# Chapter 12
# Metal Tolerant Mycorrhizal Plants: A Review from the Perspective on Industrial Waste in Temperate Region

**Katarzyna Turnau, Przemysław Ryszka, and Grzegorz Wojtczak**

**Abstract** The chapter summarizes research carried out on the role of mycorrhizal fungi in phytoremediation of heavy-metal-rich wastes in temperate regions. Symbiotic fungi are an important component of soil microbiota, especially under harsh conditions. Properly developed mutual symbiosis enhances the survival of plants in polluted areas by improving nutrient acquisition and water relations. In addition, mycorrhizal fungi were found to play an important role in heavy metal detoxification and the establishment of vegetation in strongly polluted areas. Fungal strains isolated from old zinc wastes also decrease heavy metal uptake by plants growing on metal rich substrata, limiting the risk of increasing the levels of these elements in the food chain. The effectiveness of the bioremediation techniques depends on the appropriate selection of both the plant and the fungal partners. Plants conventionally introduced in such places disappear relatively soon, while those appearing during natural succession are better adapted to harsh conditions. Symbiotic partners selected on the basis of such research are often the best choice for future phytoremediation technologies. Moreover, mycorrhizas of different types are also helpful in substratum toxicity monitoring. Further improvements can be obtained by optimization of diverse microbiota including various groups of rhizospheric bacteria and shoot endophytes.

**Keywords** Heavy metals • Industrial wastes • Phytoremediation • Phytostabilisation • Phytoextraction

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K. Turnau (✉), P. Ryszka, and G. Wojtczak
Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
e-mail: katarzyna.turnau@uj.edu.pl

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# 1 Introduction

Phytoremediation of metal contaminated areas is attracting increasing interest as a cheaper alternative to chemical methods, more friendly for environment and non-destructive to soil biota. Efficiency of this technology strongly depends on the characteristics of the given site, type of heavy metals in the substratum, climatic conditions, use of native and indigenous plants and microbiota. Our research was focused on post-flotation, heavy metal rich wastes in Southern Poland. Intensive exploitation of the metasomatic ores, found in triassic dolomites, started in the twelfth century (Szuwarzyński 2000; Strzyszcz 2003). The following eight centuries brought increased heavy metal extraction efficiency, but at the same time the risk of wind and water erosion accelerated dramatically, resulting in immediate need of phytostabilisation of the waste. Presently, the ores are subjected to flotation process. Waste material is composed of two fractions. The solid part is used to form tailing ponds into which the liquid phase is dumped. Modern flotation technologies resulted in lower content of heavy metals in the waste, but the material is much more susceptible to wind and water erosion. The toxicity of the waste substratum itself is relatively low as its pH is ranging from 7 to 8, but the waste particles can cause serious atmospheric pollution and subsequently can increase soil toxicity in the surrounding area. The dusts originating from the area often contain above the threshold levels for Zn, Pb, Tl and Cd (Dmowski 2000). Biological reclamation faces several serious difficulties, e.g. plant growth inhibition (Strzyszcz 2003). The density of particles within the sediment is much higher than in natural soils making it impermeable for water and air. This is disadvantageous during both dry and wet periods: even increased precipitation cannot assure appropriate infiltration, while too low porosity disables water recharge by capillary rise from the deeper layers. At the same time such material easily undergoes water or wind erosion (Strzyszcz 2003) and the heap slopes tend to slide down making stabilization efforts even harder. These processes accelerate when the exploitation is finished. When additional watering is stopped the situation changes dramatically. Chemical composition of the waste material is another factor complicating the reclamation of such places, namely low levels of basic ions like $Na^+$, $K^+$, $Mg^{2+}$ and $Cl^-$, almost complete lack of organic matter and significant deficiency of N and P. On the contrary, the carbonate content of the waste substratum exceeds 75% and is usually accompanied by high levels of $Ca^{2+}$ and sulphate ions. Additionally, the substratum contains high levels of heavy metals although alkaline pH of waste substrata highly reduces the availability of potentially toxic elements to plants. The major concern is to keep these metals in place and avoid their transfer into areas with lower pH values.

