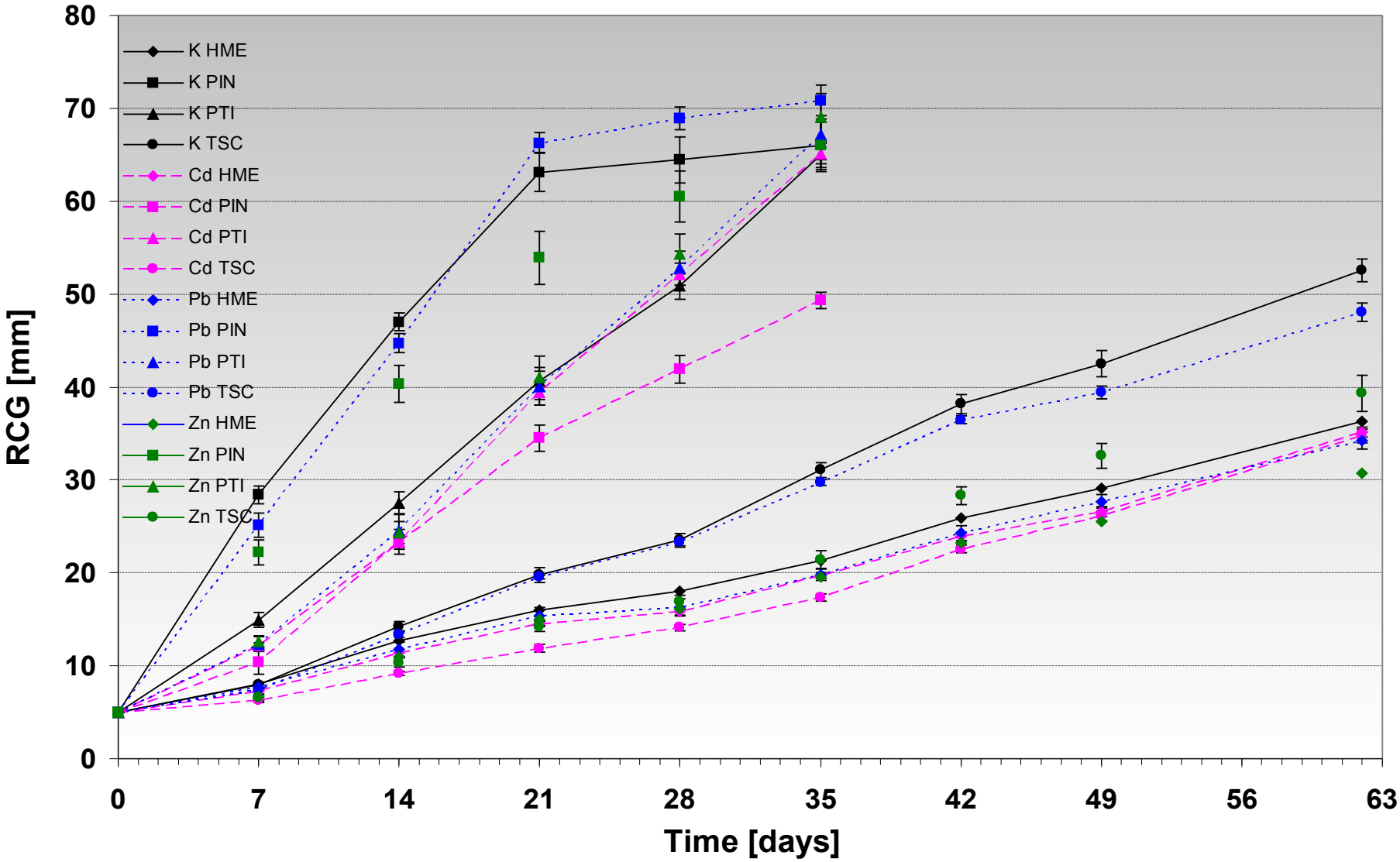


*Fig. 8.8. Growth of chosen ectomycorrhizal strains on agar media amended with Cd or Pb or Zn. Control group (K) was left free of HMs. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=5).*

### 8.1.2.2. HMs uptake

Examined fungal isolates significantly differed in the ability to accumulate HMs in their tissues. Results showing accumulation of individual HMs by strains of given treatment are shown in figures 8.9-8.11.

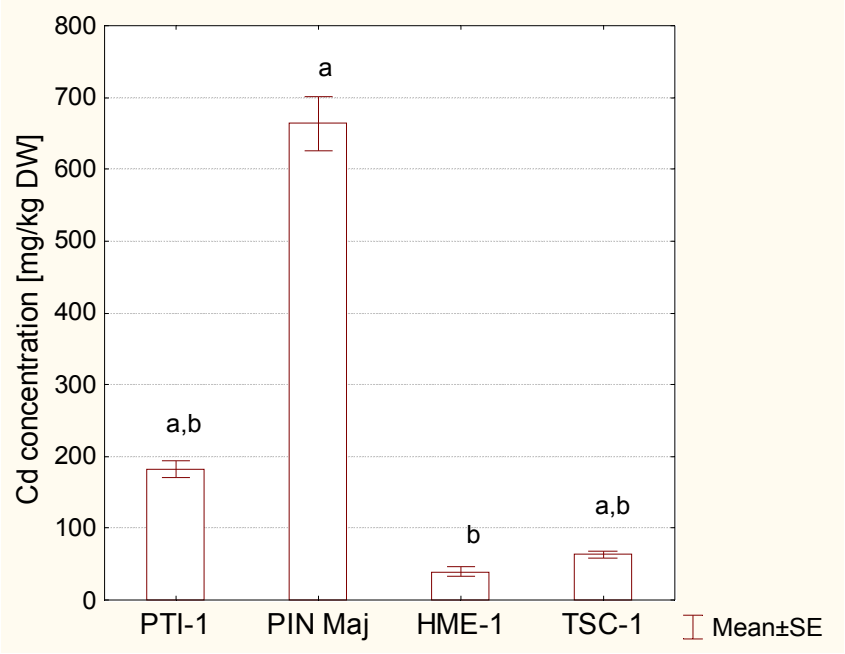


*Table 8.9.Accumulation of Cd [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment Cd. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=4). Different letters denote statistical difference at p<0.05.*

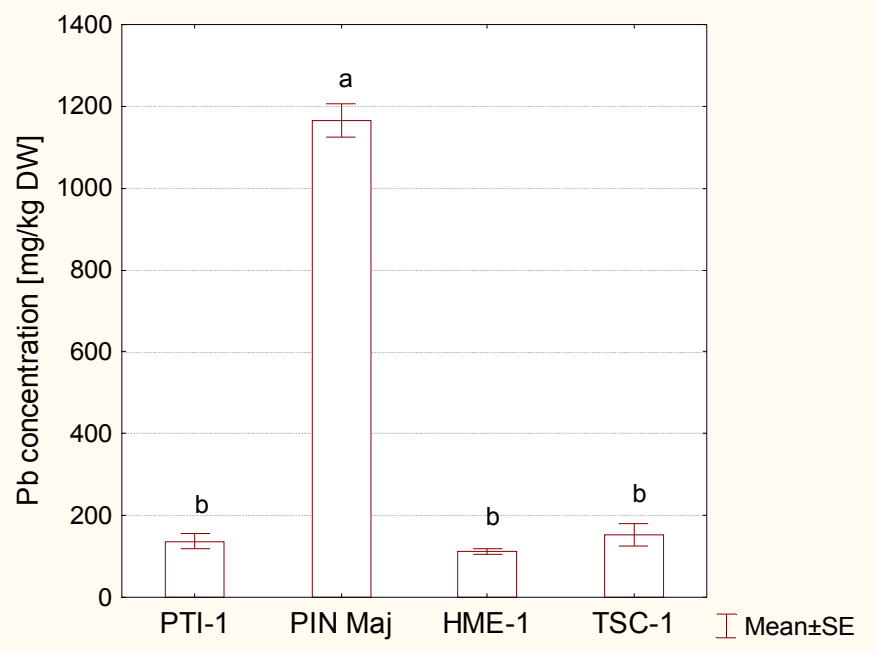


*Table 8.10.Accumulation of Pb [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment Pb. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=4). Different letters denote statistical difference at p<0.05.*

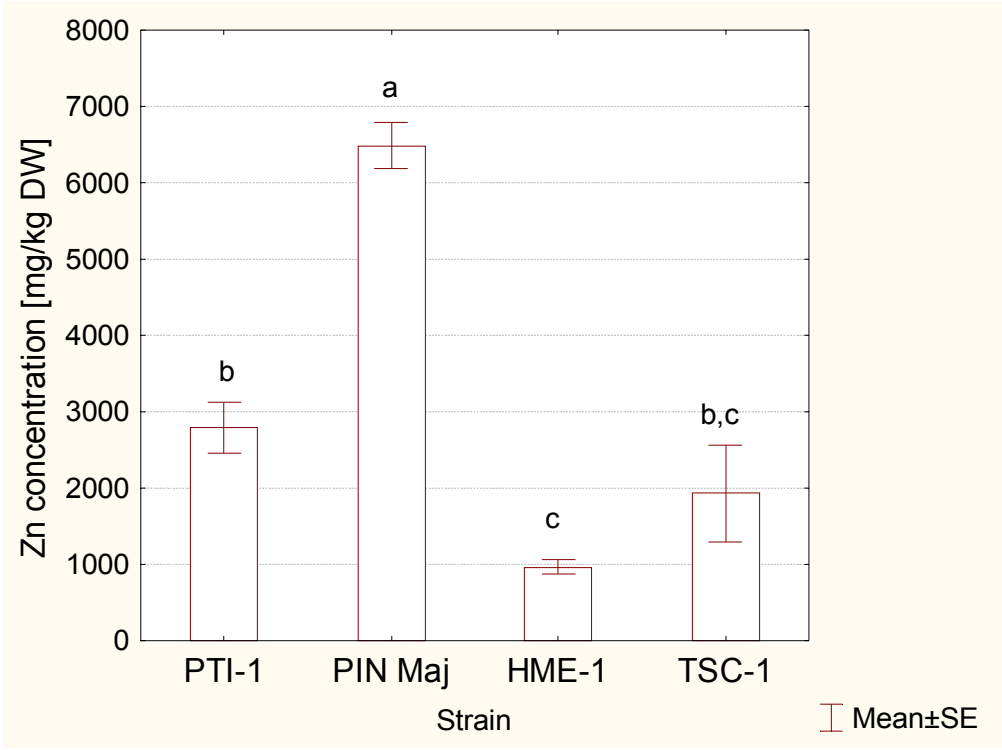


*Table 8.11.Accumulation of Zn [mg/kg dry weight] in mycelium of ectomycorrhizal strains in treatment Zn. Explanation of fungal strains abbreviations and HM amendment of agar media is given in Materials and Methods. Data are expressed as means ± SEM (n=4). Different letters denote statistical difference at p<0.05.*

### 8.1.2.3. SOD activity

SOD activity was evaluated as number of SOD units per mg of fresh weight of mycelium. Chosen isolates differed significantly in SOD activity when grown on control medium (figure 8.12.). Logarithmically transformed data were used to assure normal distribution. Differences between treatments were evaluated for each strain separately. Only PIN Maj and TSC-1 showed significant differences (table 8.3.).

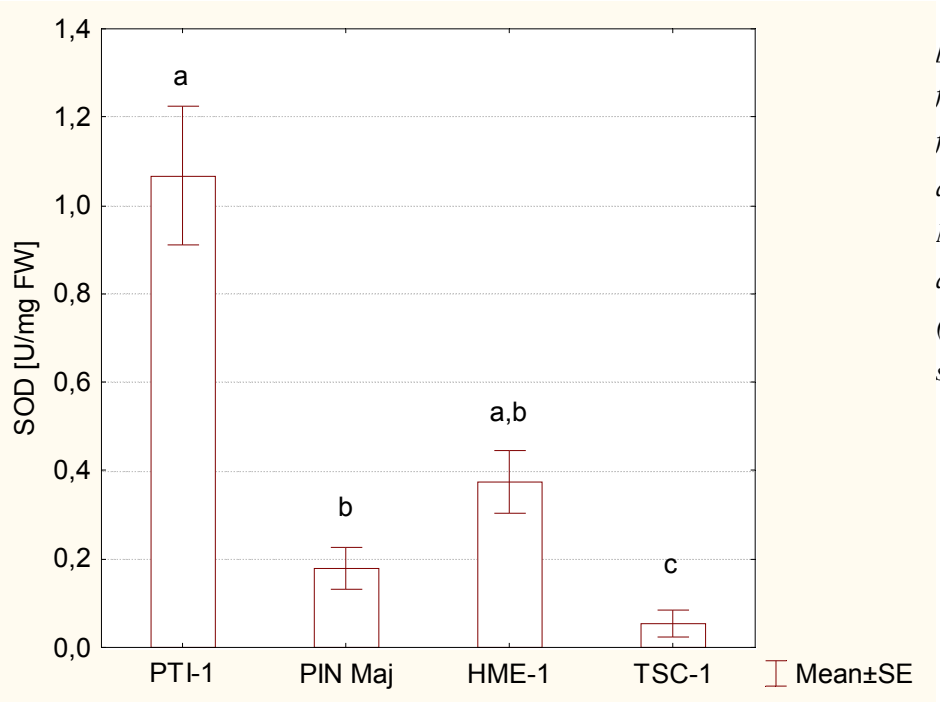


*Figure 8.12. SOD activity [U/mg fresh weight] in selected fungal strains. Explanation of fungal strains' abbreviations and treatments is given in Materials and Methods. Data are expressed as means ± SEM (n=4). Different letters denote statistical difference at p<0.05.*

| Isolate | Treatment | N | SOD [U/mg FW] Mean ± Std.Err. | | | statistics |
|---|---|---|---|---|---|---|
| PTI-1 | C | 4 | 1,07 | ± | 0,16 | NS |
| | Cd | 4 | 2,25 | ± | 1,37 | |
| | Pb | 4 | 1,35 | ± | 0,56 | |
| | Zn | 5 | 0,72 | ± | 0,11 | |
| PIN Maj | C | 4 | 0,18 | ± | 0,05 | a,b |
| | Cd | 4 | 0,25 | ± | 0,02 | a |
| | Pb | 4 | 0,10 | ± | 0,05 | b |
| | Zn | 5 | 0,12 | ± | 0,02 | b |
| HME-1 | C | 4 | 0,37 | ± | 0,07 | NS |
| | Cd | 4 | 0,31 | ± | 0,04 | |
| | Pb | 4 | 0,40 | ± | 0,01 | |
| | Zn | 4 | 0,33 | ± | 0,04 | |
| TSC-1 | C | 4 | 0,05 | ± | 0,03 | b |
| | Cd | 4 | 0,26 | ± | 0,17 | a |
| | Pb | 4 | 0,01 | ± | 0,01 | b |
| | Zn | 4 | 0,05 | ± | 0,02 | a,b |

