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Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects

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Abstract High concentrations of heavy metals in soil have an adverse effect on micro-organisms and microbial processes. Among soil microorganisms, mycorrhizal fungi are the only ones providing a direct link between soil and roots, and can therefore be of great importance in heavy metal availability and toxicity to plants. This review discusses various aspects of the interactions between heavy metals and mycorrhizal fungi, including the effects of heavy metals on the occurrence of mycorrhizal fungi, heavy metal tolerance in these micro-organisms, and their effect on metal uptake and transfer to plants. Mechanisms involved in metal tolerance, uptake and accumulation by mycorrhizal hyphae and by endo- or ectomycorrhizae are covered. The possible use of mycorrhizal fungi as bioremediation agents in polluted soils or as bioindicators of pollution is also discussed.

Key words Arbuscular mycorrhizae · Ectomycorrhizas · Ericoid mycorrhizas · Heavy metals · Soils

Introduction

Pollution of the biosphere with toxic metals due to man-made activities poses a major environmental and human health problem. In addition to metals of geochemical origin, sometimes reaching high concentrations (Jeng and Bergseth 1992), the sources of metals in soil are diverse, including burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments, the use of pigments and batteries (Darbon et al. 1992). These metals are commonly called heavy metals, although this term strictly refers to metallic elements with a specific mass higher than 5 g cm^{-3} able to form sulphides (Adriano 1986). Trace metals would be a more correct term, since the latter is based only on concentration ($<0.1\%$ in soil or 100 mg kg^{-1} in dry matter of biological samples). However, the term heavy metal is generally used and accepted in environmental studies, and it will be employed here. Some of the metals are micronutrients necessary for plant growth, such as Zn, Cu, Mn, Ni and Co (Marschner 1995), while others have no known biological function, such as Cd, Pb and Hg.

Metals in soil are present as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides, or they may form part of the structure of silicate minerals (indigenous soil content). The toxicity of metals in soil depends on their bioavailability, defined as their ability to be transferred from a soil compartment to a living organism (Juste 1988). According to Berthelin et al. (1995), metal bioavailability is a function not only of their total concentration but also of physico-chemical (e.g. pH, Eh, organic matter, clay content) and biological (e.g. biosorption, bioaccumulation and solubilization) factors. Different techniques are used to estimate the bioavailability of metals in soil, including chemical extractions (Sauerbeck and Styperek 1985) and biological tests using plants or micro-organisms (Hertz 1991).

High metal concentrations in soil are toxic to bacteria and fungi. Metal tolerance in soil micro-organisms has been studied in the context of removing metals from polluted soils, but also to provide a biological understanding of the adaptation of living organisms to extreme environments. The effect of soil micro-organisms

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on the availability of metals in soil has also been studied extensively, e.g. in a geochemical context (Krumbein 1983). The metal-solubilizing potential of microorganisms has even attracted biotechnological interest as it represents the biological basis for microbial leaching and biohydrometallurgy (Torma 1988; Rossi 1990). Mycorrhizae are integral, functioning parts of plant roots and the fungi involved provide a direct link between soil and roots. The influence of mycorrhizae on plant nutrition is thought to be greater for elements with narrow diffusion zones around plant roots, such as P and most heavy metals (Lambert et al. 1979). Therefore, in interactions between mycorrhizae and metals, two aspects must be considered (Fig. 1): the effect of metals on arbuscular mycorrhizal (AM) fungal populations and their tolerance to metals, and the effect of the fungi on the availability and transfer of metals to the plant. This review will present different aspects of interactions between heavy metals and ectomycorrhizae, AM, and ericoid mycorrhizal fungi. Heavy metals are considered here only as toxic elements at high concentrations and not as micronutrients.

Mycorrhizal fungal development in heavy-metal-polluted soil

Initial colonizers of heavily disturbed soils are often plant species which can tolerate extreme conditions and tend to be non-mycotrophic (Shetty et al. 1994). These species increase the soil organic matter content, improve the soil microclimate and are conducive to the establishment of plant species of stronger mycorrhizal dependence.

Ectomycorrhiza

Little is known about the density and vitality of ectomycorrhizae and their contribution to the fungal biomass and activity in polluted soils. In general, it is believed that pollution inhibits mycorrhiza development (Schneider et al. 1989; Perrin and Estivalet 1990). Some work has been done on ectomycorrhizal fungi colonizing heavily polluted areas. Monitoring field studies in Sweden carried out along a complex heavy metal pollution gradient revealed that the number of fruitbodies and fruiting species decreased strongly with increasing pollution (Rühling and Söderström 1990). Heavy metal stress may also lead to a decrease in fruitbody abundance for some species and an increase for others that are sometimes rare in non-polluted ecosystems (Rühling et al. 1984). However, fruitbody frequency may not necessarily reflect the actual activity of the organisms in the soil system, or their functional structure.