# 2 Plant Reaction to Heavy Metals

The major toxic effects of transition metals appear to result from: (i) generation of reactive oxygen species (ROS), e.g. by the Fenton reaction (e.g. Gallego et al. 1996; Keightley et al. 2004; Kieffer et al. 2008); (ii) damaging cellular components and interfering with metabolic processes; (iii) binding heavy metals to SH-groups of enzymes and inactivation of their catalytic domains. Individual organisms respond to heavy metals by: (i) prevention of heavy metal uptake (exclusion); (ii) absorption of heavy metals and attenuation of the toxic effect by chelation, covalent bonding, dilution, compartmentalization, extrusion, etc. (amelioration); (iii) production of physico-chemical barriers to protect crucial organs from toxicity (avoidance) (Baker 1987). The toxic effect of metals that entered the cells can be counteracted by: (i) complex organic molecules such as metallothioneins and phytochelatins that are synthesized by the organisms (Cobbett and Goldbrough 2002; Hall 2002); (ii) heavy metal transporters, a broad group of different proteins such a CPx-ATPases for Cu or Cd, ABC- transporters for Cd-transport into the vacuole, ZIP-transporters (ZRT-, IRT-related proteins for Fe or Zn) and Nramp transporters (Hall 2002). Recent advance in proteomic studies discovered also the induction of an array of proteins that are expressed under stressed condition such as chitinases (Van Keulen et al. 2008), the heat shock protein HSP60 (Rios-Arana et al. 2005), proteins of the sulfur metabolism pathway (Roth et al. 2006) and cysteine synthase (Yang et al. 2007).

Not only individual plants, but also whole communities and populations respond to heavy metals. This response involves: (i) succession that includes changes in species composition and diversity and (ii) natural selection that leads to communities of higher tolerance to stress (Baker 1987; Fitter and Hay 1987; Salisbury and Ross 1992). Such processes take long time (Bradshaw and McNeilly 1991; Hoffmann and Parsons 1991). Succession occurs faster than selection and uses a broader range of metabolic processes. Plant populations are capable of the so called "phenotypic plasticity" meaning a range of variation in phenotypes expressed by a single genotype under different environmental conditions (Hoffmann and Parsons 1991). The phenotypic plasticity involves diverse aspects of plant life such as closure of stomata, development of roots, shoots and leaves, flowering and metabolic rate. Depending on genetic variation in species/population, natural selection changes the plasticity level under heavy metal stress and results in selection of resistant genetic variants. Studies by Wierzbicka and Panufnik (1998), Wierzbicka and Potocka (2002), Załęcka and Wierzbicka (2002), Pielichowska and Wierzbicka (2004), Baranowska-Morek and Wierzbicka (2004) and Olko et al. (2008) are the best examples of research of such processes that were observed in plants colonizing 100-years-old wastes rich in heavy metals in Poland.

## 3 Revegetation Technologies Non Assisted by Microorganisms

So far, there are no large scale documented applications of restoration techniques including microorganisms in Central and Eastern Europe. The slopes are often covered with a 20 cm layer of humus, or other material such as spoils from hard coal mining (Aldag and Strzyszcz 1980; Strzyszcz 1983) or material excavated during mining. Such material can be relatively rich and theoretically can be used to grow plants. Clean soil is usually avoided as it is very expensive if the area is big.

Such material is often sliding down the steep slopes and needs to be stabilized in various ways. The introduction of such a layer allows for easier establishment of the vegetation by blocking the factors limiting plant growth and it may diminish the transfer of heavy metals into the plant material. Several experiments were carried out to evaluate the influence of diverse soil amendments to improve the physico-chemical properties of the waste substratum. Among them were bentonite, bituminous emulsion, gigtar and vinacete (Trafas 1996). At least in the first few years following the application, the results were encouraging.

Among trees, the most commonly introduced are birches, pines and poplars. Especially the last ones, belonging to phreatophytes, under moderate climate are genetically predisposed to rooting at up to 12 m (Negri et al. 2003). Roots of this species can be important in improvement of the water supply due to so called "hydraulic lift", the process of water movement from relatively moist deeper layers and its release into the shallower layer (Richards and Caldwell 1987; Caldwell et al. 1998). The role of hydraulic lift in moderate environment was discussed by Dawson (1993). It is expected that this phenomenon can result in improved rhizosphere processes, especially activity and life span of fine roots and associated microorganisms (Lacombe et al. 2009). This might be also the mechanism by which the mobilisation of nutriens can be improved (Caldwell et al. 1998).

Comparatively well establishing on metal rich industrial wastes are shrubs such as *Hippophaë rhamnoides*, *Eleagnus angustifolius*, *Robinia pseudoacacia* and *Physocarpus opulifolius*. Most often used are grasses including: *Festuca ovina*, *F. rubra, Phleum pratense, Poa pratensis, Lolium perenne, Dactylis glomerata, Arrhenatherum elatius, Bromus inermis* and *Festuca pratensis*. The grasses are often supplemented with *Medicago sativa* and *Trifolium repens*. According to long term observations carried out on zinc wastes' populations of introduced non-woody species, their number dramatically decreases with time and almost none of them is left on the 20–30 years old wastes. Much more stable are plants that appear on the wastes spontaneously, but, it takes a long time till they establish and form stable communities (Ryszka and Turnau 2007).