*Table 8.3. Difference of SOD activity [U/mg FW] between treatments within fungal strains. Explanation of fungal strains' abbreviations and treatments is given in Materials and Methods. Data are expressed as means ± SEM (n=3). Different letters denote statistical difference at p<0.05. NS -not significant.*

## 8.2. DUAL

### 8.2.1. Mortality and mycorrhizal colonisation

The experimental design consisted of 10 repeat for each treatment,t to prevent experimental failure in case of higher mortality due to presence of HMs and other factors. Total mortality reached 17,5% and 22,5% in the end of expermient for willows and poplar, respectively. Moreover, only plants successfully inoculated by the given fungal strains and free from contamination were analysed. Overview of the available plants per treatment is summarized in table 8.4.

| | willows | | | | poplars | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | mortality | pots contaminated | unsuccessful colonisation | pots left for analyses | mortality | pots contaminated | unsuccessful colonisation | pots left for analyses |
| C | 3 | 3 | 0 | 4 | 1 | 0 | 0 | 9 |
| AE | 0 | 1 | 6 | 3 | 2 | 0 | 0 | 8 |
| AM | 0 | 0 | 8 | 2 | 3 | 0 | 0 | 7 |
| ECM | 2 | 0 | 5 | 3 | 2 | 3 | 1 | 4 |
| GCL | 3 | 6 | 1 | 0 | 4 | 6 | 0 | 0 |
| GIN | 2 | 0 | 7 | 1 | 0 | 1 | 0 | 9 |
| HME | 0 | 1 | 1 | 8 | 3 | 0 | 5 | 2 |
| PIN | 4 | 4 | 2 | 0 | 3 | 4 | 1 | 2 |

*Table 8.4. Mortality, contaminated pots, cases of unsuccessful colonisation and pots left for analyses given in number of replicates for willows and poplars and for all experimental treatments respectively (C-control, GCL - G. claroideum inoculation, GIN - G. intraradices inoculation, HME - H. mesophaeum inoculation and PIN - P. involutus inoculation AM - arbuscular (both GIN+GCL) inoculation, ECM - ectomycorrhizal (both PIN+HME) inoculation, AE - mixed AM and ECM inoculation).*

Treatments with less than 3 replicates were omitted from further analyses. The number of treatments suitable for analyses was reduced within willows to C, ECM, HME and AE treatments. Treatments left for analyses within poplars include C, AE, AM, ECM and GIN. All following results will be presented separately for willows and poplars.

Microscopic analysis of willow roots revealed presence of two introduced ECM morphotypes as well as presence of arbuscular fungi, differing among treatments. AM colonisation was found in all treatments except GCL and AE treatment. In C, ECM, HME and PIN treatment AM colonisation was considered contamination, therefore pots within these treatments, infected by AM were excluded from further analyses. As for the ECM colonisation, only *H. mesophaeum* represented by morphotype H (figure 8.14.) was succesfully introduced in HME, ECM and AE treatments while inoculation by *P. involutus* failed. Colonisation of willows by *H. mesophaeum* ranged from 20% to almost 70% being significantly lower in AE treatment compared to HME treatment (figure 8.13.). Apart from *H. mesophaeum* one other morphotype (morphotype 12 shown in figure 8.15) was distinguished. It occurred in treatments PIN, AE and massively contaminated almost whole GCL treatment.

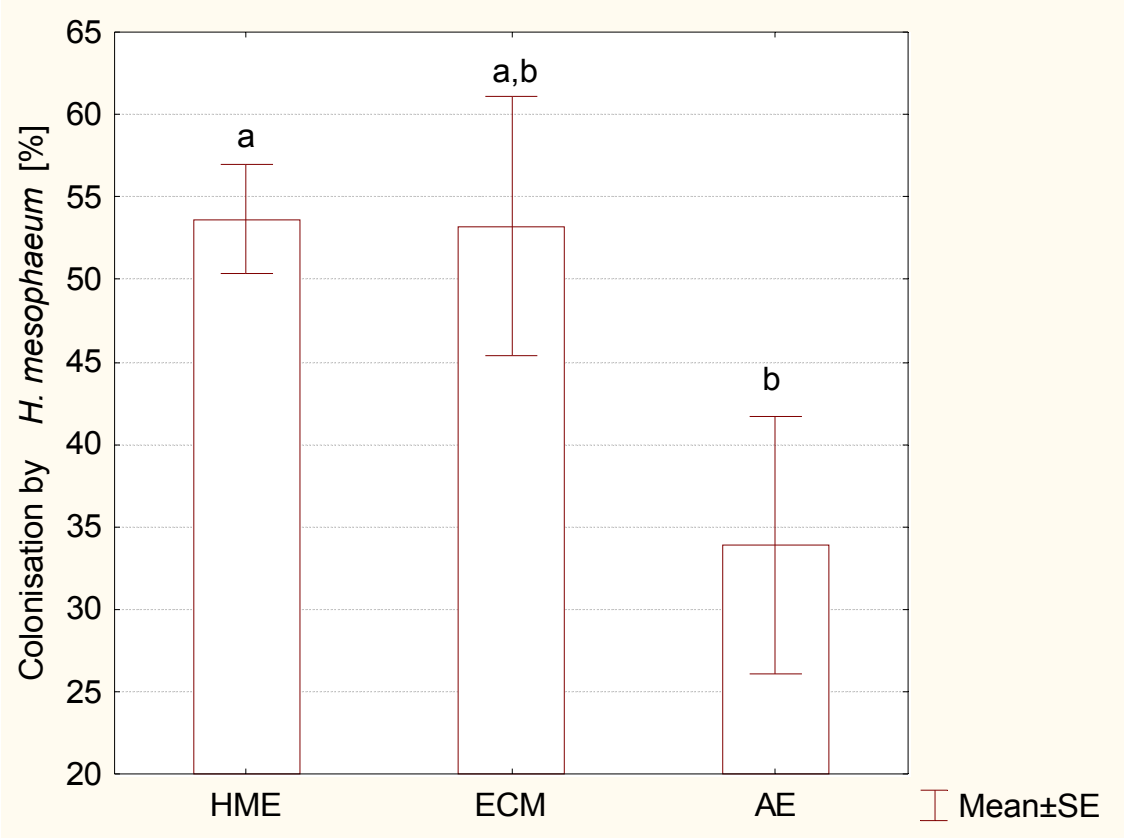


*Figure 8.13. Colonisation of willows by H. mesophaeum. Explanation of treatments abbreviations is given in Materials and Methods. Control treatment not included in analysis. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$.*

Microscopic analysis of poplar roots revealed presence of several ECM morphotypes as well as presence of arbuscular fungi, differing among treatments. AM colonisation was found in all treatments which were inoculated by arbuscular fungi. There was one additional case of AM contamination in ECM treatment. As for the ECM colonisation, both *H. mesophaeum* represented by morphotype H and *P. involutus* represented by morphotype P (figure 8.16.) were introduced in ECM and AE treatments and PIN treatment respectively. However inoculation by *P. involutus* failed due to only 2 valid replicates in PIN variant. Colonisation of poplars by *H. mesophaeum* ranged from less than 2% to almost 12% showing no significant difference between treatments. Colonisation by AM fungi ranged from less than 55% to 93% also showing no significant difference between treatments. Apart from *H. mesophaeum* and *P. involutus* 2 other distinct morphotype were distinguished. These were found in PIN treatment (morphotype 2 shown in figure 8.17.) and both GCL, GIN and ECM treatment (morphotype 1 shown in figure 8.18.). Figure 8.19. shows cross section of mycorrhizal root tip (morphotype H) obtained from poplar HME treatment, as observed under microscope.


*Figure 8.14. Morphotype H representing H. mesophaeum, Morphotype is characterised by bright brown coloured mycorrhizal root tip, which is usually short, not branching with dense extraradical hyphae emanating from it, often capturing small soil particles.*


*Figure 8.15. Morphotype 12 - bright beige not fully developed, but easy to distinguish compared to non-mycorrhizal root with dense root hair network.*


*Figure 8.16. Morphotype P attributed to P. involutus - deep brown colour, with dense extra-radical mycelium emanating from rather short and swollen mycorrhizal root tip.*


*Figure 8.17. Morphotype 2, characterized by beige colour smooth fungal sheath with rarely emanating short hypahe.*


*Figure 8.18. Morphotype 1- rather swollen brownish morphotype, of intrmediate length, extraradical hyphae emanate to some extent. Furthermore, adjacent to branching site of the root, AM fungal spore is present. It is most probably of G. claroideum origin.*

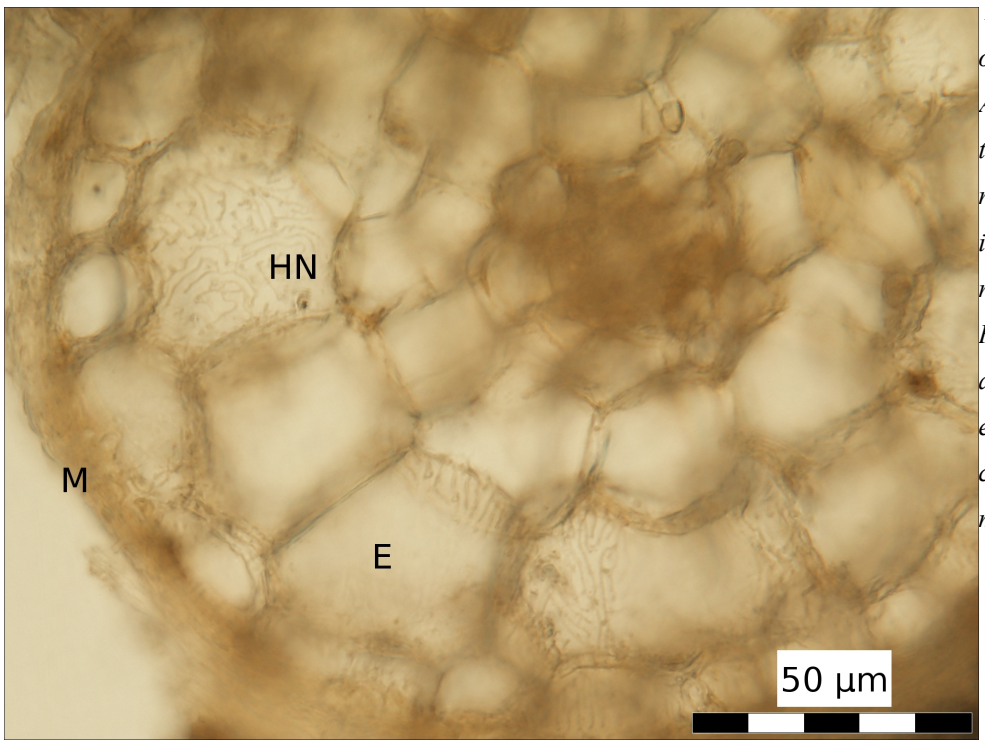


*Figure 8.19.Cross section of mycorrhizal root tip. Assigned to morphotype H, this mycorrhiza probably represents H.mesophaeum in association with poplar root, in HME treatment. Hartig net hypheae (HN) are visible between epidermal cells (E), covered by not hyphal mantle.*

### 8.2.1.1. Molecular identification of the ECM strains

In order to confirm the identity of morphotyped EcM fungi, DNA was isolated from the root tips of distinguished morphotypes and used for subsequent PCR-based analysis of internal transcribed regions of ribosomal DNA cassete. The obtained sequences were compared to the sequences collected in Genbank (NCBI, megablast search). The results are summarized in the table 8.5. Most of the identified fungi belong to ascomycetous endophytes that are commonly isolated together with mycorrhiza-forming basidiomycetes. Peziza species are considered to have saprotrophic lifestyle and the occurence of Peziza sp. in morphotype 2 suggests that already decaying root tip was used in this case. Apart from morphotype P found in PIN treatment in poplars, representing P. involutus no other morphotype was characterised molecularly. In summary, these results suggest that for identification of mycorrhiza-forming basidiomycetous fungi use of specific basidiomycetous primers (ITS1F-ITS4b) is advanteagous. Unfortunately I have not managed to run such analysis until the termination of the Thesis.

| **Treat-ment** | **Morpho-type** | **Sequence** | **Identity (most similar sequences – Accesion number)** |
|---|---|---|---|
| PIN poplar | 2b | TGATTTGAGGTTACCCGATTTAGCAATGGGTGG-GTTCAGGCAAGTATAAATATGGTAAACTCAGCGACTTT ATTATTATTACGCCTGGTTCAGATATAAACTATACTGCC ATATACATTTCTGAAGGGTACCTCCATGATAGAGGTGC CTCTCCAAACAAATCCAGGCATTACAAAATAATGCTTG AGAATATGGGGAGCTAACGCTCAAACAGGTATGCCCTA TGGAATACCATAGGGCGCAATGTGCGTTCAAAGATTCA ATGATTCACGAGATTCTGCAATTCACATTACTTATCGCA TTTCACTGCGTTCTTCATCGATGCAAGAGCCTAGAGAT CCGTTGTTGAAAGTTTTATTTCATTTTCAAGACACAAA ATTCAGACTATATATTTCACAAACAGGTTTTAGCAAGT GGTCTTGCTACTGGCAGCTCTCTGGAAAGTGTGGTTC ACACAAGCCTGAGGTTTTGCCTAGAGGTTTATAAATCT TACCTCCAAGCAAAACAGTCCAGTAGAAGCAACAGG GTAGATAAAACAATGGGATATAAAAAACTAACATAGTT TTTCAATAATGATCCTTCCGCAGGTCACCCTACGGAAG | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Pezizomycetes; Pezizales; Pezizaceae; ***Peziza sp.***<br><br>(GU990362.1, GU969261.1, GQ267495.1, EU819461.1 |
| ECM willow | Hc | CTGATCCGAGGTCACCTTAGAAATGGGGT-TGTTTTACGGCGTAGCCTCCCGAACACCCTTTAGCGAA TAGTTTCCACAACGCTTAGGGGACAGAAGACCCAGCC GGACGATTTGAGGCACGCGGCGGACCGCGTTGCCCAA TACCAAGCGAGGCTTGAGTGGTGAAATGACGCTCGAA CAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTG CGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGC CAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTAT TAATTAAGTTTACTCAGACTGCAAAGTTACGCAAGAGT TTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGG GTCGCCCCGGAGGCAACAGAGTCGGACAACAAAGGG TTATGAATAACCAGGCCGAAGCCCGGGCGTTCTTGTAA TGATCCCTCCGCAGGTTCACCCTACGGAA | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Leotiomyceta; Dothideomyceta; Dothideomycetes Dothideomycetidae; Capnodiales; **unresolved**<br><br>(AF222833.1, AF222830.1, EU622926.1, AJ300333.1) |
| GCL willow | 12b | CTGATCCGAGGTCACCATGATTGTTTGTGGGGATTGT-GGGCGGGACGATCCAGGGCCACGACGAGAGATTGCTC TTACTACGTCGGTGTCCACATGGACCATCCGCCATTGC ATTTCGGAGGGGAACCCACCCAAAGGGCACGCTCTTC CTCCAAGTCCAAGCAAACATGCTTGAGTCGGTTTTAAT GACGCTCGAACAGGCATGCCTCCCGGAATGCCAGGAG GCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAAT TCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCT TCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAG TTTTAAGTTTTCTCATTGAGACAATTCAGACAGATATCA TTGAAAGCAATGGTTTGATGTAGACCTCCCGTGGGTTC CCCACGTTCGGGGGGTGTGGCCTTGGCCAACCACCCA CGGAAGCAACAGGTAAAATACACAGATGGGTGTCATA ATACGCGCTGGGAGCACTTATCACTTTTAATGATCCTTC CGCAGGTTCACCCTACGGAAG | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Leotiomyceta; Lecanoromycetes; Lecanoromycetidae; Lecanorales; **unresolved**<br><br>(GQ240937.1, FJ626917.1, EF484935.1, GQ205368.1) |