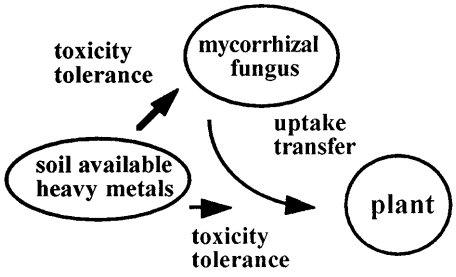


Fig. 1 Relationship between bioavailability of heavy metals in soil and their effects on plants and mycorrhizal fungi

AM fungi

In mine spoils heavily polluted with metals (up to 8.3% Zn and 863 $\mu g\ g^{-1}$ Cd), Gildon and Tinker (1981) found 35% colonized roots of clover (growing naturally on these soils). Diaz and Honrubia (1993) used *Medicago sativa* to show that mine spoils and waste sediments containing Zn and Pb (up to 236 and 456 mg kg^{-1} DTPA-extractable Zn and Pb, respectively) had a mycorrhizal infection potential although the number of AM fungal spores was lower than in an adjacent soil not altered by mining activity. Large variation in spore densities was observed in mine spoils in Canada (390–2070 spores in 100 g dry substratum; Zak et al. 1982). Mycorrhizae were also observed in slender wheatgrass [*Agropyron trachycaulum* (Link)] planted on a subalpine coalmine spoil and on oil-sand tailings amended with peat containing AM fungal propagules, but not when the same species was planted on the unamended oil-sand tailings, revealing them to be devoid of AM fungi (Zak and Parkinson 1982). These results suggested poor or absent mycorrhizal inoculum in some of the mine spoils, which could explain the lack of mycorrhizal colonization. However, mycorrhizal rather than non-mycorrhizal grasses could colonize polluted mining sites (Shetty et al. 1994), suggesting that heavy metal tolerance or other beneficial effects were conferred by the mycorrhizal symbiosis.

Mycorrhizal *Festuca rubra* and *Calamagrostis epigejos* occurred on coastal dunes contaminated by atmospheric deposition from a blast furnace (Dueck et al. 1986). On a Zn- and Cd-contaminated site close to a zinc refinery, *Agrostis capillaris* was extensively colonized by AM fungi, while a low percentage of colonization was observed beside an old copper mine, suggesting a fungitoxic effect of copper (Griffioen et al. 1994). With the exception of seasonal variation, there was no significant difference in mycorrhizal root colonization between three populations of *A. capillaris* growing on a sandy soil polluted by a smelter and on limestone-derived clay with or without metals of natural origin (Ietswaart et al. 1992).

High levels of mycorrhizal colonization were also observed in agricultural soils contaminated with metals of different origins, including atmospheric deposition from a smelter and sludge amendments (Weissenhorn et al. 1995a, b); occasionally, mycorrhizal colonization was delayed (Koomen et al. 1990).

Metal-tolerant *Oxalis acetosella* plants colonizing acidic forest soils with low pH (characterized by accumulation of organic matter) treated with Cd-, Zn- and Pb-containing industrial dusts showed even higher mycorrhizal colonization than on non-treated soils (Turnau et al. 1996a).

The fact that mycorrhizal colonization occurred in most of these in situ observations suggests metal tolerance of AM fungi. Unfortunately, the availability of the metals was rarely measured, making it difficult to compare the results of the different studies (Haselwandter et al. 1994). In fact, there was no correlation between total Cd concentration in sludge-amended agricultural soils and mycorrhizal colonization of maize roots (Weissenhorn et al. 1995a), while other soil parameters such as organic matter, pH, cation exchange capacity and phosphorus content were correlated with mycorrhizal colonization. Neither was any relationship found between mycorrhizal colonization and metal concentration in soil or roots (Weissenhorn et al. 1995b). However, mycorrhizal infectivity of different heavy-metal-polluted soils and non-polluted soils was negatively correlated with amounts of NH_4NO_3-extractable Cd and Zn (Leyval et al. 1995), indicating the importance of measuring bioavailable/extractable rather than total metal concentrations in soils.

Microcosm experiments using soils artificially polluted with metals (salts or sludges) have shown a reduction or complete inhibition of AM colonization in the presence of metals (Gildon and Tinker 1983; Graham et al. 1986; McGee 1987; Chao and Wang 1991; Vidal et al. 1996), but the AM fungi used in these experiments did not originate from metal-polluted soils (which limits any interpretation that can be made).

Heavy metal uptake and accumulation by mycorrhizal fungi

Ectomycorrhizal fungi

Field observations by Gast et al. (1988), Lepsova and Mejstrik (1988) and Turnau (1991) showed that ectomycorrhizal fungi can accumulate high metal contents in their fruiting bodies, and that metal accumulation varied between species. These values exceed those in vascular plants (Byrne et al. 1976). Gast et al. (1988) showed large differences between metals, with very high accumulation for Cd, exclusion for Pb and a narrower range of concentrations for Zn and Cu, suggesting regulation of uptake for the latter two, which are essential elements. The concentration ratio (metal concentration in fungal biomass divided by soil concentration) varied from 2 to 1000 for Cd, with a maximum value observed for *Amanita muscaria*, 0.2–50 for Zn, 0.2–100 for Cu and 0.002–0.5 for Pb. *A. muscaria* also accumulated extreme concentrations of Vd up to 1000-fold (Bayer and Kneifel 1972). For Hg, some ectomycorrhizal fungi were shown to accumulate up to 60 times the concentration that was present in the soil of a mining area (Bargagli and Baldi 1984). On the basis of research where soil factors were considered, Gast et al. (1988) concluded that species differences, and not soil factors, are the primary determinants of metal levels in fungi.

Metal uptake and accumulation in the mycelium of ectomycorrhizal fungi have also been studied in axenic culture experiments where metals were added as soluble salts (Colpaert and Van Assche 1992a). Under these conditions, the fungal/soil concentration ratios were around 200 and 80 for Cd, and 40 and 30 for Zn of non-tolerant and metal-tolerant isolates of *Suillus bovinus,* respectively.