## 4 Why Mycorrhizal Fungi Are Important in Restoration of Metal Rich Wastes

In natural soil, nutrients are most abundant in the surface layer. The industrial waste substratum, if not fertilized or covered with the humus layer, is originally uniformly poor. The nutrient distribution becomes patchy when decaying plant or animal material such as feces are deposited. Also dead microorganisms (bacteria and fungal hyphae) can be an important source of nutrients (Tibbett 2000). Plant roots have to reach these patches, at the same time competing for nutrients with other organisms. While plants growing in nutrient-rich soils do not need efficient root systems, the so-called "hidden half" of the plant has to be built very efficiently to survive under industrial waste condition. In this nutrient-poor environment the mycorrhizal association proves to be very useful. Mycorrhizal fungi enhance the root absorption area up to 47-fold (Smith and Read 1997) and provide access to nutrients and water otherwise not accessible for plants (Cui and Nobel 1992; George et al. 1992; Nadian et al. 1997). The fungi also enhance the substratum structure by formation of the hyphal net (Jasper et al. 1989; Smith et al. 1998; Jeffries et al. 2003). In general fungi are known to be able to accumulate significant amounts of heavy metals (Gadd 1993) varying from a few percentage to 20% of dry mass (Tobin et al. 1984) suggesting that microbial biomass may affect the mobility of metals in the soil system. Processes such as metabolism dependent (bioaccumulation) or independent (biosorption) can be involved in removal of metals from the wastes (Gadd 1993). In the second case, both living and dead biomass can be active (Volesky and Holan 1995). Cell wall components of fungal mycelium contain free amino, hydroxyl, carboxyl and other groups (Gadd 1993), that can be very efficient in binding heavy metals. The ability to chelate and retain heavy metals within cell walls is so high that some saprobic fungi producing large biomass are used as commercial biosorbants (Mullen et al. 1992; Kapoor and Virarghavan 1995; Morley and Gadd 1995). The similar role of arbuscular mycorrhizal fungi was demonstrated (Joner et al. 2000; Gonzales-Chavez et al. 2002, 2004). Janouskova et al. (2006) showed that extramatrical mycelium of AMF is capable to accumulate ten to twenty times more Cd per unit biomass than tobacco roots. Abuscular mycorrhizal fungi (AMF) produce glomalin (glomalin-related protein, GRSP), first believed to be a hydrophobin, later identified as likely a 60-kDa heat shock protein homolog (Gadkar and Rillig 2006; Rillig et al. 2007) that seems to be efficient in sequestering Cu, Cd, Zn (Gonzales-Chavez et al. 2004, 2009; Cornejo et al. 2008) and also Fe, Pb, Mn (Chern et al. 2007; Vodnik et al. 2008). Immunolocalization of glomalin in the AMF mycelium and spores has shown the presence of glomalin mainly in the inner cell wall layer L2 and L3 while it is less abundant in the outer layer L1 (Purin and Rillig 2008). This result might be, however, an artifact due to chemical fixation of the material and release of the glomalin in a similar way as heavy metals are released during chemical fixation (as explained by Turnau and Kottke 2005). Interestingly, this substance is also present outside the fungal structures and being deposited in the environment (Driver et al. 2005), similarly to compounds produced by saprobic fungi and active in formation of nanoparticles. Manceau et al. (2008), in his pioneer studies on the distribution of Zn and Cu in roots and mycorrhizal hyphae, used techniques such as micro-extended X-ray absorption fine structure (μEXAFS) spectroscopy with the addition of micro-X-ray fluorescence (μXRF). Besides GRSP also phyllosilicate were shown to chelate Zn. The sequestration and spatial distribution of Zn was studied with μSXRF (micro synchrotron based X-ray fluorescence) and μEXAFS by Sarret et al. (2003) and these techniques showed that while in roots Zn is mainly associated with malate, in the mycelium it is chelated by phyllosilicate that is most probably found on the surface of the mycelium and not inside. Both results need further studies.

According to Joner and Leyval (1997) the efficiency of protection against heavy metals depends on the given AMF isolate, but generally the transfer from the soil into the plant is restricted by metal immobilization in extraradical mycelium.

These authors have also shown that no inhibition of mycelium growth was observed even at 20 mg of $NH_4NO_3$-extractable Cd $kg^{-1}$ of substratum. MeT-like sequences were identified in the arbuscular fungus *Gigaspora rosea* (Stommel et al. 2001) although the metal sequestration capacity and actual MeT-like nature was not determined till recently. The identification and functional characterization of an MeT-encoding gene from *G. margarita* was demonstrated by Lanfranco et al. (2002) and in addition the differences in gene expression in symbiotic and pre-symbiotic stages were shown.

Heavy metal distribution within mycorrhizal roots compared to nonmycorrhizal were investigated by various groups using techniques such as EELS, EDAX, SIMS, LAMMA and PIXE. In general, the cortex where the fungal structures are formed was selectively enriched with Fe, Ni and Zn (Turnau et al. 1993; Kaldorf et al. 1999; Orłowska et al. 2008). Ultrastructural localization of metals in extramatrical mycelium and spores (Gonzales-Guerrero et al. 2008) confirmed that heavy metals were mainly localized within cell wall and in the vacuoles. Cu mainly accumulated in the spore vacuoles while Cd in vacuoles of the mycelium. (Ferrol et al. 2009) studying extraradical spores suggested that Cu might be accumulated within some spores in high concentrations, and this might be a novel mechanism to store excess of this element.