| Treat-ment | Morpho-type | Sequence | Identity (most similar sequences – Accesion number) |
|---|---|---|---|
| ECM poplar | 1a | ACTGATCCGAGGTCACCATGATTGTGTGGTGGGTTTGT GGGAGGGTCGATCCCTGGCCACGAGCATAGAGTGCTC TTACTACTTCTGTGTCCACATGGACCATCCCCCATTGGA TTTCGGACGGGAACCCTCCCAAGAGCCACGCTCTTCC TCCCAGTCCTGGTCCCTTGCTTGACTCCGTTTTAGTGA CGCTCAAACGTGGATGACTCCCTGAATGCCAGGAGAC GCAATGCGTCGAAAAACTTTCGATGACACACTGCATT CTGCAATTCACTGAACTTATCACATTTGCCTGACTTCTT CAACGATGCGAGAACCTGCATTTTTTATTGTATGAAAG TTTTCAATATTCTCATTGCTACAATTCATATAGATATGCT TGACAGCAATGGTTTGATGTAGACCTCCTGTGGGTTCC CCAAGTTCAGGGGGTGTGGCCTTGTTCAACATCCCCG GGAAAAAACACAGTGCTTTACTCGTATGGATGTCACA ATACGCGCTGGTAGCACTTATCACTTTTAATGATCCTTC CGCACGCTCAACCTACGGAACAACCCAGTCAAAACTC GATAATGATCCTTCCGCAGGTTCACCCTACGGAAG | Fungi; Dikarya; Ascomycota; **unresolved**<br><br>(GQ205368.1, EF484935.1, GU553372.1) |
| ECM poplar | Ha | ACTGACCCGAGGTCAACCTTAGAAATGGGGTTGTTTT ACGGCGTAGCCTCCCGAACACCCTTTAGCGAATAGTTT CCACAACGCTTAGGGGACAGAAGACCCAGCCGGACG ATTTGAGGCACGCGGCGGACCGCGTTGCCCAATACCG AGCGAGGCTTGAGTGGTGAAATGACGCTCGAACAGG CATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTT CAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGA ACCAAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATT AAGTTTACTCAGACTGCAAAGTTACGCAAGAGTTTGA AGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGGGTC GCCCCGGAGGCAACAGAGTCGGACAACAAAGGGTTA TGAATAACCAGGCCGAAGCCCGGGCGTTCTTGTAATG ATCCCTCCGCAGGTTCAACCTACGGAAACGGA | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Leotiomyceta; Dothideomyceta; Dothideomycetes Dothideomycetidae; Capnodiales; **unresolved**<br><br>(AY625062.1, AJ300333.1, AF393704.2) |
| PIN poplar | 2a | GGGGGGGGACAGGCAAGTATAAATATGGTAAACTCAG CGACTATAAATTATTACGCATGGTTCAGATATAAACTAT ACTGCCATATACATTTCTGAAGGGTACCTCCATGATAG AGGTGCCTCTCCAAACAAATCCGGGCATTACAAAATAA TGCTTGAGAATATGGGGAGCTAACGCTCAAACAGGTA TGCCCTATGGAATACCATAGGGCGCAATGTGCGTTCAA AGATTCAATGATTCACGAGATTCTGCAATTCACATTAC TTATCGCATTTCACTGCGTTCTTCATCGATGCATGAGCC TAGAGATCCGTTGTTGAAAGTTTTATTTCATTTTCAAG ACACAAAATTCAGACTATATATTTCACAAACAGGTTTT AGCAAGTGGTCTTGCTACTGGCAGCTCTCTGGAAAGT GTGGTTCACACAAGCCTGAGGTTTTGCCTAGAGGTTTA TAAATCTTACCTCCAAGCAAAACAGTCCAGTAGAAGC AACAGGGTAGATAAAACAATGGGATATAAAAAACTAA CATAGTTTTTCAATAATGATCCTTCCGCAGGTC | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Pezizomycetes; Pezizales; Pezizaceae; ***Peziza sp.***<br><br>(GU990362.1, GU969261.1, GQ267495.1, DQ974687.1) |
| HME willow | Hb | TGATCCGAGGTCACCTTAGAATGGGGTTGTTTTACGGC GTACCTCCCGAACACCCTTTAGCGAATAGTTTCCACAA CGCTTAGGGGACAGAAGACCCAGCCGGACGATTTGAG GCACGCGGCGGACCGCGTTGCCCAATACCAAGCGAGG CTTGAGTGGTGAAATGACGCTCGAACAGGCATGCCCC CCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATT CGATGATTCACTGAATTCTGCAATTCACATTACTTATCG CATTTCCCTGCGTTCTTCATCGATGCCAGAACCAAGAG ATCCGTTGTTAAAAGTTTTAATTTATTAATTAAGTTTAC TCAGACTGCAAAGTTACGCAAGAGTTTGAAGTGTCCA CCCGGAGCCCCCGCCCGAAGGCAGGGTCGCCCCGGA GGCAACAGAGTCGGACAACAAAGGGTTATGAATAACC AGGCCGAAGCCCGGGCGTTCTTGTCATGATCCCTCCG CAGAGTTCACCCTACGGAAGC | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Leotiomyceta; Dothideomyceta; Dothideomycetes Dothideomycetidae; Capnodiales; **unresolved**<br><br>(FN539069.1, GU065534.1, HM037657.1, GU566258.1) |

| Treat-ment | Morpho-type | Sequence | Identity (most similar sequences – Accesion number) |
|---|---|---|---|
| GCL poplar | 1b | CTACCTGATTTGAGGTCAGCTTTCGAGTGTCGGTCTGT TCGACGAGAGATTCCTCACCGCTGGCTTCACGACACC CGCTTAGCACTCCAGCCACGACAATCATTATCCCGTCG AACGCCGTTCAGGCCTGGAGTTAGGGCGTTGCACCAT CGCTAATGCTGTTAGGGAGAGCCGAAGACCGTAGGGT CGGCCGGCCCCTCCCAAAATCCAGGCCAAACCTGGGA ACAAATCGAGGGAAGGATGAGAATTAAATGACACTCA AACTTGCATGCTCCTCGGAATACCAAGGAGCGCAAGG TGCGTACAAAGATTCGATGATTCACTGAAAATCTGCTA TTCACATTACTTATCGCAATTCGCTGCGTTCTTCATCGA TGCGAGAGCCAAGAGATCCGTTGCCTGAAAGTTGTAT TTCGTTCTTTACCATCCCCGACCAGGCAACGAGCCAA GCCGGTCGACGCTTTGTGACATTCCTAGACATACTTAG AGTTTTATGAAGACATATATCCCCCTCGCAGAGACCTA CAATGGGTGCACGGGTGTGAAGAAAGGTTGTCCGAA GGAAAGGCAAAGGAGGACTAAGGAGAACTCGGAACG TGCACATGCCTTCGTGTCCAAAGGCCAGCGACAATCC CTACGATCTACCAAACTTCTCTCGGTCGCCTCTCCCCG GATTGCTTTTCGATAATGATCCTTCCGCAGGTCACCCTT ACGGAA | Fungi; Dikarya; Ascomycota; Saccharomyceta; Pezizomycotina; Leotiomyceta; Dothideomyceta; Dothideomycetes Dothideomycetidae; Capnodiales; **unresolved**<br><br>(FN539069.1, GU065534.1, HM037657.1, GU566258.1) |
| PIN poplar | P | CTACCTGATTTGAGGTCAGCTTTCGAGTGTCGGTCTGT TCGACGAGAGATTCCTCACCGCTGGCTTCACGACACC CGCTTAGCACTCCAGCCACGACAATCATTATCCCGTCG AACGCCGTTCAGGCCTGGAGTTAGGGCGTTGCACCAT CGCTAATGCTGTTAGGGAGAGCCGAAGACCGTAGGGT CGGCCGGCCCCTCCCAAAATCCAGGCCAAACCTGGGA ACAAATCGAGGGAAGGATGAGAATTAAATGACACTCA AACTTGCATGCTCCTCGGAATACCAAGGAGCGCAAGG TGCGTACAAAGATTCGATGATTCACTGAAAATCTGCTA TTCACATTACTTATCGCAATTCGCTGCGTTCTTCATCGA TGCGAGAGCCAAGAGATCCGTTGCCTGAAAGTTGTAT TTCGTTCTTTACCATCCCCGACCAGGCAACGAGCCAA GCCGGTCGACGCTTTGTGACATTCCTAGACATACTTAG AGTTTTATGAAGACATATATCCCCCTCGCAGAGACCTA CAATGGGTGCACGGGTGTGAAGAAAGGTTGTCCGAA GGAAAGGCAAAGGAGGACTAAGGAGAACTCGGAACG TGCACATGCCTTCGTGTCCAAAGGCCAGCGACAATCC CTACGATCTACCAAACTTCTCTCGGTCGCCTCTCCCCG GATTGCTTTTCGATAATGATCCTTCCGCAGGTCACCCTT ACGGAA | Fungi; Dikarya; Basidiomycota; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Boletales; Paxilineae; Paxillaceae; **Paxillus involutus**<br><br>(AM084700.1, EU488711.1, EU346879.1, FJ746640.1) |

*Table 8.5. The results of comparison of sequences obtained froom different ectomycorrhizal morphotypes with sequences collected in Genbank (NCBI, megablast search). Explanation of treatments abbreviations is given in Materials and Methods.*

### 8.2.2. Willows

#### 8.2.2.1. Plant growth, biomass production, above-ground architecture

Plants displayed significant differences in wooden biomass accumulated. ECM and AE treatment accumulated significantly more stem biomass than C treatment, this is true considering either dry or fresh weight (figures 8.19. and 8.20. respectively). Furthermore control treatment had the lowest dry-fresh weight ratio in stems (figure 8.21). Dry and fresh weight of leaves and their ratio, as well as total above-ground biomass was not affected by fungal treatment.

Plant height was significantly altered by fungal treatments (figure 8.22.). Unlike biomass the measurements of height were done regularly once a month through the whole growing season. The effect of treatment depended on the time of measurement. Last 3 measurements (circa 3 months) the trends were consistent with C treatment being significantly shorter than HME, ECM and AE

treatments. Number of branches (including the primary one), and their total length at the end of the experiment was not significantly affected by any fungal treatment.

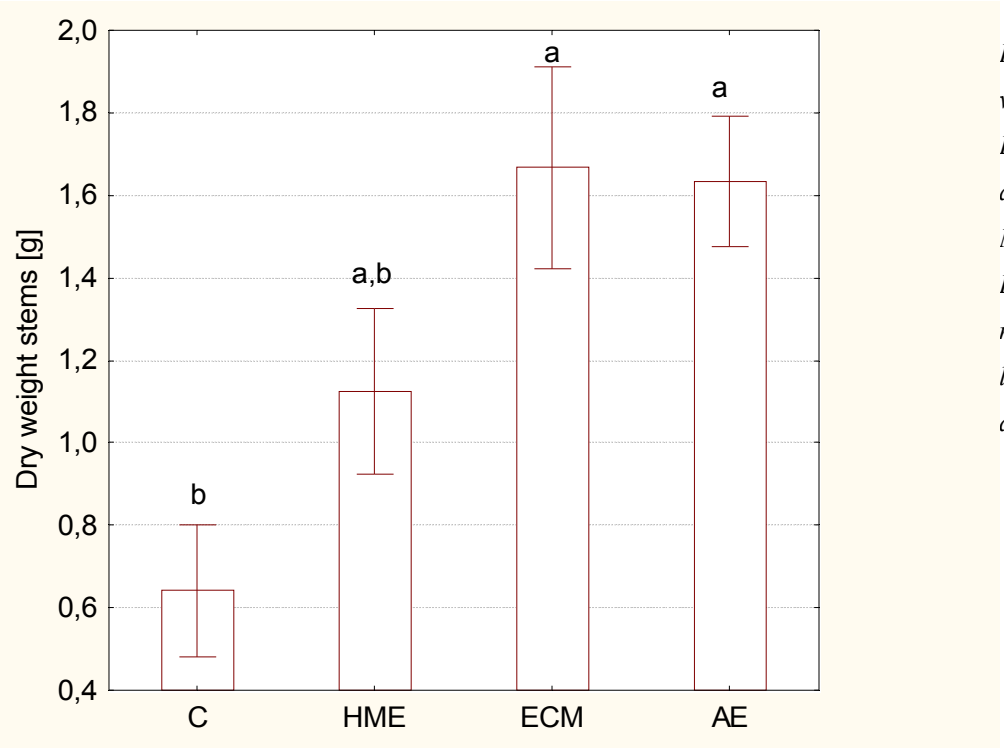


*Figure 8.19. Dry weight of willow stems [g]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