Ectomycorrhizal fungi can also solubilize minerals, including metal-containing rock phosphates, by production of organic acids or proton extrusion. In this way, these fungi may increase the availability of the metals in the rhizosphere (Leyval et al. 1993).

AM fungi

Since AM fungi cannot be cultivated without a host plant, it is more difficult to demonstrate the intrinsic metal uptake by their hyphae. Using culture systems which separate extraradical hyphae from roots, it has been shown that extraradical hyphae can accumulate and translocate ^{65}Zn, to a degree that may differ between species (Cooper and Tinker 1978; Bürkert and Robson 1994). Adding ^{109}Cd to a hyphal compartment, Joner and Leyval (1997a) showed that extraradical hyphae may transport Cd from soil to roots.

Heavy metal tolerance of mycorrhizal fungi and possible mechanisms

All organisms, including micro-organisms, can achieve resistance to heavy metals by "avoidance" when the organism is able to restrict metal uptake, or by "tolerance" when the organism survives in the presence of high internal metal concentrations (Joho et al. 1985; Baker 1987; Turnau et al. 1996b). The first mechanism involves e.g. reduced uptake or increased efflux, formation of complexes outside cells and organic acid release. In the second situation, metals are chelated intracellularly through the synthesis of ligands such as metallothioneins, polyphosphates, and/or compartmentation within vacuoles. Heavy-metal-tolerant and -sensitive individuals can be distinguished by their growth performance on metal-contaminated substrates.

Ectomycorrhizal fungi

Since many ectomycorrhizal fungi can be grown in pure culture, metal tolerance has been tested as mycelial growth on axenic media containing increasing concen-

trations of single heavy metals. Metal tolerance differs among species. For example, in one study *A. muscaria* and *Hebeloma crustuliniforme* proved more tolerant to Cd and Hg than other fungi (Willenborg et al. 1990). In another study, *A. muscaria*was the most tolerant of all fungi tested with Cd and Zn (Colpaert and Van Assche 1992a). Thompson and Medve (1984) observed differences in Al and Mn tolerance between species and isolates. *S. bovinus* and *S. luteus* from metal-contaminated soils were less inhibited by increasing metal concentrations than isolates from unpolluted soils (Colpaert and Van Assche 1992a). According to Wilkinson and Dickinson (1995), however, metal-tolerant fungi can also be isolated from unpolluted soils, and wide genetic variation exists for metal tolerance within ectomycorrhizal populations. Interactions between metals may also influence their toxicity. For example, the presence of Zn in the medium reduced Cd toxicity to ectomycorrhizal fungi growing in vitro (Colpaert and Van Assche 1992a).

Different mechanisms have been suggested for metal tolerance of ectomycorrhizal fungi, involving adsorption or accumulation of metals due to ion exchange, formation of complexes, precipitation or crystallization (Mullen et al. 1992). *A. muscaria* accumulates extreme concentrations of Vd via specific metal-binding proteins (Bayer and Kneifel 1972). Similar mechanisms are believed to account for high accumulation of e.g. Cd in *Rozites caperata,* Cs in *Cortinarius* spp., Cd and Th in *A. muscaria* and Tl in *Tricholoma album* (Tyler 1980; Seeger and Schweinshaut 1981; Bakken and Olsen 1990). Production of metallothionein-like proteins by some ectomycorrhizal fungi, like *Pisolithus tinctorius*, in pure culture has been shown using histochemical staining (Morselt et al. 1986). However, Galli et al. (1993) did not detect metallothioneins in the mycelium of *Laccaria laccata* although Cd induced an increase in sulphate assimilation and cysteine formation. In *Paxillus involutus,* biosynthesis of polyamines, involved in the maintenance of membrane integrity, was increased by Pb exposure but was little affected by Zn exposure (Zarb and Walters 1995, 1996). Biosorption of heavy metals to fungal structures may reduce the intracellular accumulation of metals and their effect on cytoplasmic processes (Brown and Wilkins 1985a; Denny and Wilkins 1987). The latter authors showed that Zn was mainly located extrahyphally, bound to the cell wall or extrahyphal slime of *P. tinctorius*. This was confirmed by Tam (1995). The fungal cell wall has been suggested as the main barrier protecting fungal hyphae against uptake of potentially toxic metal species. Fungal cell wall components, such as chitin, and pigments (Fig. 2–4) like melanin, can bind heavy metals (Galli et al. 1994; Turnau et al. 1994a, 1996b). Colpaert and Van Assche (1992a) observed an increased density of mycelium upon exposure to high Cd concentrations.