Mycorrhizal fungal activity also influences qualitatively and quantitatively the microbial populations of the mycorrhizosphere. They are accompanied by bacteria such as legume symbiotic nodular bacteria, plant growth promoting rhizobacteria (PGPR), mycorrhiza helper bacteria (MHB) and saprobic fungi. As these organisms influence plants either by interactions with abiotic and biotic components of the soil, or by stimulating plant growth through the production of vitamins and hormones, they should be included in the optimisation of the restoration processes. Unfortunately, so far very little has been done in this respect.

## 5 Role of Arbuscular Mycorrhizal Fungi in Revegetation of Metal Rich Wastes

Mycorrhizal symbiosis is an important component of industrial waste habitats as the conditions are extremely harsh for plant and microbial growth. Inappropriate management of such areas results in low availability of mycorrhizal inoculum within the deposited substratum, originally devoid of AMF propagules. The first possible source of mycorrhizal inoculum is the soil or humus transferred from another area to cover the waste material. Fertilization aiming to improve the growth of freshly sown grasses is detrimental to most or all members of Glomeromycota. Although the transferred soil contains a seed bank including AM hosts, they are usually unable to survive and die shortly after germination. The exceptions are *Molinia caerulea* (Poaceae) and *Anthyllis vulneraria* (Fabaceae). These plants are accompanied by various rhizosphere microorganisms such as AMF, diazotrophs and rhizobacteria (Reinhold-Hurek and Hurek 1998; Hamelin et al. 2002) and they are well adapted to dry and nutrient poor substrata. These two species are able to form stable patches of vegetation, although their area is not increasing fast due to the prevalence of vegetative reproduction under this particular situation. Under restoration practices commercial cultivars of grasses are usually sown on the introduced soil in favour of high biomass production at high fertilizer input. Being independent upon mycorrhizal symbiosis, such plants are able to grow there as long as the introduced soil is not removed by water erosion. Such grasses disappear either leaving uncolonized areas or spontaneous plant colonizers completely displace them (unpublished data).

Zn-Pb heaps are an ideal object of plant natural succession studies. The oldest parts are located at its basis and its age decreases with height enabling further estimations of age of the plant communities. The first plants appearing on the bare surfaces of wastes were nonmycorrhizal members of Caryophyllaceae (*Cerastium* spp., *Silene vulgaris*) and Brassicaceae (*Cardaminopsis arenosa*). They were, however, incapable of forming a dense cover of the waste substratum. These were followed by facultative mycorrhizal species. Grasses such as *Molinia caerulea, Agrostis gigantea, Bromus inermis, Calamagrostis epigejos, Corynephorus canescens, Dactylis glomerata, Festuca tenuifolia* and *F. trachyphylla*, all turned out to be very expansive and promising in terms of phytostabilisation; similarly to other members of this particular plant family they were also shown to be strongly colonised by mycorrhizal fungi when collected from the zinc wastes (Ryszka and Turnau 2007), with some species (e.g. *Festuca tenuifolia*) exhibiting twofold to fivefold higher difference in mycorrhizal colonization and arbuscular richness when compared to unpolluted sites. Nevertheless, concerning plant growth, mycorrhizal dependence was not equal among all grass species. In fact, only 45% exhibited increased growth following mycorrhization, while 38% did not react at all and a negative effect was observed in the remaining 17%. This results are in accordance with Newsham and Watkinson (1998) who showed that grasses can respond differently to mycorrhizal symbiosis. The observed negative effect can be attributed to increased transfer of carbon compounds from the host to the fungus, low photosynthetic activity and increased activity of the mycelium. Such situation was already shown in the case of *Lolium perenne* (Buwalda and Goh 1982). It is not always possible to observe a direct positive influence of AMF symbiosis. Some effects of symbiosis are visible only after a long time, in subsequent generations or may concern seedlings survival rates.

It is noteworthy that exceptionally low levels of mycorrhizal colonization in some grasses were observed in individuals in places flooded by fresh waste liquid from broken pipes. This shows that the waste material is harmful when deposited, although, due to the neutral pH of the substratum, the toxicity is not extremely high. The main source of stress under these conditions is rather extreme water and nutrient deficiency. This was clearly shown in the case of zinc mound located in Bolesław (Southern Poland), where a part of the waste was covered with material excavated from metal ores, but containing similar metal content to post-flotation substratum. The unprocessed substratum contained much more N and P and had much higher water holding capacity. Therefore, a much better development of plants and higher viability of AMF spores (Ryszka and Turnau 2007) was observed.