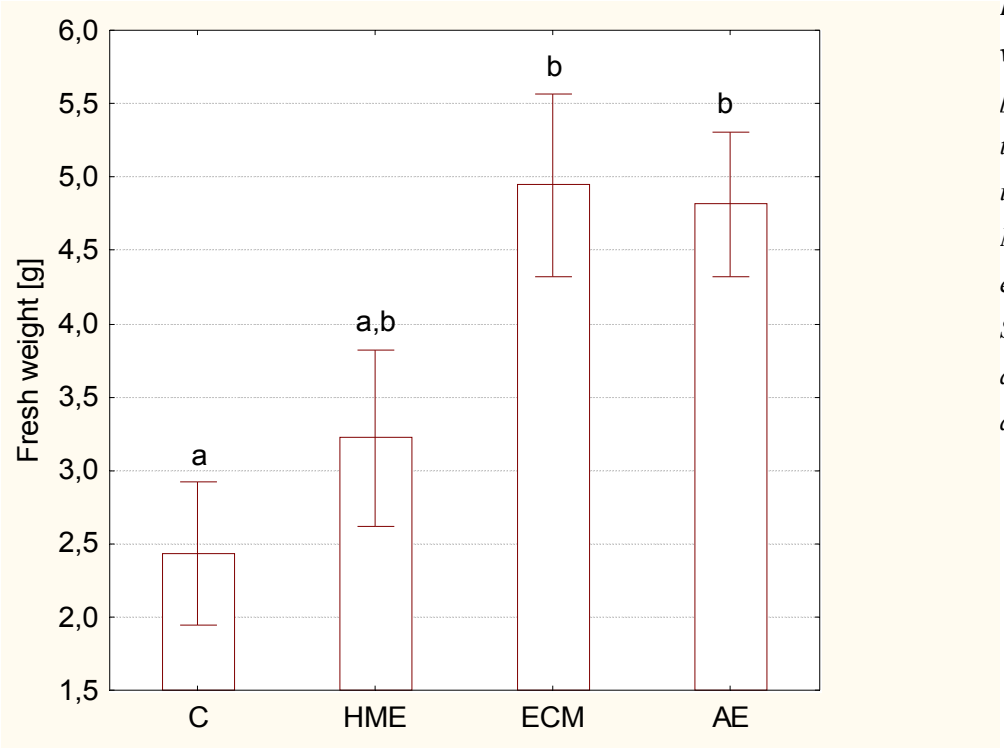


*Figure 8.20. Fresh weight of willow stems [g]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

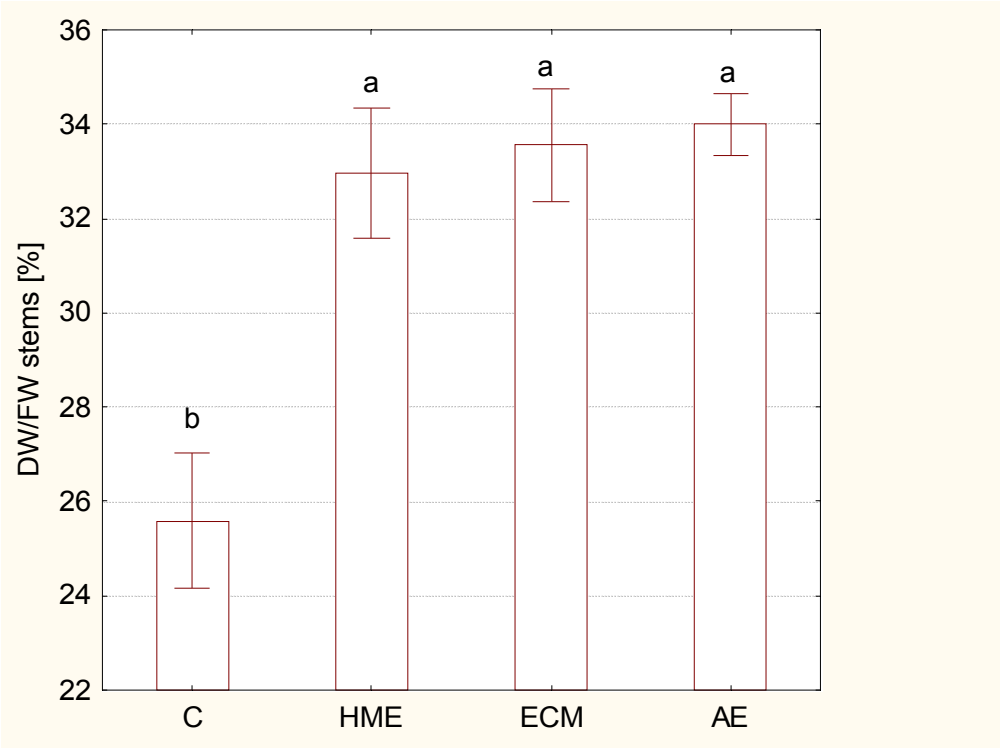


*Figure 8.21. Dry-fresh weight [%] of willow stems. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

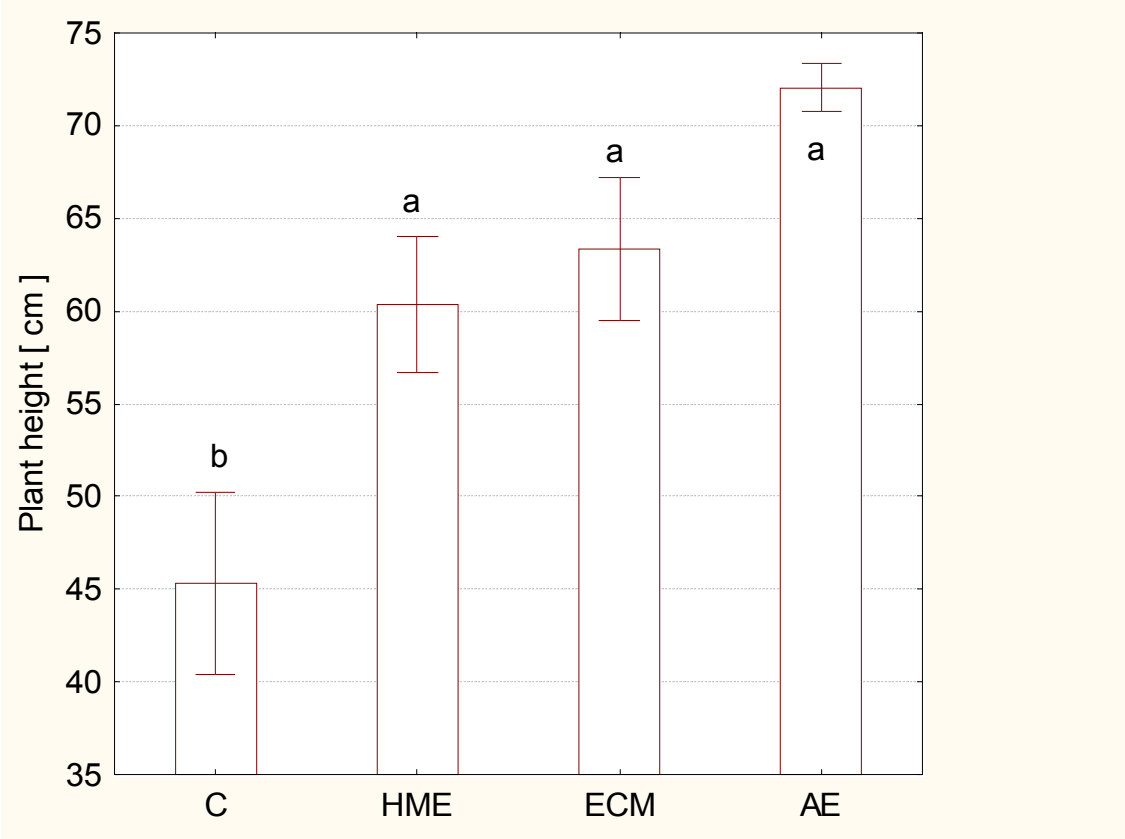


*Figure 8.22. Height of willow plants at the end of experiment [cm]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

### 8.2.2.2. HMs uptake

Stem HM concentrations did differ significantly for Cd and Pb (figure 8.23.), but not for Zn. Cd stem concentration was lower in all fungal treatments compared to control. Pb stem concentration was lower in HME and ECM treatment compared with control. Although HM concentrations differed in several cases, HM content in stems (total amount of HM accumulated in stems) was affected by fungal treatments only for Pb, where AE treatment increased Pb stem content compared to HME treatment (figure 8.24.). Neither one of metal leave concentration, metal leave content or total above-ground metal content was not affected by fungal treatments for all HM studied.

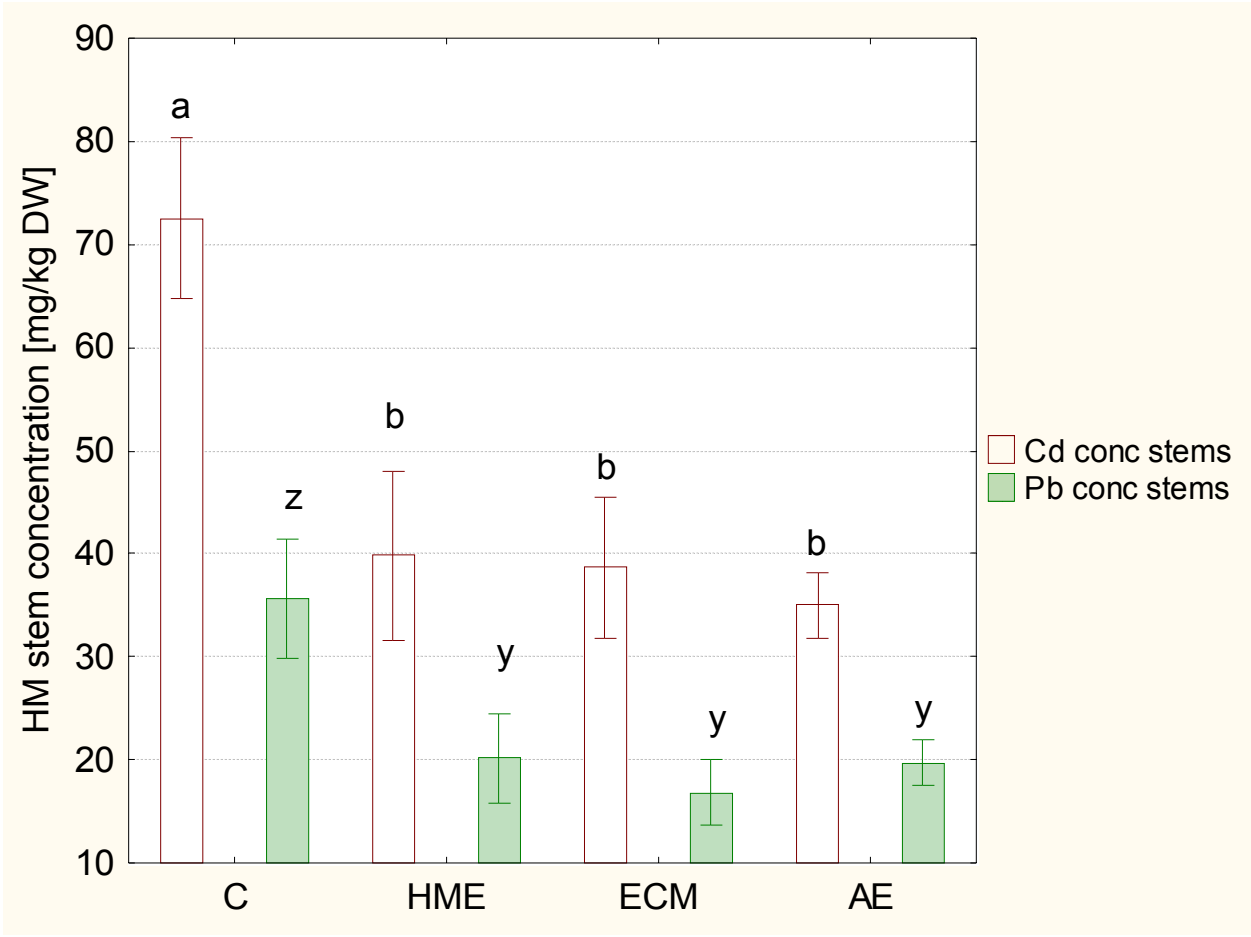


*Figure 8.23. Willow stem concentrations of Cd and Pb [mg/kg dry weight]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$ for each HM.*

### 8.2.2.3. Interactions between factors

Significant correlation was found between several measured factors. Cd, Pb and Zn stem concentrations were correlated with each other. HM stem concentration (Cd, Pb and Zn) negatively correlated with stem dry weight, and stem dry-fresh weight ratio. Furthermore Cd and Pb stem concentrations negatively correlated with plant height in the end of experiment. In leaves Cd concentration positively correlated with Zn concentration.

Several measured factors correlate, including HM stem concentration (for all Cd, Pb and Zn) with each other. HM stem concentration (for all Cd, Pb and Zn) negatively correlates also with stem dry weight, and stem dry-fresh weight ratio. Furthermore Cd and Pb stem concentrations negatively correlaltes with plant height in the end of experiment. In leaves Cd concentration positively correlated with Zn concentration.

### 8.2.3. Poplars

#### 8.2.3.1. Plant growth, biomass production, above-ground architecture

Plants did not display significant differences in wooden biomass accumulated, this was true considering for dry weight. GIN treatment significantly decreased fresh weight compared to AE and ECM treatments (figures 8.25). Control treatment had the lowest dry-fresh weight ratio in stems that was significantly different from that found in AM and GIN treatment (8.26). Dry and fresh weight of leaves and their ratio, as well as total above-ground biomass were not affected by fungal treatment.

Plant height was significantly altered by fungal treatments. Treatments had different effects during the time of experiment resulting in GIN treatment being significantly shorter than ECM and AE treatments (figure 8.27). Since poplars produced only few side branches, all of which died off during experiment, number of branches (including the primary one) was not affected by any fungal treatment, and their total length corresponded with plant height.