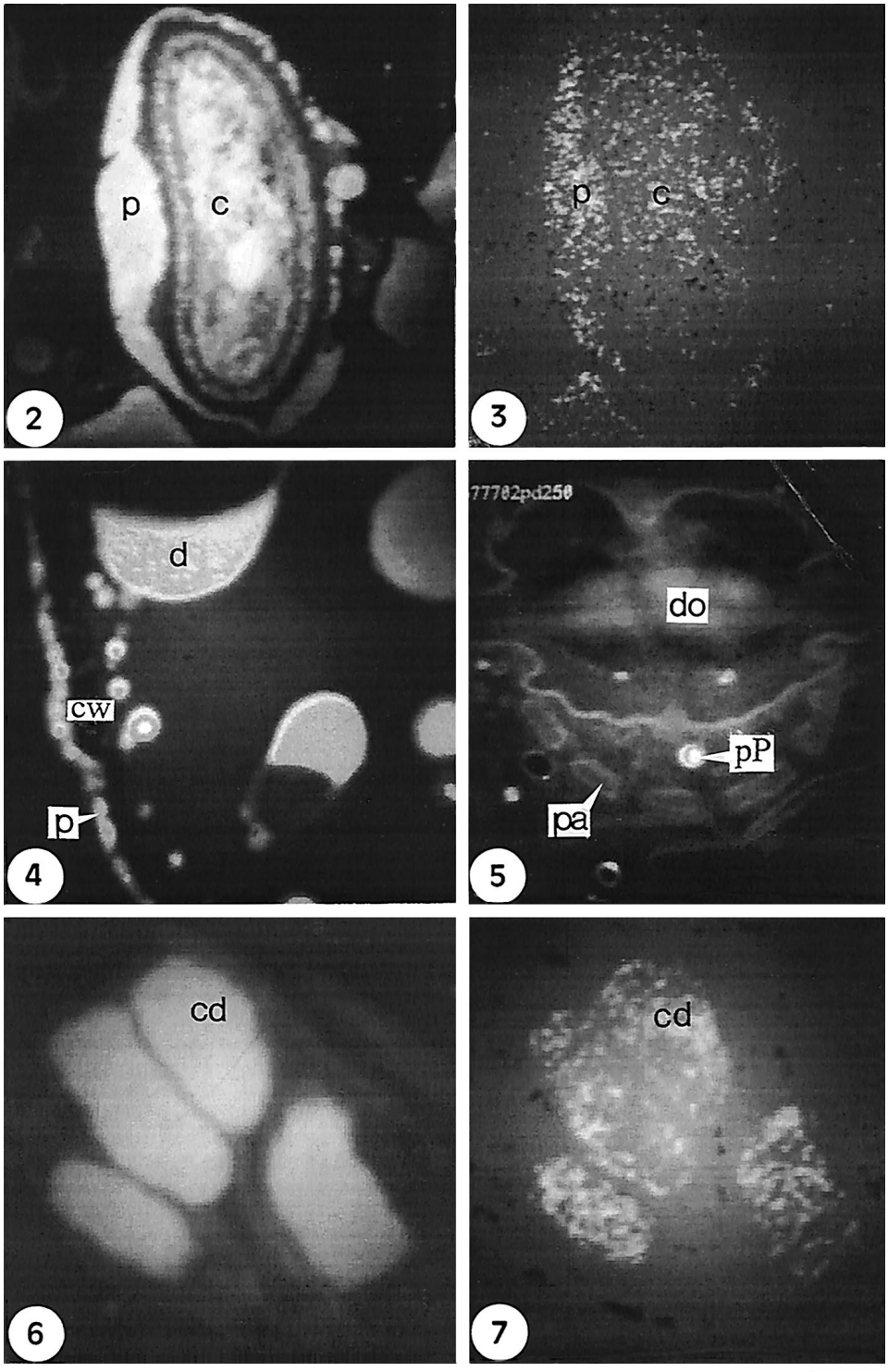
The formation of crystals containing heavy metals in mycelium cultures has been shown by energy dispersive X-ray scanning electron microscopy (Turnau et al. 1995). The nature of the crystals is unknown, but they could be related to organic acid secretion, which can increase in the presence of heavy metals (Turnau et al. 1995), and calcium oxalate crystals have been observed in ectomycorrhizal fungi (Cromack et al. 1979). Using glutaraldehyde-fixed material, heavy metals are often, but not always, localized within so-called polyphosphate or metachromatic granules. Stress alleviation through metal accumulation in polyphosphate granules in hyphae of *P. tinctorius* was found for Cu and Zn, but was not evident for Al, Ni, Cd, Cr or Hg (Tam 1995). According to Orlovitch and Ashford (1993), however, the granular appearance of vacuolar phosphate-rich material is an artefact of specimen preparation. Using freeze-substituted mycelium they showed that polyphosphate is present in vacuoles of living hyphae in a soluble form and is precipitated to form granules by various treatments, including fixation in glutaraldehyde (Fig. 5). As they discuss, the presence of heavy metals may also lead to the formation of granules prior to chemical fixation. However, the existence of aluminium polyphosphate complexes in *L. laccata* has been proved in chemically unfixed material using nuclear magnetic resonance (Martin et al. 1994). Such a detoxification mechanism may have some disadvantages for the fungus, since the turnover of polyphosphate might be important. Sequestration of polyphosphate with metals might also affect transport of other nutrients, and it could be critical in some seasons, or during other P-limiting conditions.

Using electron energy loss spectroscopy (EELS) and electron spectroscopic imaging (ESI), heavy metals in *Pisolithus arrhizus* mycelium were localized mainly within pigmented material deposited on the surface (Figs. 2–4; Turnau et al. 1994b), and Cd, Cu, Ti, N and S were found associated with P-rich amorphic or spherical material in the vacuole (Figs. 6, 7) which contained cysteine-rich proteins (Figs. 8–10). When dead or senescent mycelium was observed, the hyphal septal pores were blocked by electron-dense material, and heavy metal levels were elevated within the cytoplasm and the vacuole remains. As discussed by Turnau and Dexheimer (1995), this process was due to an increased activity of acid phosphatase, resulting in the liberation of HPO_4^{2-}, which can precipitate stoichiometrically with metallic ions.