Several decades of spontaneous succession led to the creation of communities consisting of very rare and interesting species such as *Biscutella laevigata, Silene vulgaris, Gypsophila fastigiata, Cerastium arvense* and *Armeria maritima subsp. halleri* (Grodzińska et al. 2000). The last species is considered to be a metallophyte (Szafer 1959). Among the listed species, the most astonishing was documenting of mycorrhizal symbiosis in *Biscutella laevigata* (Orłowska et al. 2002) since this plant belongs to Brassicaceae (Cruciferae) family, widely accepted to consist of non-mycorrhizal species. Well developed arbuscules were found within the thin lateral roots, while the main (thick) root remained uncolonized. According to DeMars and Boerner (1996), members of this particular plant family can possibly be colonised by AM fungi, but for unknown reason the arbuscules which are the most important criterion of a functional mycorrhiza do not develop under glasshouse conditions. This hypothesis was indeed confirmed by Orłowska et al. (2002) who during a 2-year cultivation assay was not able to observe arbuscules. On the other hand, arbuscules were found in *B. laevigata* growing in the wild, both in Tatra Mountains (Orłowska et al. 2002) and Alps (H. Bothe personal communication).

Other members of plant communities appearing on the Zn-waste consist of strongly mycorrhizal species. In the younger waste areas *Festuca ovina, Plantago lanceolata*, and *Viola tricolor* are the most common plants; they may serve as a source of mycorrhizal inoculum. Other species like *Thymus pulegioides* and *T. serpyllum* form dense tufts on bare ground, while members of Fabaceae possess the ability to form triple symbiosis with both AMF and nitrogen-fixing bacteria. Other plant species, less common or visible but still worth noticing are *Gentianella germanica* and *Linum catharticum*. Both strongly mycorrhizal and considered as extremely rare in the wild. It is also worth pointing out that within a given plant community on industrial waste one can find species originating from entirely different environments. *Molinia caerulea, Carex flacca, Sanguisorba officinalis, Valeriana officinalis, Phragmites australis* – all are almost exclusively found in swamp-like habitats. Those plants are mostly mycorrhizal and often resistant to heavy metals due to increased content of Si in their tissues (Kabata-Pendias and Pendias 2001). They can easily survive periodical changes of water level. Another group belongs to those typical for xerothermic grassland species: *Poa compressa, Scabiosa ochroleuca, Sedum acre, Dianthus carthusianorum, Linum catharticum, Thymus pulegioides* – most developing mycorrhizal associations.

In terms of practical applications, perennial plants are important for restoration as they can cover the area in a relatively short time, although it is limited by low viability of seeds produced on zinc wastes.

Fungal symbionts can be used furthermore in biomonitoring of ecosystem soil quality and effectiveness of restoration practices (Turnau and Haselwandter 2002; Leyval et al. 2002; Orłowska et al. 2002). Such applications require an appropriate plant host and *Plantago lanceolata* seems to be a good choice. This plant is not only widespread, but also tolerant to increased levels of Pb (Wu and Antonovics 1976), Sb (Baroni et al. 2000) and PAHs (Bakker et al. 1999). *P. lanceolata* is mycorrhizal in greenhouse conditions (Walker and Vestberg 1994) and can be propagated vegetatively (Wu and Antonovics 1976). Mycorrhizal parameters like relative mycorrhizal colonisation, relative arbuscular formation and arbuscule richness can be used to assess the differences between restored and the non-restored areas, under the condition that the same fungal strain is used. Additionally, the fungal strains used must not be adapted to conditions present on industrial wastes. The main limitation of such approach is therefore a narrow group of fungal strains that can be used in the analyses. This can be easily overcome with *P. lanceolata* as a host as it can host a broad spectrum of AM species. With this system it was possible to indicate the toxicity of various sites of Polish industrial wastes (Orłowska et al. 2005b). Our results closely corresponded to those obtained by means of chemical analysis of heavy metal "availability" (i.e. metals extraction in $Ca(NO_3)_2$ and $NH_4NO_3$). A slightly different system for testing soil toxicity also employing AMF was developed by Leyval and was based on *Glomus mosseae* spore germination rates, but it seems that both approaches lead to similar results. Soil quality can be also accessed via examination of several morphological changes in the mycelium such as thick septa, thickenings of the mycelium and changes in shape of arbuscules (Orłowska et al. 2005b).

## 6 Abundance and Diversity of Arbuscular Mycorrhizal Fungi in the Wastes

The number of spores in the substratum of different age vary from 0 to 100 spores per 100 g in revegetated areas. The restoration practices, including scarification and planting trees or covering the original substratum with material excavated from the part of mine that has a higher nutrient content (N and P) results at least in doubling the number of spores and the percentage of alive spores (E. Orłowska personal communication).