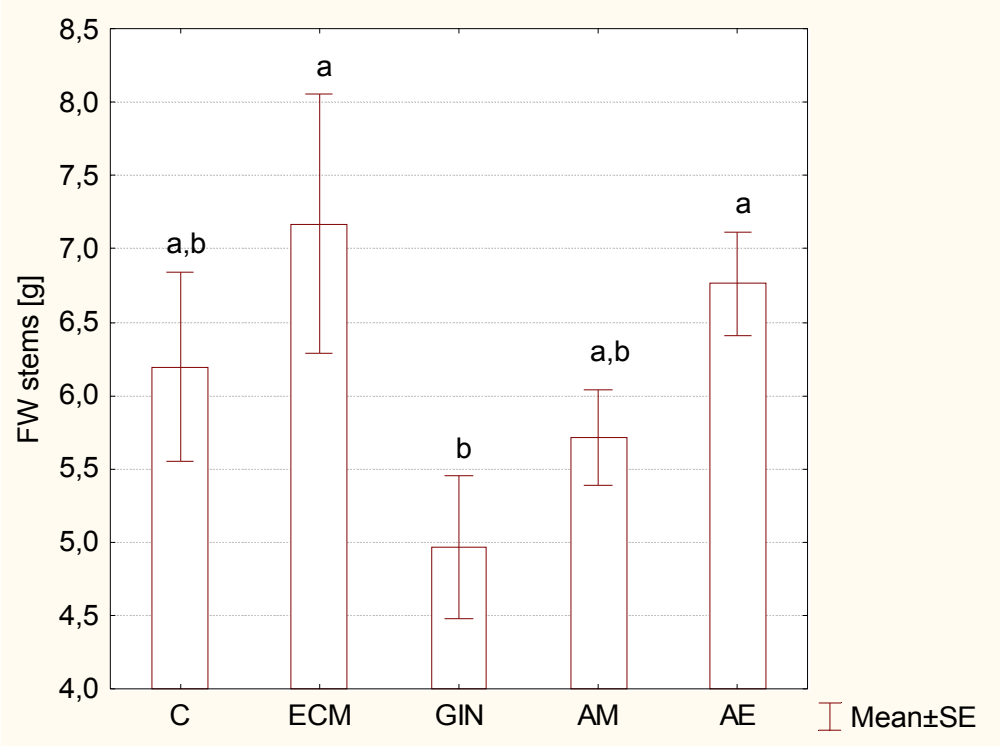


*Figure 8.25 Fresh weight of poplar stems [g]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

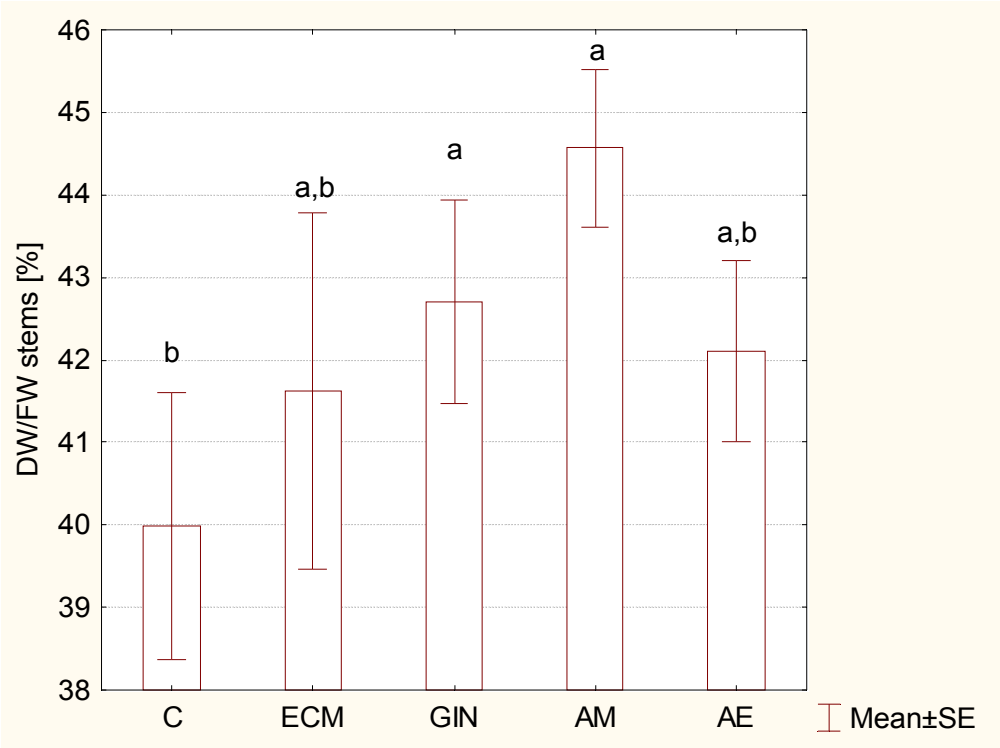


*Figure 8.26. Dry-fresh weight [%] of poplar stems. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

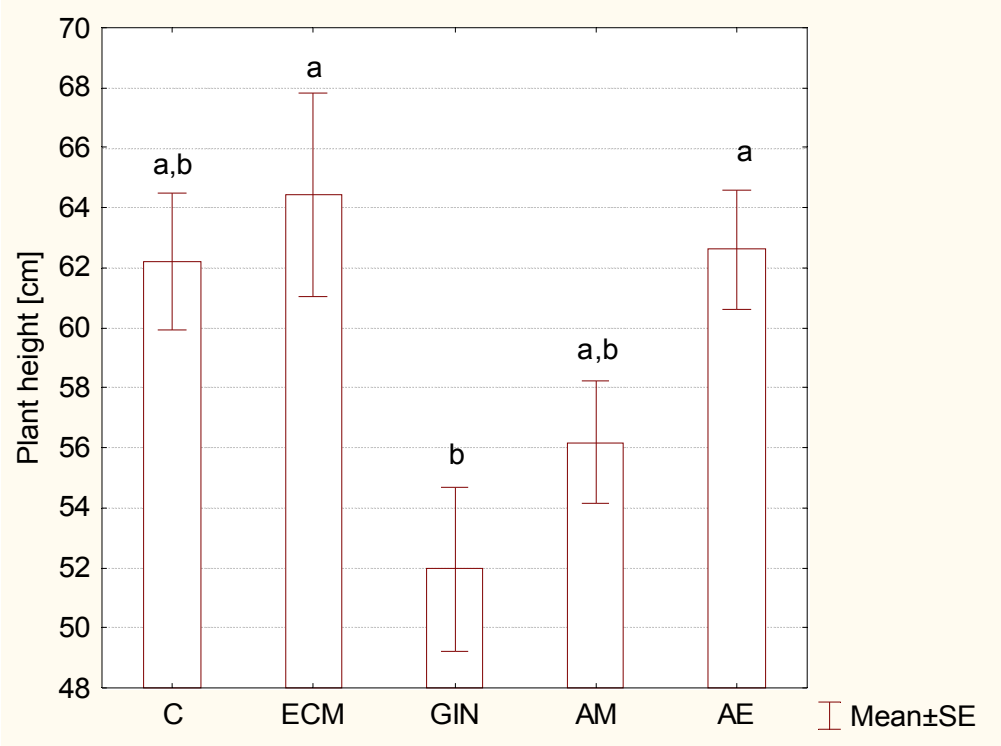


*Figure 8.27 Height of poplar plants at the end of experiment [cm]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

### 8.2.3.2. Photosynthesis and photosynthetic pigment content

Photosynthetic activity showed no significant differences between fungal treatments in the first measurement (14th week after start of the experiment) but in the second measurement (19th week after start of the experiment) ECM treatment had lower photosynthetic activity than AE treatment (figure 8.28). Stomatal conductance was significantly lower in GIN treatment compared with control and ECM treatment in the first measurement (figure 8.29). In the second measurement stomatal conductance displayed differences as well, however this time among different treatments (figure 8.30); only conductance of ECM treatment was significantly lower from controls.

Chlorophyll A, chlorophyll B, and total carotenoid concentration in leaves showed no significant differences in both measurements. However chlorophyll A /chlorophyll B ratio was higher in all fungal treatments compared with control treatment in first measurement (figure 8.31). Only AM treatment retained higher chlorophyll A /chlorophyll B ratio to second (8.32.) measurement, it was significantly higher than those from all other treatments.

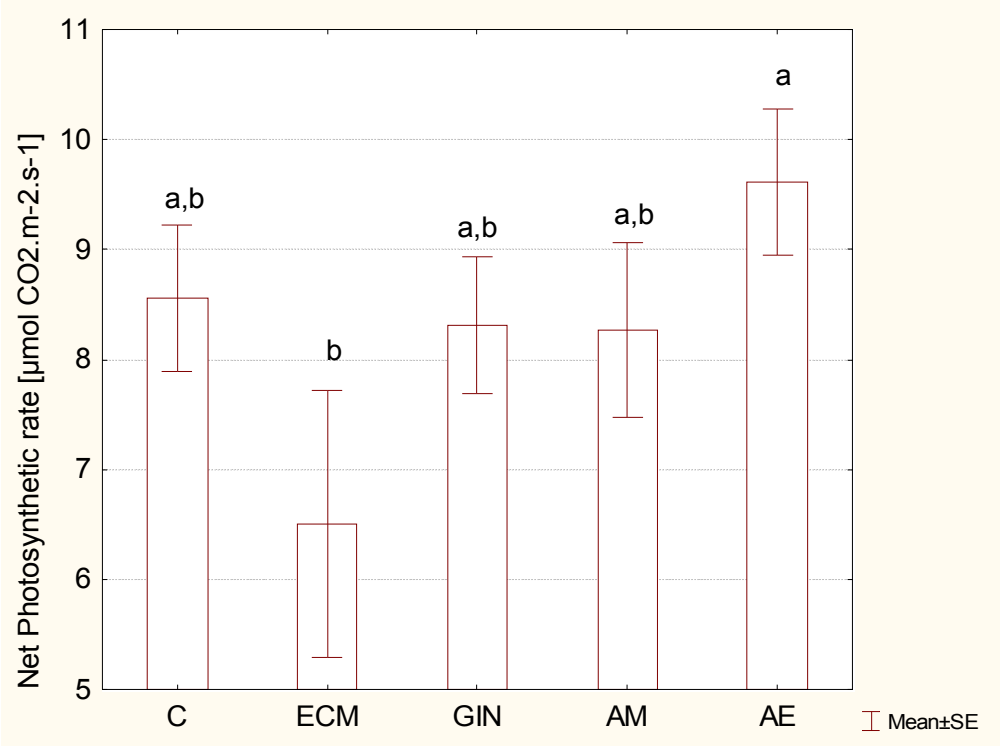


*Figure 8.28. Net photosynthetic rate of poplar plants in the second measurement [µmol $CO2.m^{-2}.s^{-1}$]. Explanation of treatments abbreviations and further details are given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$.*

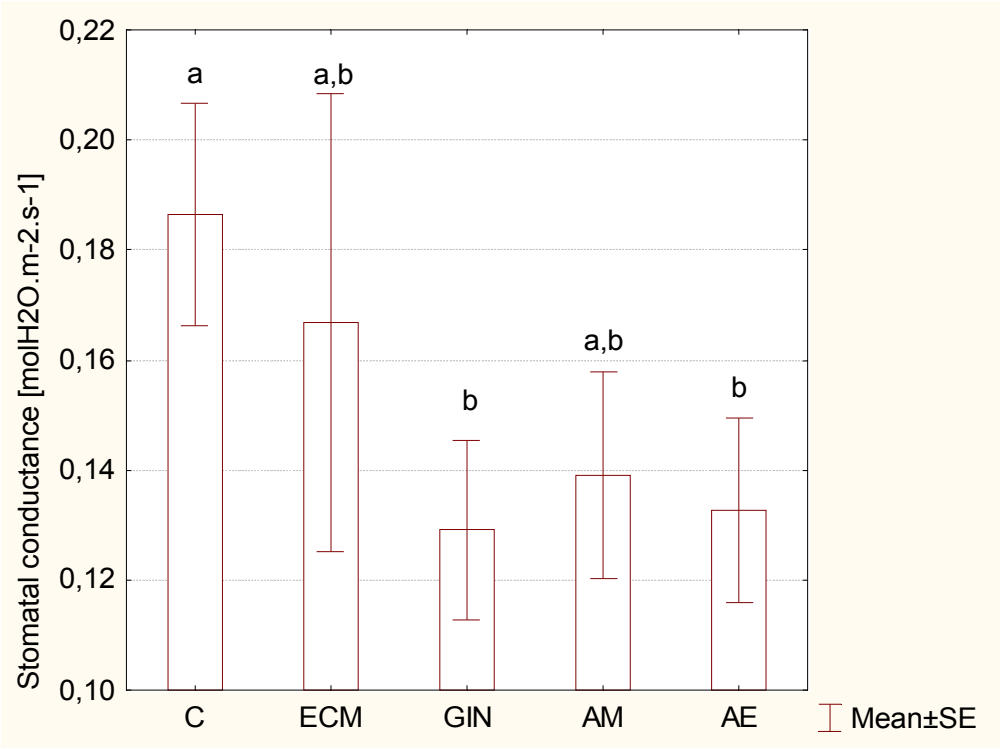


*Figure 8.29. Stomatal conductance of poplar plants in the first measurement [molH2O.m$^{-2}$.s$^{-1}$]. Explanation of treatments abbreviations and further details are given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

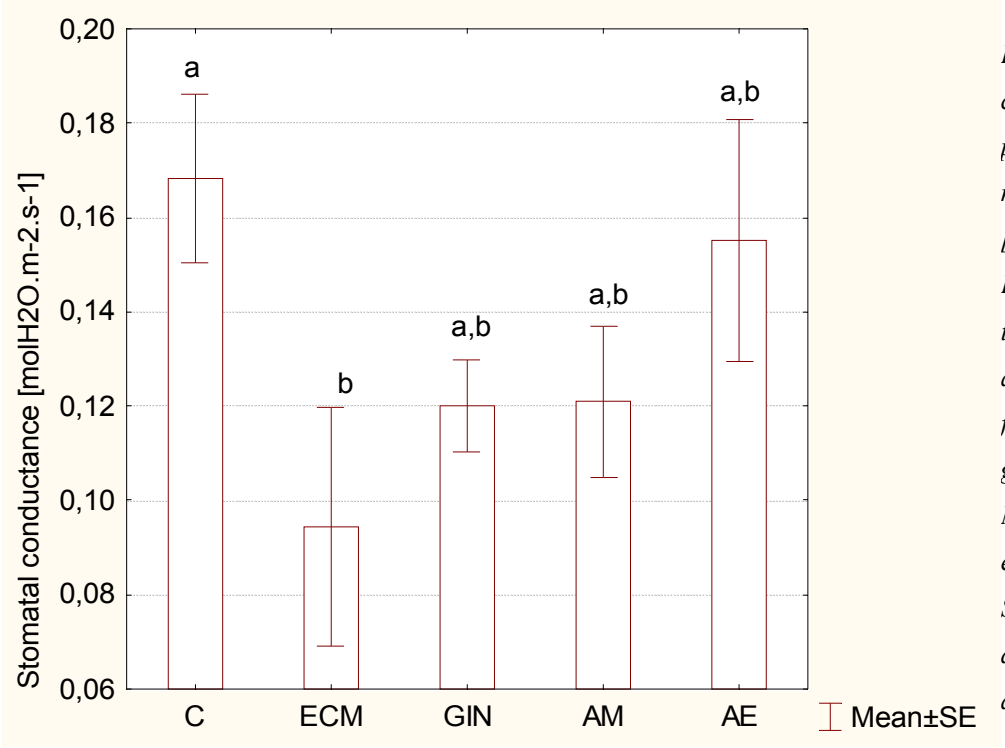


*Figure 8.30. Stomatal conductance of poplar plants in the second measurement [molH2O.m$^{-2}$.s$^{-1}$]. Explanation of treatments abbreviations and further details are given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