Gast et al. (1988) suggested a transport/regulation system at the cell membrane for essential elements such as Cu and Zn, and an exclusion mechanism for Cd for some ectomycorrhizal species such as *P. involutus*. Tolerance mechanisms are probably metal specific since e.g. Zn tolerance does not elicit Cu tolerance (Colpaert and Van Assche 1987).

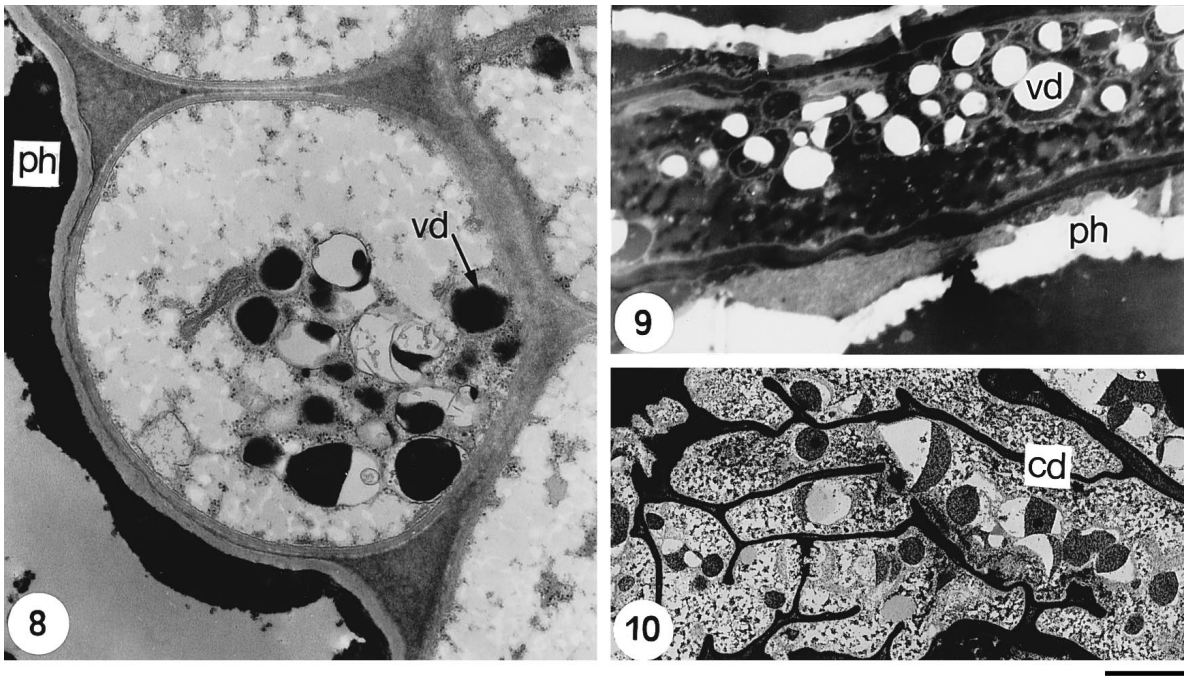
AM fungi

For AM fungi, Cd-tolerant isolates have been isolated from contaminated soils (Gildon and Tinker 1981,



Figs. 2–7 Electron spectroscopic (ES) image micrographs revealing localization of heavy metals within mycelium of ectomycorrhizal fungi (*c* cytoplasm, *cd* cysteine-rich material deposited within vacuoles, *cw* cell wall, *d* amorphic deposition within vacuole, showing similar element distribution as within pigment material, *do* dolipore septum, *p* pigment, *pa* parenthesome, *pP* polyphosphate granule). **Figs. 2–4** *Rhizopogon roseolus.* **Fig. 2** ES image at energy loss of 250 eV revealing presence of a pigment layer on the hyphal surface (*bar* 1 μm). **Fig. 3** Distribution of Cd in the same area as shown in **Fig. 2**, revealing Cd in the pigment layer and in the cytoplasm (*bar* 1 μm). **Fig. 4** Distribution of Al (showing similar pattern to heavy metals) within vacuolar, amorphic, phosphate-rich material and within the pigment layer deposited on the surface of the cell wall (*bar* 0.5 μm). **Figs. 5–7** *Pisolithus arrhizus*. **Fig. 5** Presence of P (accompanied by Ca) within a polyphosphate granule passing through a dolipore septum [element distribution presented as combination of the binary element distribution image (grey)] (*bar* 0.25 μm). **Fig. 6** ES image at 250 eV of amorphic, vacuolar material, rich in P (but not polyphosphate), characterized by similar levels of Cd, Cu, Ti but without Ca (*bar* 0.5 μm). **Fig. 7** Distribution of Cd in area shown in **Fig. 6** (*bar* 0.5 μm)