The diversity of AMF on metal rich substrata in Europe is mostly limited to species belonging to genus *Glomus*. The diversity of AMF on industrial wastes seems to be very dependent on the number of conditions including substratum characteristics and the presence of carriers that could transfer the spores into the waste. In a recent study by Gonzales-Chavez et al. (2009) on a Cd rich slag heap in Mexico, spores of *Gigaspora, Glomus. Scutellospora* and *Acaulospora* were found. Spores and even sporocarps were found in vegetal residues and in animal feces. As suggested by the authors, the propagules were efficiently spread by mesofauna. The slag was spontaneously colonized by invasive plant species that were almost all colonized by AMF. This seems to be, however, an extraordinary situation. While studying wastes from Poland the spread of fungal inoculum is very slow, although, when the propagules are already on the waste, spreading might be enhanced by animals such as ants. AMF diversity in Polish wastes was studied in the Boleslaw calamine area and on the zinc wastes of ZG Trzebionka. Morphological analysis of spores from 100-year-old calamines has shown the presence of *G. aggregatum, G. constrictum, G. fasciculatum, G. pansihalos* and *Entrophospora* sp. (Pawlowska et al. 1996). Nested polymerase chain reaction (PCR) with taxon-specific primers was used to identify the species *G. moseae, G. intraradices, G. claroideum, G. gerdemannii, Glomus occultum* (Turnau et al. 2001) and morphological analysis added *G. fasciculatum* and *G. aggregatum* to the list of AMF species of zinc wastes of ZG Trzebionka (Turnau et al. 2001). Several, but not all of these fungi are by now available in monocultures. The roots of *Fragaria vesca* from Trzebionka waste were used to evaluate which fungus is the most effective in mycorrhizal colonisation. The most frequent fungus was *G. gerdemannii*, which, however, was not obtained in trap cultures. Slightly less frequent were *G. claroideum* and *G. occultum* while the least common were *G. intraradices* and *G. mosseae*. The easiest to get in trap cultures was *G. claroideum*. These studies were followed by the localisation of heavy metals using rhodizoniate (Turnau 1998; Turnau et al. 2001). This stain was used to evaluate the differences between fungal species in their ability to absorb metals. The experiment has shown that fungi isolated from industrial wastes such as *G. intraradices* and *G. clarum* reduce metal uptake into the roots and shoots of *P. lanceolata*, while compared to strains originating from soils of low HM content (Orłowska et al. 2005a). In addition, in spores of a *G. intraradices* isolated from zinc wastes of ZG Trzebionka, characteristic depositions within periplasmic space, between the inner layer of the wall and plazmolemma, were observed. The accumulation of heavy metals within these depositions were confirmed by EDS analysis coupled with SEM. The method was also used to estimate the percentage of the mycelia that show increased metal accumulation. In the case of *Euphorbia cyparissias* (Turnau 1998) about 80% of the intraradical mycelium showed an increased content of heavy metals. However, at the same time, the number of arbuscules was slightly lower than in mycelium containing lower levels of these elements. On the contrary, *Fragaria vesca* roots from the same industrial wastes that were selected for molecular studies on fungal identity, when stained with rhodizoniate, have shown that increased metal accumulation is visible only in case of *G. mosseae* that is one of the least efficient colonizers (below 10%). These results clearly show that there are differences not only between fungi but also between plant species in their preference concerning symbiotic associations. Under laboratory conditions, for practical reasons, we usually inoculate the plants with one species, while under field conditions the plants are inhabited by several different strains or species. Studying the metal uptake by plants is one of those examples that has to be carried out first with the use of the single species.

## 7 Metal Accumulation in Plants Growing on Industrial Wastes

The response of the plant to soil pollution is known to vary depending on the AMF isolate and species (Streitwolf-Engel et al. 1997; van der Heijden et al. 1998). AMF were already reported to both decrease and increase HM uptake by plants (Leyval et al. 1997; Khan et al. 2000). Therefore, the selection of appropriate plant and fungal genotypes, can provide a potential for decreasing health hazards during crop production and improve sustainable agriculture and phytoremediation technologies, including phytoextraction (Turnau and Mesjasz-Przybylowicz 2003; Jurkiewicz et al. 2004). To demonstrate the importance of the selection of the appropriate plant variety, 15 cultivars of *Zea mays* were cultivated on Zn-Pb waste substratum with *Glomus intraradices*. Heavy metal content in plant material varied significantly between the varieties. A different experiment, in which *Plantago lanceolata* was grown in pots filled with Zn-Pb substratum demonstrated the influence of various AMF strains on Cd, Zn and Pb uptake (Orłowska et al. 2005a). Heavy metal uptake clearly depended on the fungal strain used. Plants inoculated with AMF strains originating from soils not affected by heavy metals had higher metal concentrations in tissues than plants inoculated with strains from polluted areas. Similar results have been obtained by Sudova et al. (2008) while comparing selected fungal isolates and plant clones from contaminated and noncontaminated substrates. Also results by Redon et al. (2008) confirmed that fungi from polluted soils increase metal tolerance of plants by increasing plant biomass and reduce metal toxicity. Fungi isolated from polluted sites were also found to be effective to reduce shoot Cd concentration. In *Medicago truncatula* also the interactions with rhizobacteria were influencing metal transfer. As suggested by Lingua et al. (2008), metal uptake can differ depending on the use of particular fungal strains and particular plant genotypes involved in the plant-fungal interaction. These findings are very important as plants growing on wastes often contain high levels of metals in aboveground tissues, and this can create the risk of metal transfer into the food chain, as the industrial wastes are often inhabited by a broad range of wild animals. The introduction of proper fungi can decrease metal uptake into the shoots, while the available pool of metals could be stored within the root system.