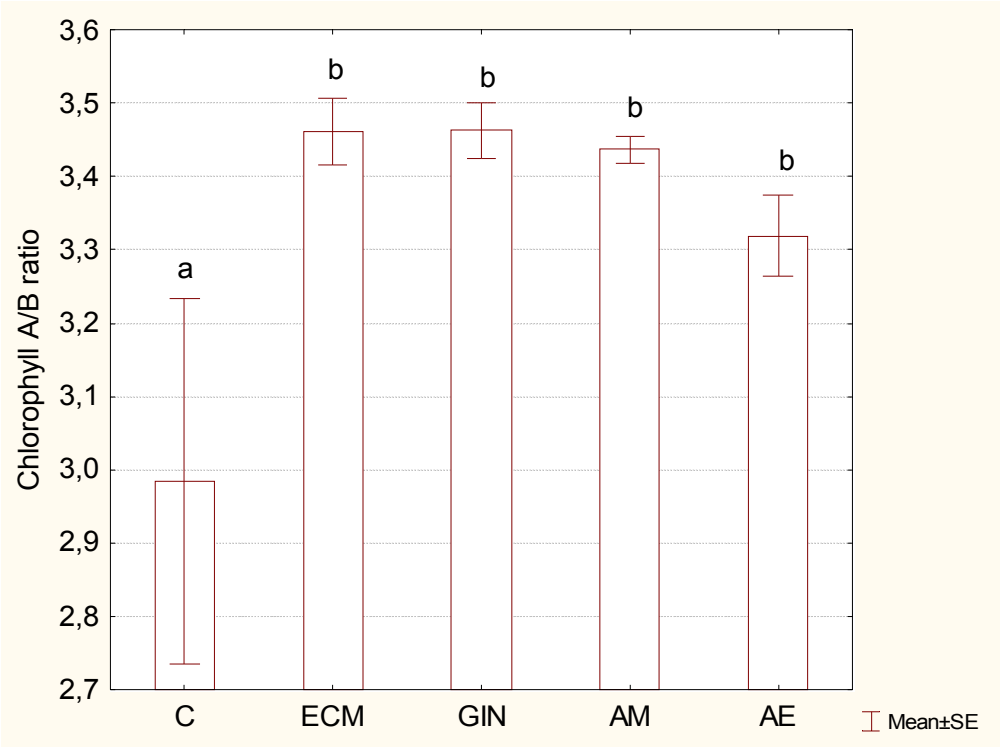


*Figure 8.31. Chlorophyll A/ chlorophyll B ratio in first measurment in poplars. Explanation of treatments abbreviations and further details are given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

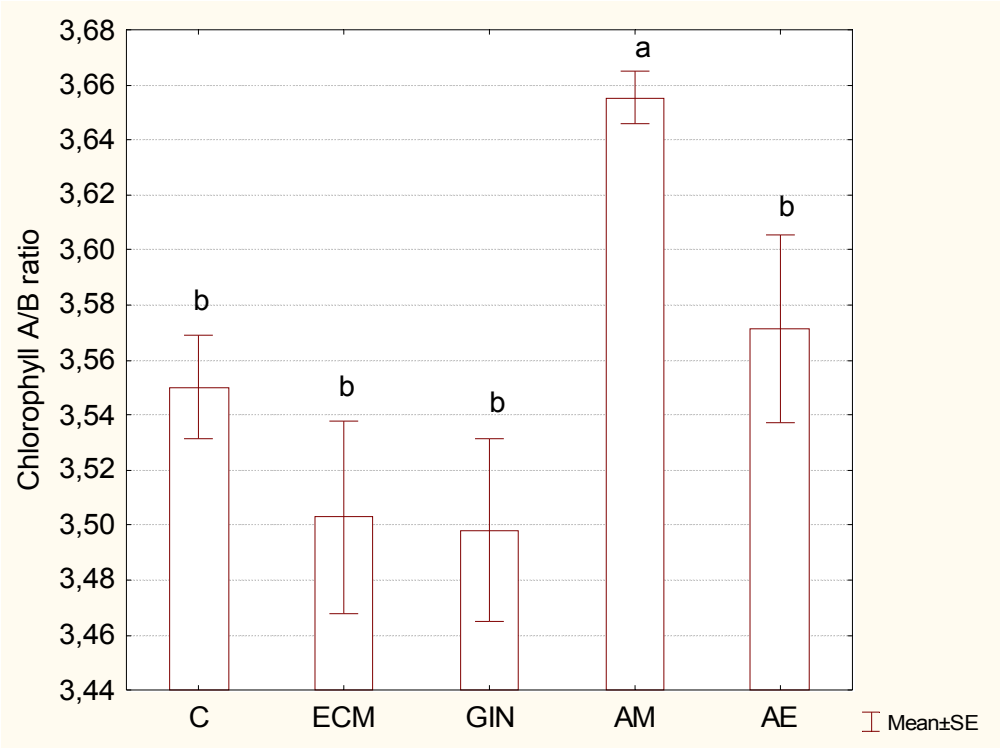


*Figure 8.32. Chlorophyll A/ chlorophyll B ratio in poplars in second measurment. Explanation of treatments abbreviations and further details are given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at p<0.05.*

### 8.2.3.3. Raman spectroscopy measurements

Representative Raman leaves spectra of poplar plants from 3 different treatments - GIN, ECM and C are shown in Figure 8.33. Raman spectra of poplar leaves show four major bands corresponding to the water, waxes and carotenoid content (cuticular waxes are first of all characterized by the bands corresponding to the aliphatic moiety /bands at 2882, 2845 and about 1456 cm-1/; two bands at about 1530 and 1160 cm-1 correspond to the carotenoids and the relatively weak but broad band at 3220 cm-1 is attributable to the water stretching vibration modes). Principal component analysis (PCA) was performed to elucidate contribution of studied components found in Raman spectra. The results demonstrate that both mycorrhizal treatments contain more carotenoids and more water in the upper leaf tissues compared to control (figure 8.34).

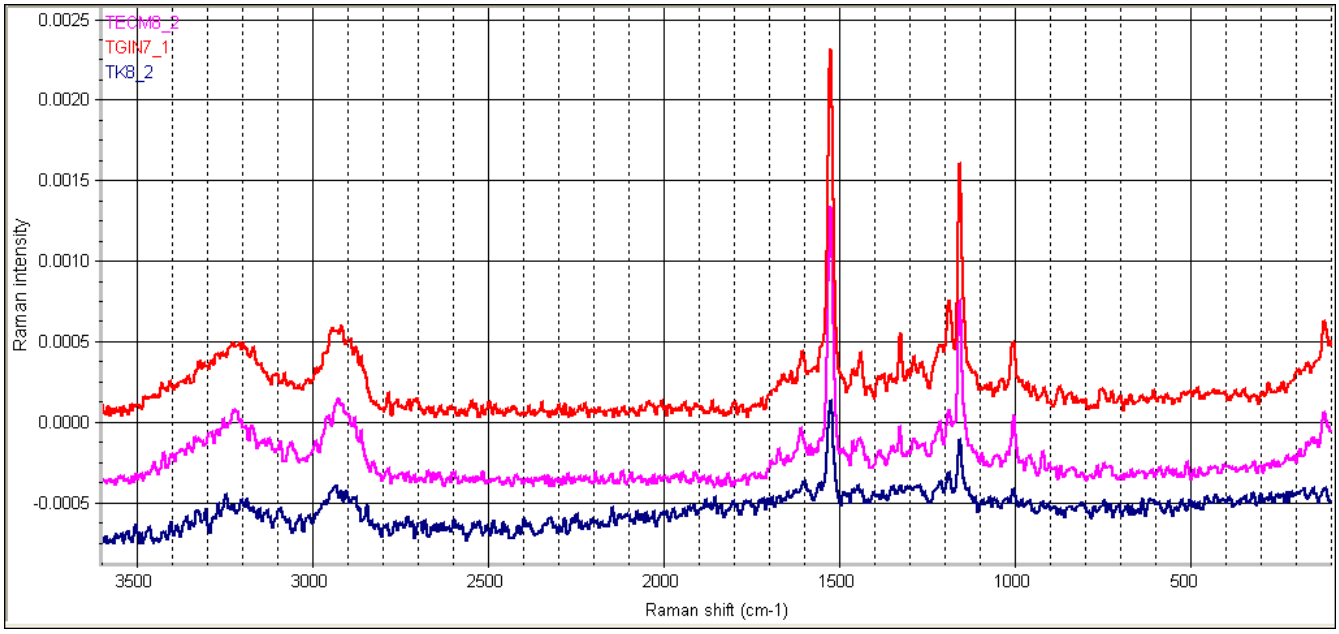


*Fig. 8.33. Selected Raman spectra measured on leaves of control (blue), ECM (violet) and GIN (red) plants. Attribution of spectral bands to chemical moieties is given in Materials and methods. Leaf cuticular waxes are first of all characterized by the bands corresponding to the aliphatic moiety (bands at 2882, 2845 and about 1456 cm-1). Two bands at about 1530 and 1160 cm-1 correspond to the carotenoids and the relatively weak but broad band at 3220 cm-1 is attributable to the water stretching vibration modes.*
*C - control treatment, ECM -treatment inoculated by H. mesophaeum and P. involutus, GIN - tretment inoculated byG. intraradices.*

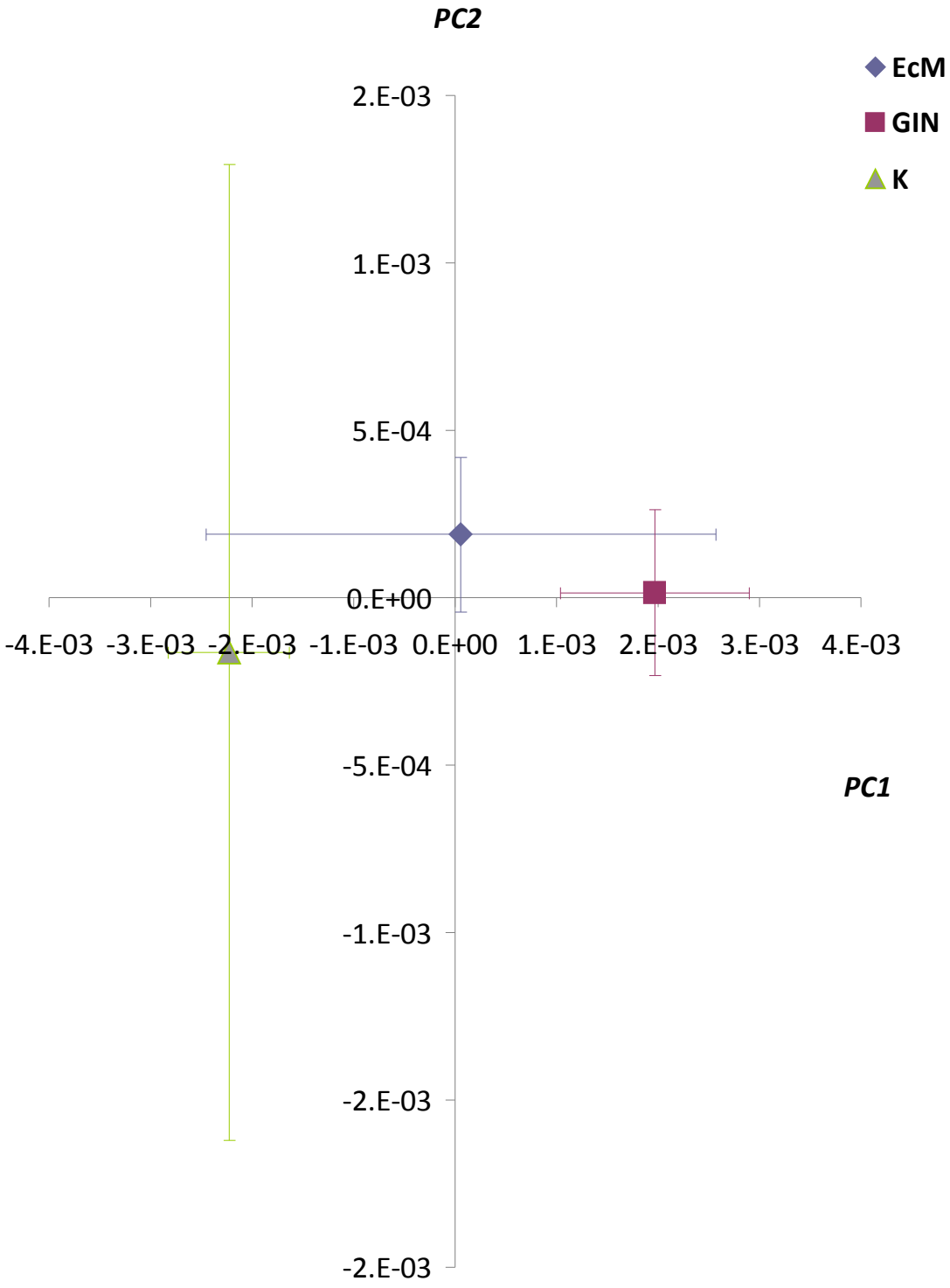


*Fig. 8.34.: Principal component analysis (PCA) of Raman spectra from control, ECM and GIN plants. Error bars correspond to standard deviation of each group with respect to principal components (PC) 1 and 2. Carotenoids and partly cuticular waxes contribute to PC1, whereas backround deformations related to leaf humidity contribute to PC2. C - control treatment, ECM -treatment inoculated by H. mesophaeum and P. involutus, GIN - tretment inoculated byG. intraradices.*

### 8.2.3.4. HM uptake

Stem concentrations showed no significant differences. Stem HM content was also not affected considering Cd and Zn. The only significant difference was observed in Pb content, which was lower in ECM treatment compared with AE, AM and GIN treatments (figure 8.35).

Neither Zn nor Cd leave concentration or content were affected by fungal treatments. Pb leave concentration was higher in GIN treatment compared with control treatment (figure 8.36.). Total above-ground biomass HM content was decreased by ECM treatment for Cd and Pb compared to controls, increased by GIN treatment for Pb compared to controls and showed no significant differences for Zn compared with controls (figure 8.37.).