1983; Weissenhorn et al. 1993, 1994). Metal tolerance has been tested regarding spore germination ability in sand amended with Cd solution and in heavy-metal-contaminated soils (Weissenhorn et al. 1993, 1994). *Glomus mosseae* P2 (BEG 69) isolated from a metal-polluted soil was more tolerant to Cd than a *G. mosseae* isolate (BEG 12) from a non-polluted soil. In these experiments, Cd reduced spore germination more than hyphal extension once a spore had germinated. Since AM fungi cannot be cultivated without a host plant, metal tolerance cannot really be evaluated through fungal growth on axenic media. Using a compartmented system separating roots and extraradical hyphae, Joner and Leyval (1997b) showed that hyphal length was unaffected by up to as much as 100 mg kg^{-1} Cd added to the soil of the hyphal compartment and that juvenile



Figs. 8–10 Transition electron micrographs of sections of *Paxillus involutus*/*Pinus sylvestris* mycorrhizae collected from plots treated with heavy metals (*cd* cysteine-rich material deposited within vacuoles, *ph* phenolic material within plant vacuoles, *vd* vacuolar depositions). **Fig. 8** Section observed after staining with uranyl acetate and lead citrate: amorphic, vacuolar depositions, shown previously to contain heavy metals, are visible as dark material (*bar* 1 μm). **Fig. 9** ES image at 250 eV of a similar region with very bright vacuolar deposition rich in P (*bar* 1 μm). **Fig. 10** Gomori-Swift reaction of Hartig net with strong staining of vacuolar depositions revealing proteins rich in cysteine (*bar* 1.7 μm) Bars: 8, 9–1 μm; 10–1.7 μm

spores were observed. Sporulation and spore germination of non-mycorrhizal fungi are also more affected than hyphal development by Cd (Babich and Stotzky 1977; Ross 1982), suggesting that distinctions should be made between concentrations that inhibit spore germination and those that inhibit fungal growth.

The mechanisms of heavy metal tolerance in AM fungi have not yet been elucidated. Co-tolerance to Cd and Zn was suggested for one isolate from a soil polluted with long-term application of Zn-polluted sewage sludge (Weissenhorn et al. 1994). Increased Cd tolerance of a mixed fungal culture was observed in a soil amended with Cd salts, but only 1 year after amendment of Cd (Weissenhorn et al. 1994). Increased metal tolerance has also been observed for AM fungi isolated from a soil naturally high in metals (Leyval et al. 1995). The stability of metal tolerance in AM fungi and ectomycorrhizal fungi has not been checked, although preliminary results show that growing a metal-tolerant *G. mosseae* on a metal-free substrate did not influence metal tolerance (C. Leyval, unpublished results).

Effect of mycorrhizal fungi on metal transfer to plants

Solubilized heavy metals enter the root via apoplastic and symplastic pathways (Salt et al. 1995), using energy-dependent specific transmembrane metal ion carriers or channels. Non-essential metals compete with essential ones for these carriers. However, uptake mechanisms for metals in mycorrhizal roots have not been described. Once metals have entered the hyphae they can be immobilized or transferred to the root, and once in the root, sequestered there or translocated to the shoot. According to Salt et al. (1995), metal translocation from root to shoot takes place in the xylem after symplastic crossing of the casparian strip to the endodermis. Organic acid complexes, such as Cd-citrate, and phytochelatins may play a role in metal transport in the xylem. When complexed, the translocation rate of metal cations such as Zn, Cd and Cu in the xylem is enhanced (Marschner 1995).

Ectomycorrhizal fungi

The effect of ectomycorrhizae on metal uptake by trees has been reviewed by Wilkins (1991). He concluded that mycorrhizae can reduce metal concentrations in shoot tissues, although some fungi are inefficient and others affect growth or uptake alone. This general feature has been found for Zn, Ni and Cu. Experiments on metal uptake from soil by *Pinus banksiana* and *Picea glauca* (Dixon and Buschena 1988) showed that *S. luteus* reduced Cd, Ni, Pb and Zn concentrations in needles except at the highest concentrations of metal added to soil which prevented mycorrhiza formation. Mycorrhizal colonization led to a reduction in zinc shoot

concentration and an increased zinc accumulation in the root system (Brown and Wilkins 1985b). Under low external Zn concentrations, *S. bovinus* caused increased Zn uptake in roots and needles of *Pinus sylvestris,* while at high external concentrations the same fungus reduced the needle concentration (Bücking and Heyser 1994). This effect was increased when *S. bovinus* was pretreated with Zn for 6 months, but it was not observed when another ectomycorrhizal fungus was used, suggesting that ectomycorrhizal fungi differ in their ability to reduce translocation from root to shoot. Non-mycorrhizal *P. sylvestris*, however, also accumulated heavy metals in its roots, thus protecting the shoot against toxic tissue concentrations. Among four fungi forming ectomycorrhiza with birch seedlings in sand culture, *Scleroderma flavidum* reduced the Ni concentration in the stem, while the Cu concentration was not affected (Jones and Hutchinson 1988a). Colpaert and Van Assche (1993) compared nine ectomycorrhizal strains for their effect on Cd uptake in *P. sylvestris* at a relatively low Cd concentration added to the substrate. With all mycobionts, the Cd concentration in needles and stems was lower than in non-mycorrhizal plants, while it was not significantly affected in the roots.