The success of the introduction of the selected fungus into the non-polluted or moderately polluted soils could be low due to the presence of native fungi that are better adapted to the particular soil characteristics. However, zinc wastes at the beginning of the succession are often devoid of AMF and inoculation seems to be reasonable. Still, the practical questions related to the application of the AMF await further experiments and studies. There were so far several experiments showing weak points of the technology. Among them the use of annual plants has been shown not to be the best solution; promising perennial plants are already known but they require optimization of propagation techniques in order to obtain suitable amounts of material. The metal content of the waste is not the most important problem for the revegetation, other issues such as poor water holding capacity, water/wind erosion and mineral deficiency have to be addressed. The water holding capacity can be improved e.g. by the use of water-binding gels ("hydrogels") applied simultaneously with the inoculum, which prevents the inoculum from being removed by the wind. The application of inorganic fertilizers should be replaced by compost or manure. The use of sewage sludge might be a good choice, but may led to increased toxicity (e.g. Cu). Therefore, the dosage needs to be optimized in order to sustain the development of the belowground microbial consortia.

## 8 Introduction of Plants from Xerothermic Grasslands into the Metal Rich Wastes: New Approach

The establishment of an appropriate plant cover of reasonable diversity in order to reduce erosion and further contamination of areas surrounding the wastes requires further efforts (Turnau et al. 2008). On the basis of long-term observations it was observed that among plants that spontaneously colonize heavy metal rich wastes are species that normally occur on calcareous or/and sandy xerothermic grasslands, such as *Potentilla arenaria, Hieracium pilosella, Anthyllis vulneraria*. In 2003, first attempts were carried out to experimentally introduce *Anemone sylvestris* and *Primula veris* into the Zn-Pb wastes in Poland. Seedlings of these plants accompanied by microbiota and original soil from xerothermic grasslands were introduced into the wastes, and were found to establish successfully. During further studies, almost 20 xerothermic grassland species were first grown in the laboratory on industrial substratum and their survival rates were monitored. These experiment showed that most of the plants introduced as seeds are not able to germinate and develop on industrial wastes. The seedlings have to be cultivated on a mixture of clean soil and industrial waste substratum and equipped with mycorrhizal fungi – this is the most important condition for mycorrhizal species. Non-inoculated plants moved to field conditions of the waste die within a few days or weeks. The inoculum used in this experiment originated either from the xerothermic grasslands or from industrial wastes. Plants colonized by mycorrhizal fungi established well on the experimental plots (Turnau et al. 2008). The results suggest that inocula from xerothermic grasslands are well suited for improving plant growth on industrial wastes. Although in several cases the photosynthetic activity of plants from the waste was lower than in plants growing at natural sites, almost all plants survived and produced seeds. In all experiments the plant vitality was estimated on the basis of chlorophyll *a* fluorescence. This method was useful to show differences between the various waste substrata, different inocula and coexisting plant species. The interactions between mycorrhizal and non-mycorrhizal plants were studied under greenhouse conditions and at least no negative effect of this coexistence was observed (Turnau et al. 2008). The next step of this experiment was to check whether the plants growing on industrial wastes are accumulating heavy metals that would create a health risk for potential grazers. This was done using shoots of plants that survived already the third vegetation season, using Total Reflection X-ray Fluorescence (TXRF). The data were compared to plants that were collected in xerothermic grasslands (Turnau et al. unpublished). Among all introduced plants, three grass species (*Melica transsilvanica, Bromus inermis, Elymus hispidus*) and one legume (*Anthyllis vulneraria*) turned out to be the most suitable for phytostabilisation. The highest metal accumulation among all plants was found in *Verbascum thapsus*, one of few species that efficiently started to produce seeds that germinated successfully. Higher levels of heavy metals (Zn, Y, As, Pb, Cu) in plants grown on the waste were usually accompanied by higher Ca, suggesting a possible role of this element in detoxification mechanisms.