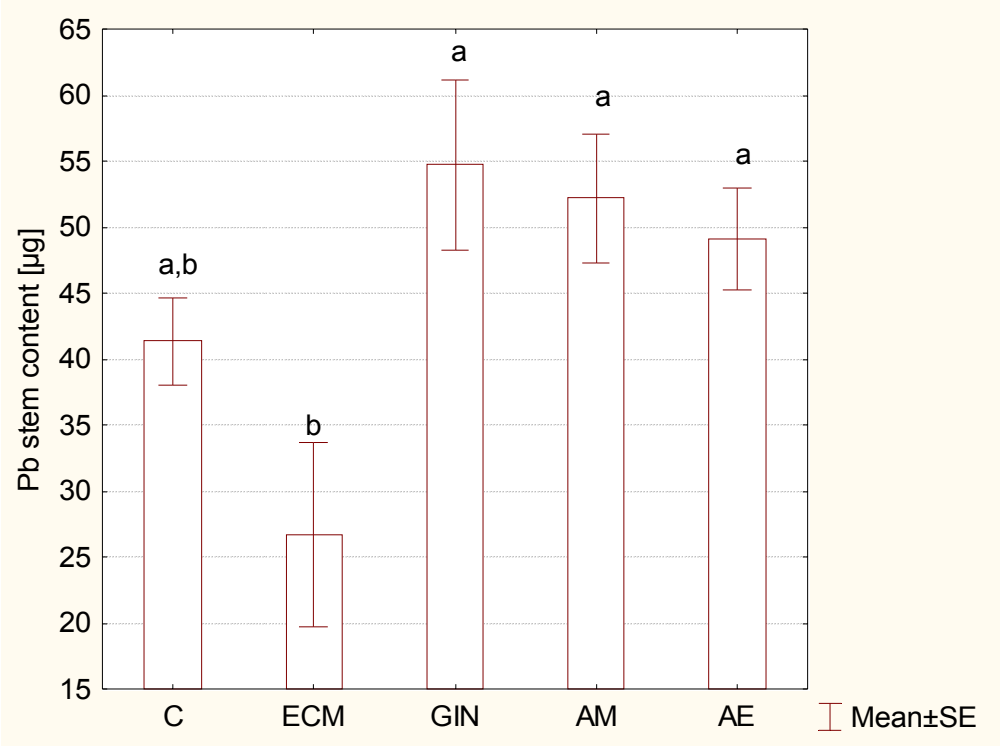


*Figure 8.35. Poplar stem content of and Pb [µg]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$.*

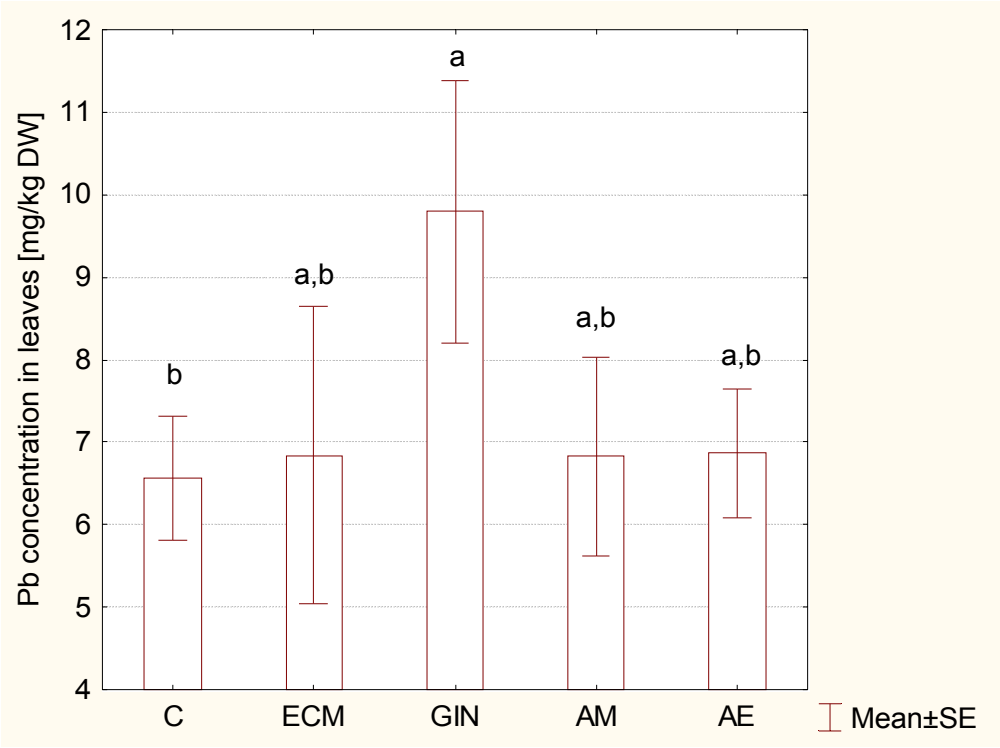


*Figure 8.36. Poplar stem concentration of and Pb [mg/kg of dry wight]. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$.*

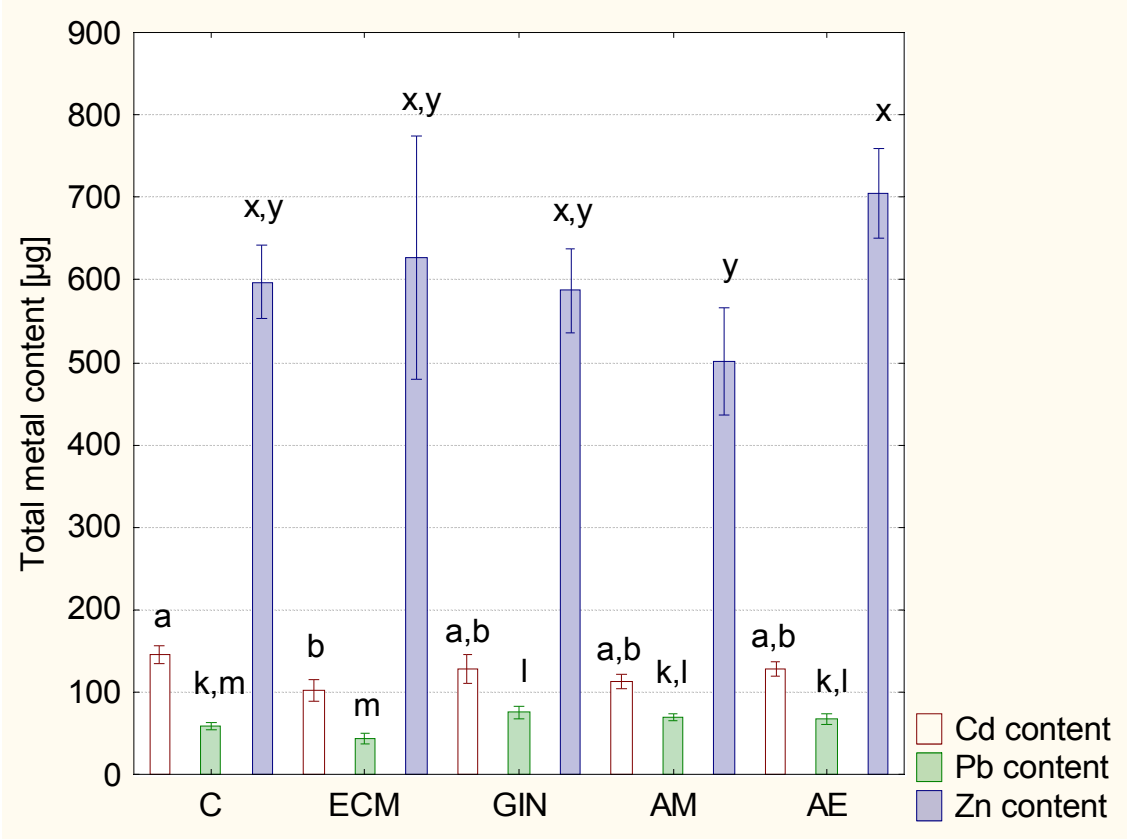


*Figure 8.37. Total above-ground content of HMs [µg] in poplars. Explanation of treatments abbreviations is given in Materials and Methods. Data are expressed as means ± SEM. Different letters denote statistical difference at $p<0.05$ for each HM separately.*

#### 8.2.3.5. Interactions between factors

Several measured factors correlate, including HM stem concentration (for all Cd, Pb and Zn) with each other. HM stem concentration (for all Cd, Pb and Zn) negatively correlates also with stem dry weight, and stem dry-fresh weight ratio. Furthermore Cd and Pb stem concentrations negatively correllates with plant height in the end of experiment. In leaves Cd concentration positively correlated with Zn concentration.

## 9.Discussion

The experimental design consisted of two successive experiments, which aimed to assess:
1) firstly the possibility to cultivate selected fungal strains, to examine their tolerance to HM, accumulation of HM in their mycelia and their SOD activity (experiment INVIT); and 2)
secondly physiological responses to HM and HM uptake by willows and poplars inoculated by AM fungi and EcM fungal strains selected from previous experiments (based on their metal accumulation tolerance and ability to grow *in vitro*) either individually or in combination (experiment DUAL). Hence the discussion follows the same pattern.

### 9.1. INVIT experiment

#### 9.1.1 INVIT part A

In soil, HM can exist in many forms including free ions, (in)soluble complexes, exchangealbe ions, organically bound metals or precipitated compounds such as oxides, carbonates, hydroxides or they may form a part of silicate minerals. Their toxicity depends rather on their bioavailability than their total concentration (Lasat 2002). Bioavailability is defined as HM ability to be transferred from a soil compartment to a living organism. In this experiment, HM bioavailability was estimated by their extraction with weak acid (0.11M acetic acid), what is considered as a relevant procedure (Leyval 1997). However, one must keep in mind, that even this approach represents simplification of reality and does not take into account the enormous complexity of soil substrate. This lack of complexity is desired on one hand, providing better control over growth conditions, but on the other hand it may result in slight differences in fungal behaviour compared with that in soil, where, for example, fungal hyphae may avoid more polluted microhabitats (Pawlowska 2004). Indeed, I observed different growth rates of several strains grown in agar media (experiment INVIT) and in HMs polluted soil amended with low-glucose MMN solution (data not included in this work). Furthermore, fungal isolates were grown axenically - again ensuring controlled conditions, while neglecting something almost essential – plant-symbiont interactions.

To assess HM tolerance - comparison of fungal growth between control treatment (without HM) and treatments with HM are evaluated. Colony growth can be described either by fungal biomass production or colony diameter in time (Ray 2005, Hartley 1997b). It was reported that later parameter is more sensitive to HMs and was used in this study (Colpaert and van Assche 1992). A model extracting RCGmax (maximal colony diameter) and T1/2 (time when colony reaches RCGmax/2) derived from growth curves fitted by Weibull function (López 2004) was used.

#### 9.1.1.1. RCGmax and T1/2 on control media - fungal ability to grow in axenic cultures

Fungal isolates showed different abilities to grow axenically as expected based on previous works with fungal cultures *in vitro* (Kernaghan 2002, Kim 2003, Ray 2005). Fungal strains differed in both parameters (RCGmax and T1/2). Both the ability to produce reasonable biomass (related to RCGmax) and the rate at which this happens (related to T1/2) must be considered when selecting fungi suitable for inoculation.

#### 9.1.1.2. HMs uptake

HMs uptake (expressed as HM tissue concentration) showed similar trends for HMs studied, implying that if given isolate accumulates high amounts of one metal, it will probably accumulate other as well. This hypothesis is supported by significant correlations found in both HM treatments. Possible mechanism behind these findings may include influx by less specific divalent cation transporters, which are assumed to prevail over high specific transporters at high metal concentration (Gadd 1993). In accordance with other studies *P. tinctorius* and *P. involutus* were reported to accumulate high concentrations of Zn and Cd, respectively (Bellion 2006, Tam 1995).

#### 9.1.1.3. HM metal tolerance

Some isolates did not respond to HM treatment in any of the two parameters (RCGmax and T1/2) analysed, others were affected either in RCGmax or T1/2 or both again varying whether the response occurred in one or both HM treatments. The overall variability of results may be attributed to inter- as well as intraspecific differences in HM tolerance as previously observed in several studies with ectomycorrhizal fungi such as *P. involutus*, *P. tinctorius* or *Suillus luteus* (Adriaensen 2006, Colpaert 2000, Muller 2007, Ray 2005).Though increased fungal tolerance to HMs in autochtonous strains was shown, it is not a strict rule for all fungi (Colpaert 2000, Colpaert 2004). For example *P. involutus* can survive on polluted sites even without specific genetic adaptation to the pollution, in deed this observation is supported by results of this work (Colpaert 2004). More than half isolates in this work displayed growth decrease probably due to increased overall HMs concentration in L medium compared to the K and the C treamtents. Although the overall HMs content in media is higher in the L treatment than in the K treatment, the proportion of HMs added is not similar, for example Pb content in the L treatment is even lower than that in the K treatment. This would favour Pb sensitive strains and disadvantage strains sensitive to Cd or Zn in the L treatment compared to the K treatment (with 3 fold

higher Pb concentration). However, no such case was observed as RCGmax were either similar or lower in the L treatment compared to the K treatment. Comparing colony growth parameters may be a good indicator for selecting tolerant strains, however, this does not take into account the relative change of the parameters between isolates. Therefore comparison of RCGmax of each treatment relative to RCGmax of control of given fungal strain were compared between strains for each treatment separately (RCGmax C for K and for L). This was done for T1/2 as well (T1/2 C). Comparisons of these relative parameters did not result in any significant differences in the K treatment what would imply that selected isolates respond similarly to HM treatment such as the K treatment. In the L treatment some of the strains displayed variations in both parameters. More profound differences in the L treatment compared with the K treatment are most probably result of increased HMs toxicity based on HMs concentrations. Nevertheless, lack of significant differences and those displayed in the L treatment imply that fungal strains differ more in their response to higher overall HM concentration. One more possible mechanism explaining more profound differences observed in the L treatment compared to that on the K treatment is at ease: a positive feedback mechanism discussed by Kim et al. explaining that faster growing fungi may have an advantage over those slowly growing since increased production of hyphae may represent more metal binding sites - immobilising them bound to cell wall (Kim 2003). Furthermore, tolerance to one metal element does not imply tolerance to all metals, as the tolerance to various metals may be governed by various genes (Bladuez 2000). Different proportions of HMs in the K and the L treatments may contribute to differential HMs interactions as found for various concentrations of Cd, Zn, Pb and Sb and their combinations in growth media (Hartley 1997b).

Out of 20 isolates studied, PIN Maj and PTI-1 were always ones of 6 fungal isolates accumulating most HM element in both treatments, which is in agreement with previous findings that these two species are capable of high HMs accumulation (Ballion 2006, Ray 2005). On contrary HME-1 and TSC-1 were among others accumulating the least amount of HM in both treatments. These 4 isolates were relatively easy to cultivate *in vitro*. HMs tolerance of above mentioned fungi except PIN Maj. as displayed in the K treatment was high for PTI-1,TSC-1 and HME-1 leaving both studied parameters unaffected compared to controls. Although HME-1 grew very badly on L treatment rendering data not suitable for analysis, it grew well on both the K and the L soils amended with low-glucose MMN media (data not presented in this work) and, thus, it was selected for further usage. Based on obtained results, 4 fungal strains (HME-1,TSC-1, PTI-1 and PIN Maj) were selected for further testing of HM tolerance mechanisms and HME-1 and PIN Maj were chosen for the DUAL experiment

Nevertheless, question is: is the accumulation of HMs by particular fungal strain a good indicator for its usability for enhanced transport of HMs to plants in phytoextraction strategy? Namely considering the fact that one of the important mechanisms of HM detoxification is their adsorption on a cell wall? I suppose it could be as the HMs accumulation was assesed in fungal colonies grown on agar media covered with cellulose foil. The foil dramatically reduces direct contact of collected mycelium with growth media, thus, decreasing the probability of HM adsorption. Furthermore, *P. involutus* was reported to accumulate and tolerate high amounts of Cd and to transport it to vacuoles (Bellion 2006). Yet the compartmentalisation of HM in fungal mycelia was not studied in the present study and could be of significant importance. For example, isolates adsorbing the bulk of HMs should be favored in use in phytostabilisation strategy as further transfer to plant tissue may be restricted (Jentschke 2000). On the other hand, isolates accumulating bulk of HMs in their vacuoles eventually cytoplasm should be used in phytoextraction strategy increasing the probability of metal transfer to a plant, as it was observed for combination of *P.involutus* and *Populus canadensis* tree (Sell 2005).