AM fungi

The effect of AM fungi on plant uptake of metals is not always clear. At high metal concentrations, some reports show higher uptake of metals by mycorrhizal plants (Gildon and Tinker 1983; Killham and Firestone 1983; Weissenhorn and Leyval 1995), while others found reduced concentrations in plants or in shoots due to mycorrhizal colonization (Schüepp et al. 1987; El-Kherbawy et al. 1989; Leyval et al. 1991; Weissenhorn et al. 1995c). In two pot experiments with maize in metal-contaminated soil, mycorrhizal colonization either increased plant biomass and decreased Cd, Cu, Zn and Mn concentrations in shoots and roots, or had no effect on growth and metal uptake, depending on root density, plant growth conditions and mycorrhizal inoculum (Weissenhorn et al. 1995c). Loth and Höfner (1995) observed that mycorrhizal colonization led to a higher uptake of Cu, Zn and Cd in oat roots from a highly contaminated soil but a reduced translocation to the aerial part. Inoculation of *Festuca rubra* and *Calamagrostis epigejo*s, grown in pots filled with sand amended with Zn sulphate at high concentration, with *G. fasciculatum* alleviated the negative effect of Zn on plant growth, but had no effect on the Zn concentration in shoots and roots (Dueck et al. 1986).

Mycorrhizal colonization reduced the shoot concentration of Cd and Zn in field-grown maize and lettuce when the soil had high concentrations of available metals (Schüepp et al. 1987). In a soil with low metal concentrations, AM fungi decreased Cd uptake but increased Zn uptake in the same plants. These results suggest that the effect of AM fungi on plant heavy metal uptake is metal specific and depends on the concentration. El-Kherbawy et al. (1989) showed that the AM effect on plant metal uptake also depends on soil pH. With increasing soil pH, the DTPA-extractable metals decreased, but at the same time AM fungi increased Cd, Zn and Mn uptake in alfalfa shoots. At a lower soil pH, mycorrhizal colonization decreased metal uptake. In both cases, mycorrhizal colonization enhanced alfalfa growth.

Most studies have been performed in pots where it is not possible to separate the effect of the fungus and of the host plant on the mobilization and uptake of metals. To differentiate fungus and host plant effects, plant containers with different compartments separating roots and extraradical hyphae have proven very useful (Bürkert and Robson 1994; Guo et al 1996; Joner and Leyval 1997a). Transport of Zn in extraradical hyphae from a sandy soil to clover was shown by Bürkert and Robson (1994), but large variations were observed between fungal species due to the different abilities of their hyphae to grow in the root-free zone. In the experiments of Guo et al. (1996) up to 37, 33 and 44% of the total Cd, Cu and Zn uptake, respectively, by bean plants was attributed to mycorrhizal transfer via hyphae from a root-free compartment. Unfortunately, hyphal length in the side compartment was not measured. Joner and Leyval (1997a) studied the transfer of Cd by extraradical hyphae of *G. mosseae* from a sandy loam to clover using ^{109}Cd. Results showed that when only extraradical hyphae had access to the root-free compartment containing labelled soil, uptake of ^{109}Cd increased by 55% (100 mg kg^{-1} added) in comparison to non-mycorrhizal plants, and that a large part of it was sequestered in the roots.

Ericoid mycorrhizal fungi

In a series of classical experiments, Bradley et al. (1981, 1982) showed that ericoid mycorrhizal colonization led to a significant decrease in metal content of the shoot and an increase in the roots of *Calluna vulgaris, Vaccinium macrocarpon* and *Rhododendron ponticum* grown in sand amended with Cu or Zn. This is a clear example of mycorrhizal protection against excess plant uptake of heavy metals, in an ecosystem where heavy metal availability may be high due to soil acidity.

Metal tolerance of mycorrhizal plants and possible mechanisms

The possible mechanisms of plant tolerance to heavy metals, recently reviewed by Marschner (1995), are comparable to the microbial mechanisms of tolerance just described, and include binding to the cell wall, active efflux or restricted influx, compartmentation in the

vacuole and chelation in the cytoplasm. There are large differences between plants in their uptake and accumulation of heavy metals, which have not been considered in studies including mycorrhizal fungi. For example, maize, rice and barley, which have been described as tolerating 100 μM Cd (Inouhe et al. 1992, 1994), showed a cytoplasmic accumulation and the presence of phytochelatin (PC), while dicotyledons tolerating 10–30 μM Cd, like cucumber, tomatoes and lettuce, accumulated Cd mainly in cell walls and did not produce PC. Florijn et al. (1993) even described among different maize inbred lines "shoot Cd excluders", and "non-shoot excluders", the distinction being related to different desorption characteristics and binding capacities in and/or outside the root. Such aspects have not been taken into consideration in experiments with mycorrhizal plants and might explain some of the controversial results.

It is striking that hyperaccumulative plants, such as *Thlaspi*, which can concentrate Ni up to 1000-fold, belong to non-mycorrhizal families. Martens and Boyd (1994) attributed plant defence against herbivores to Ni hyperaccumulation, while for Baker and Proctor (1988), it is a primitive metal tolerance mechanism, since it occurs in relatively primitive tropical plants, from which it could be concluded that mycorrhizal colonization has not evolved as a very efficient mechanism to tolerate metal toxicity. On the other hand, it has been suggested that the ability of trees and long-living plants to withstand pollution stress is due to mycorrhizal colonization of roots ameliorating metal toxicity (Wilkinson and Dickinson 1995). According to these authors, survival of trees in highly metal contaminated soils, which has been ascribed to phenotypic plasticity in plants, may be due to genetic changes in the mycorrhizal communities facilitated by the shorter life cycle of the fungi.