## 9 Attenuation of Stress by Mycorrhizal Fungi and Monitoring

The possible mechanisms involved are: (i) dilution of HM concentration by increased growth (Liu et al. 2005); (ii) barrier effect of mycorrhizal fungi; (iii) metal sequestration within fungal mycelium due to phytochelatin and/or melathionin production; (iv) precipitation of metals on the surface of extraradical mycelium; (v) altering the metabolism e.g. by increased production of proline in stress conditions (Bassi and Sharma 1993; Costa and Morel 1994; Schat and Vooijs 1997; Chen and Dickman 2005) that was shown to be a potent scavenger of ROS. As suggested by Rodriguez and Redman (2005), symbiotic fungi could potentially activate the biosynthesis of proline. None of these mechanisms were so far proven with molecular techniques. Recently, the molecular responses of AM plants to Cd at proteomic level were approached by Aloui et al. (2009). Down-regulation of several Cd stress responsive proteins was found. Out of 26 mycorrhiza-related proteins, only six displayed differences following Cd application and as suggested by the authors "a part of symbiotic program may be recruited to counteract Cd toxicity through the mycorrhiza dependent synthesis of proteins having functions putatively involved in alleviating oxidative damages". Fester and Hause (2005) suggested that increased levels of antioxidant enzymes and nonenzymatic antioxidants found in mycorrhizal plants is probably linked to arbuscule senescence, and this may protect against oxidative damage resulting from Cd presence. The effect of AMF can be further improved by introduction of organic matter into the nutrient-poor soil and saprobic organisms (Azcón et al. 2009a, b).

## 10 Use of Photosynthesis to Show Adaptation of the Plant to Survive

The cost of maintenance of AM symbiosis, similarly to symbiosis of legume plants with rhizobia, can be as high as 16% of photosynthetic carbon. As recently calculated by Kaschuk et al. (2009), rhizobia and AMF improve photosynthesis by 28% and 14% respectively and by 51% if they act together and the rate of photosynthesis increases more than the C cost of these symbioses. The authors proposed that the sink stimulation represent an adaptation mechanism that allow the plant to take advantage of the microsymbionts. This is the result of removing the limitation of rubisco activity and electron transport rates through increased leaf N and P mass fraction and removal of the triose-P utilization limitation of photosynthesis (literature cited in Kaschuk et al. 2009). Microsymbionts can become even more important under heavy metal stress, when several physiological processes such as plant growth, photosynthesis and finally the yield are reduced (Jamal et al. 2006). Excess metals affects the absorption of nutrients (Barbosa et al 2007), reduce pigment content, alter chloroplast morphology. For monitoring plants on the industrial wastes, both under field and laboratory conditions, a fast, simple and non-invasive method to measure plant vitality is needed. This can be met by the Handy PEA and analyses of the OJIP chlorophyll *a* fluorescence transients (Strasser et al. 2004), suitable also for screening the beneficial role of symbiosis (Tsimilli-Michael et al. 2000; Tsimilli-Michael and Strasser 2008; Zubek et al. 2009).

## 11 Use of Hyperaccumulators in Phytoremediation of Areas Surrounding Industrial Tailings

The discovery of hyperaccumulating plants lead to the use of plants in remediation and to establishment of the phytoremediation as a new technique. Presently, over 400 species are known to be able to accumulate heavy metals at levels several times higher than non-accumulators (Baker et al. 2000; Verbruggen et al. 2009). It is a convenient and economically feasible technique to extract precious metals such as nickel; However, the use of hyperaccumulators in places such as industrial wastes, where the content of metals is very high would take a very long time. The options and possibilities of phytoextraction have been recently reviewed by Ernst (2005). Microbes such as mycorrhizal fungi were also found to be useful in phytoextraction of nickel (Turnau and Mesjasz-Przybylowicz 2003; Wu et al. 2007; Lopes de Andrade et al. 2008).

## 12 Conclusions

Phytoremediation of heavy metal contaminated areas is a very complex aim and it cannot be reached only by using a single plant species (what is often the case of large industrial wastes). To stimulate and to build a sustainable plant community, a selected array of various plant species is needed that can stimulate the wide range of soil biota. Industrial wastes are often limited by insufficient microbial populations (Haferburg and Kothe 2007). As shown above there are many possibilities to select appropriate strains of fungi, however, for each particular waste it seems to be necessary to optimize both, AMF and plant species/varieties, clones to create sustainable plant communities. There are still many additional possibilities to improve plant growth by adding inoculation with N-fixing bacteria, actinomycetes, cyanobacteria or shoot endophytes as recently reviewed by Lafferty Doty (2008). More work is therefore needed on plant and microbe selection, optimization of irrigation and amendments, evaluation of root capability and estimation of the risk connected with various ranges of heavy metal uptake into the shoots. More care should be also paid to mixed application of herbs and trees. While trees could influence water uptake from deeper layers of substratum, herbs building abundant AMF could respond by higher substratum aggregation (Hallett et al. 2009).

Phytostabilization should be the way to protect the wastes from erosion and in the meantime further technologies concerning the recovery of metals from solids should be developed, as reviewed by Krebs et al. (1997).

Optimizing practical applications of phytoremediation may result not only in stabilization of the ground but can also create an interesting area that supports the survival of rare and interesting plants. Still, this kind of depositions should be always carefully monitored and should never be considered as safe and stable.

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