### 9.1.2. INVIT part B

Both more HMs accumulating fungal isolates (PIN Maj and PTI -1) grew faster and produced larger colonies in all treatments compared to slowly growing isolates HME -1 and TSC - 1. Based on the results of the part A of the INVIT experiment I assumed that PIN Maj and PTI-1 would accumulate more individual HM than the other two isolates. Indeed, PIN Maj was clearly the most accumulating isolate but PTI-1 did not accumulate significantly more HMs than TSC-1. Yet in the part A of the experiment, the fungus PTI-1 tended to accumulate more Zn and Cd than PIN Maj, in the part B of the experiment, the fungus PTI-1 accumulated less Pb and Zn and tended to accumulate less Cd as well. Possible mechanism behind these findings may include influx of HMs by less specific divalent cation transporters in the fungus PTI-1 in the part A of the experiment, which are assumed to prevail over high specific transporters at high metal concentration. In the part B of the experiment, specific control over selected HMs could be established in PTI-1 (Gadd 1993) This once again stresses out the relevance of possible HMs interactions (Hartley1997b).

The SOD activity remained unaffected by addition of HMs into agar media except for Cd, which caused increase of SOD activity in the PIN maj and TSC-1 strains. Induction of oxidative stress by Cd is rather well documented (Clemens 2006, Schützendübel 2002). The isolates PIN Maj and TSC-1 exhibited low “basal” SOD activity, meaning that on control media, its activity was lower compared to the other two fungal isolates. This may suggest high SOD detoxication potential in PIN Maj and TSC-1

compared to PTI-1 and HME-1. Cd-induced SOD activity was reported for *P.involutus* in an *in vitro* experiment conducted by Bellion et al. (Belion 2006). In TSC-1, Cd induced SOD activity even at low rate of accumulation suggest high potential of Cd to induce oxidative stress. PTI-1 and HME-1 apparently utilise other means of protection, against HM stress. These may include restriction of HM entering the cytoplasm, thus decreasing the risk of HM induced oxidative stress. To prevent HM from entering cytoplasm, fungi may adsorp the metal on a cell wall or, precipitate it on cell wall surface (Meharg 2003). Even if the metal enters the cytoplasm, SOD activity, which was high on control media in PTI-1 and HME-1 compared to other two isolates, does not need to play principal antioxidative mechanism, as there are many others which can be exploited. These include activation of other antioxidative enzymes (CAT, POX) or ROS scavenging by antioxidants (GSH, ascorbate etc.) (Dixit 2001).

### 9.2. DUAL experiment

Compared with field study, this experiment used sterilised soil, restricted space for root growth (Petri dish), and controlled growth conditions eliminating a vast number of possible interactions of a plant with its environment in field conditions. This approach increases interpretation and reproduction potential of the experiment, while decreasing its relevance to field conditions (Krupa 1990). Petri dishes were used as containers, decreasing the risk of airborne contamination. Despite precautions taken, contamination by yet unidentified fungi occurred. Together with mortality (20% of all pots), contamination (25.6% of all pots) contributed to exclusion of some experimental treatments not allowing us to assess all interactions planned before the beginning of the experiment. The possible source of contamination could have been airborne fungi, as all containers had a hole at the bottom. However, almost all GCL plants were colonised by 2 morphotypes belonging to probably one unidentified mycorrhizal fungus, implying contamination of inoculum prior to inoculation.

#### 9.2.1. Colonisation

Because of destructive methods used for colonisation estimation, colonisation was assessed at the end of experiment. Thus, this approach inevitably disregard possible dynamic shifts in colonisation in variants inoculated by more than 1 fungus. Indeed, such dynamics were described several times, regarding mainly shifts between AM and ECM fungi, such as the AM fungi are displaced by ECM

(Gange 2005, van der Heijden 2000).

Colonisation was not always successful mainly within willows, where half or more pots from the treatments AE, ECM and GIN were non-myocrrhizal in the end. This resulted in GIN treatment being excluded from further analyses. The possible explanation could be that selected willow clone is insusceptible to chosen arbuscular mycorrhizal partners, which is rather probable for the GIN treatment where 7 out of 8 plants were not colonised. Low AM colonisation in this experiment is in accordance with previous findings that willows are often only marginally colonised by AM fungi (van der Heijden 2000). Insusceptibility of willows to mycorrhizal partner, however, would not explain massive colonisation by *H. mesophaeum* in the HME treatment and unsuccessful colonisation of so many plants in ECM and AE. Probably other factor is involved in this case. Despite not colonising some plants in ECM and AE treatments, those colonised were colonised only by *H. mesophaeum*, implying its superiority over other inoculi within *S. alba* root system. The AE treatment trees showed significant decrease in colonisation by *H.mesophaeum* compared to the HME treatment. Possible explanation is decreased *H.mesophaeum* inoculum amount in the AE treatment compared to the HME treatment, which was reported to be relevant considering an extent of colonisation by ECM fungi (Chen 2000). However, this is unlikely too, as the ECM treatment did not shown a similar trend in colonisation by *H. mesophaeum* as the AE treatment, in spite of the same inoculum amount. Lower colonisation by *H. mesophaeum* in the AE treatment could be a result of competition over space or nutrients in early stages of development (van der Heijden 2000), however no other fungi were observed in the AE treatment at the end of the experiment. Within poplars, unsuccessful colonisation by *H. mesophaeum* in the HME treatment could be explained by poplar preference of AM over ECM fungal symbiont, as AM colonisation was always higher than 54% (Beauchamp 2005, Gardes 2003). Colonisation of poplar roots by *H. mesophaeum* prevailed in the ECM treatment with no *P. involutus* present, again implying its inoculation potential superiority in this case.

### 9.2.2. Plant growth and mycorrhiza effect

#### 9.2.2.1. Willows

In the present study, fungal inoculation altered stem biomass production in willows in the treatments AE and ECM and a trend of an increase, although not significant, was also observed in the HME treatment. These results support the general assumption that mycorrhizal fungi improve plant performance (van der Heijden 2000). Though there is a dispute whether short term experiments, such

as this one can evaluate mycorrhizal contribution to plant fitness(Smith 2009), this was not the aim of the present experiment Rather than overall fitness, this experiment attempted to evaluate mycorrhizal contribution to above-ground biomass production (as for application to SRC) and HM stress alleviation. Indeed, HMs content (unlike concentration) did not differ between control and fungal treatments (which were all colonised only by *H. mesophaeum*) and increased stem biomass acquisition correlated with decreased Pb and Cd but not Zn accumulation in all fungal treatments, suggesting that beneficial effect of *H. mesophaeum* inheres in restriction of HMs uptake. Decreased HMs accumulation due to *H. mesophaeum* colonisation, however, does not necessarily mean that this isolate is suitable for phytostabilisation strategy, as all treatments displayed higher Cd stem concentrations than those reported for soil. Although this could be attributed to high Cd mobility and/or influx via Zn transporting mechanisms (Eng 1998, Kim 2003). Furthermore, phytostabilisation strategy assumes HMs storage in underground tissues either of plant or fungal origin (Brunner 2008, Regvar 2006, Vamerali 2009). Neither root biomass, root HMs concentration, nor HMs root content were analysed in this study, leaving space for further research with given *H. mesophaeum* fungal isolate and a willow clone.

#### 9.2.2.2. Populus

No significant effect of mycorrhizal colonisation on biomass production was observed within poplar plants, which may be result of low responsiveness of selected poplar clone to selected fungal isolates (Zhu 2001). This, however, contradicts with vast number of findings, where myocrrhizal fungi improve plant performance (Sell 2005, Smith 2009). Furthermore, study on soils contaminated with HMs show strong improvement of *P. nigra* performance as effect of inoculation by two *Glomus* species (Lingua 2008), though intraspecific differences may be source of these discrepancies, as the authors used Jean Pourtet clone of *P. nigra*. Some fungal effect, however, was observed: Pb stem content was lower in the ECM treatment compared to all treatments containing *G. intraradices*. This is in some accordance with findings on willows where colonisation by *H. mesophaeum* resulted in lower HMs stem concentrations, as in the ECM treatment within poplars was colonised only by *H. mesophaeum*. Moreover, the GIN treatment resulted in increased leaf Pb concentration compared to control trees, which contributed to the total above-ground Pb content. Total above-ground metal content differed for all metals studied, with *G. intraradices*. increasing Pb uptake compared to the ECM and C treatments, and the ECM treatment decreasing Cd uptake by tress compared to control trees. These findings again imply *H. mesophaeum* suitability for phytostabilising purpose, at least for Pb and Cd. However, it is

difficult to explain increase in the Zn content in the AE treatment trees compared to the AM treatment as the AE treatment trees were colonised by *H. mesophaeum* and probably *G. intraradices* only, and no significant increase in biomass was observed. In poplars, only colonisation by *H. mesophaeum* gives coherent results in terms of decreased uptake of Pb and Cd. Evidence supports *G. intraradices* being suitable for phytoextraction of Pb: the same isolate of *G. intraradices* as used in this work increased Pb uptake by maize roots (Sudová 2007). *G. intraradices* suitability for phytoextraction may be of particular interest on both experimental sites, since they are both heavily contaminated by Pb. However, the Litavka site is also heavily polluted with Zn, making phytoextraction strategy risky in temrs of plant survival. Nevertheless, some fungi were reported to differ in plant HMs uptake modulation depending on HMs concentrations in soil - even when promoting HM uptake by plant at low HMs soil concentrations, they may act as a barrier when HMs soil concentrations exceed some point (McGrath 2001, Selosse 2003).

#### 9.2.2.2.1. Photosynthesis and photosynthetic pigments content, Raman spectroscopy

The presentthesis studied plant - mycorrhizal - HM interactions also at the level of photosynthesis (e.g. photosythetic rate, photosynthetic pigment contents). Several studies reported impairment of photosynthesis by HMs, and mycorrhizal fungi may improve plant HM tolerance alleviating this deletirious effects of HMs or simply boosting photosynthetic rate by improved nutrition, or hormonal regulation (Gu 2007, Khan 2000). Indeed, several factors were affected by mycorrhizal colonisation such as chlorophyll A to chlorophyll B ratio, stomatal conductivity in both measurments, and photosynthetic rate in the second measurement. However, results for carotenoid concentration obtained by DMF extraction do not correspond with those obtained by Raman spectroscopy. The results obtained by DMF extraction showed no differences while the results obtained by Raman spectroscopy indicated increase in carotenoid content in the ECM and GIN treatments compared to control trees. On contrary, some other factors seem to coincide: for example, water content as explained by PCA from Raman spectra, showed lower values within control plants compared to plants from the GIN and ECM treatments, indeed, such trend was observed when comparing fresh and dry leaf biomass, however, the differences in latter were not significant. Furthermore stomatal conductivity in the second measurement was lower in the ECM and GIN treated plants than in control treatment plants (being significant only between the ECM and control trees), which may be possible explanation of different water content in leaves. This implies beneficial mycorrhizal effect on plant water status or in the case of the ECM - alleviation of water stress induced by Cd accumulation in shoots as reported by

Perfus-Barbeoch et al. (2002). However, the discrepancy in carotenoid content measured in DMF and assessed by the Raman spectroscopy may be atributted to a fact that Raman spectroscopy analyses only the outer leaf tissues whereas DMF extraction involves the whole leaf volume. Furthermore, analysis of photosynthesis related factors were done twice, again disregarding much of the possible dynamics of these processes during experiment duration.

### 9.3. Will this work support further research and/or practical aplications?

The SOD analyses in the present study contributed to a yet limited number of known physiological characteristics of trees inoculated with ectomycorrhizal fungi in interaction with HMs. It surely inspires further research aimed at characterising and quantifying mechanisms involved in HMs tolerance and accumulation.

Selection of ECM partner *in vitro*, followed by the re-synthesis experiment with plant host and AM fungal symbionts involved, seems to be an appropriate approach for inoculum tuning (that is screening a number of candidates to obtain best fungal partner for a selected plant on a given location (Kernaghan 2002). Indeed, *H. mesophaeum* inoculation, resulted in willow trees in expected decrease in at least some of HM contents. As for other fungi selected, apart from the effect of colonisation of poplar by *G. intraradices*, the effects of other fungi could not be evaluated due to contaminations occurred unexpectedly in the DUAL experiment. This, however, encourages further research also with respect for optimization of methods to prevent such drawback. Furthermore, applications of plant - fungal symbioses in field conditions of short rotation coppice plantations may be challenged by several issues, including fungal HMs tolerance differing in *in vitro* and in field conditions (Selosse 2003). Furthermore, interactions with other fungi already present in soil substrate at plantations may interfere with targetted effects of carefully selected mycorrhiza partners. Nevertheless, the use of fast growing trees of the family *Salicaceae* inoculated by mycorrhizal fungi for phytoremediation in SRC plantations is a considerable promise. Apart from long term carbon storage, low cost, high impact amelioration of soil, SRC plantations can serve as a source of renewable energy, which is being demanded more and more (Pulford 2003, Vamerali 2009).

## 10. Conclusions

* Ectomycorrhizal fungi differ in their ability to grow in vitro on media amended with HMs, moreover do they differ in ability to accumulate HMs. These differences allowed us to choose fungal strains usable for further inoculation of fast growing trees serving phytoextraction and phytostabilisation strategies.

* Fungal isolates tested for the effect of HMs on the superoxide dismutase activity showed that fungal isolates exploit this mean of protection against oxidative stress to various extent, which also depends on metal element involved. Moreover, this study showed that growth media amendment with one or mixture of HMs may lead to different response of fungal metabolism, emphasizing the necessity to study also possible interactions between HMs when assessing their effect on organisms.

* Due to unforseen complications (especially contamination), experimental design was rather reduced, not allowing us to answer all the questions asked. Nevertheless colonisation of willow trees by *H. mesophaeum* imply possible use of these two in phytostabilisation techniques, as it increased stem biomass acquisition and decreased Pb and Cd stem concentrations. In poplars similar effect was found with *H. mesophaeum* and opossite with *G. intraradices*. Provided successful inoculation, fungi chosen by *in vitro* screening may yield expected results in phytoremediation techniques.

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