Within the mycorrhizal root, the mechanisms of tolerance or of alleviation of the metal stress by the fungi cannot be separated from the plant tolerance. But metal sensitivity of mycorrhizal fungi in axenic culture is not necessarily correlated with the sensitivity under symbiotic conditions (Colpaert and Van Assche 1992b).

Ectomycorrhizal plants

Most published reports suggest that the cell walls of extramatrical hyphae are the main binding sites for heavy metals in ectomycorrhizal-fungi (Denny and Wilkins 1987; Galli et al. 1994). The survival of an ectomycorrhizal fungus in a Cd-polluted substrate depended to a great extent on the density of extramatrical mycelium produced by the mycorrhizae (Colpaert and Van Assche 1993), reducing the amount of Cd diffusing through an individual hypha, and reducing the Cd supply to the host. Colpaert and Van Assche (1987) suggested that ectomycorrhizal fungi producing extensive extramatrical mycelium, such as *Suillus*, would be better than other fungi at excluding metals from the host. An increased density of mycorrhizal mycelium around the root may also be induced by Cd (Darlington and Rauser 1988). Denny and Wilkins (1987) attributed the alleviation of Zn toxicity in mycorrhizal *Betula* to the adsorption of Zn to the hyphal surface, thus the reducing Zn concentration in the soil solution and, thus, plant uptake. Metals were adsorbed at electronegative sites of the hyphal cell wall, especially on extraradical hyphae, and in extrahyphal polysaccharide slime.

Root protection by the fungal mantle has also been suggested by Dixon and Buschena (1988). A filtering effect within mycorrhizae of *Rhizopogon roseolus*/*P. sylvestris* selected from calamine wastes was demonstrated using a transmission electron microscope equipped with EELS (Turnau et al. 1996a). A gradient of heavy metals was observed, with the highest level in the outer part of the fungal mantle and the lowest level in the Hartig net. This phenomenon was associated with electron-opaque substances, probably pigments, excreted mainly in the outer region of the mantle (Fig. 4). A similar, but less evident filtering effect was found in mycorrhizae of *P. involutus*/*P. sylvestris* (Turnau et al. 1993a) from highly polluted plots.

The whole fungal biomass produced by mycorrhizal fungi, including sporophores, extramatrical mycelium, mantle mycelium, even dead biomass, can complex metals and reduce their availability and toxicity to the host plant (Colpaert and Van Assche 1993). Rapid turnover of fungal tissue may also contribute to protecting the host plant against metals (Colpaert and Van Assche 1993).

Other hypotheses for plant protection against metal toxicity by ectomycorrhizal fungi concern the modification of the rhizosphere by the mycorrhizal fungi (Dixon and Buschena 1988), the interactions between heavy metals and anions (Gildon and Tinker 1983), the precipitation of metal oxalates in intercellular spaces of the fungi or the host plant and the restriction of apoplastic transport by the casparian strip (Harley and Smith 1983).

The compatibility between the fungal isolate and the host plant appears to be more crucial in the alleviation of metal toxicity than the fungal tolerance to the metal (Denny and Wilkins 1987). On the other hand, a preculture of *S. bovinus* on a medium enriched with Zn increased its beneficial effect on shoot Zn concentration (Bücking and Heyser 1994). Since the concentrations of P and Ni in stems and roots of mycorrhizal *Betula papyrifera* were correlated, and since P occurs mainly as polyphosphate located in the fungal mantle, Jones and Hutchinson (1988a) suggested that Ni binding in phosphate-rich material could be the detoxifying mechanism. Such a mechanism involving interaction with P has been described for non-mycorrhizal plants: Mo uptake by tomato plants via phosphate binding (Heuwinkel et al. 1992), Zn phytate-rich granules observed in maize and wheat root cells (Van Steveninck

et al. 1993), and also in the plant compartment of mycorrhizae (Turnau et al. 1993a, b, 1996b). Cytochemical analyses indicate that element detoxification by ectomycorrhizal fungi may also involve metal binding to vacuolar proteins or peptides (Turnau et al. 1994a) (Figs. 8–10).

Amongst selected ectomycorrhizae collected in soils with high surface levels of Pb, Cd and Zn, the highest mean concentration of Zn was found in *Cortinarius semisanguineus*, and the highest level of Pb and Cd in *Russula* and *Suillus* spp., but Pb was almost excluded in mycorrhizas (Berthelson et al. 1995), suggesting that metal accumulation in ectomycorrhizae is fungal and metal dependent, as observed previously for the fungi alone.

Finally, no relationship was observed between axenic growth of ectomycorrhizal fungi in the presence of Ni and Cu and their effect on metal concentration in birch seedlings (Jones and Hutchinson 1988b), demonstrating that screening of metal-tolerant mycorrhizal fungi under axenic conditions will not necessarily select fungi that will confer protection in a functional symbiosis. *Thelephora terrestris* was the most Cd-tolerant species in vitro but one of the most sensitive in symbiosis, while *Scleroderma citrinum* was less sensitive as a symbiont than as a free-living organism (Colpaert and Van Assche 1992b, 1993). Among nine ectomycorrhizal strains tested by Colpaert and Van Assche (1993), three were isolated from a metal-polluted site and were metal tolerant, but their effect on Cd concentration in pine needles and stems did not differ significantly from the same species isolated from non-polluted soils.

It seems clear that for uptake of and protection against heavy metals, fungi have evolved two different mechanisms: one operates at low external metal concentrations and is relatively specific for individual metals, and one which is relatively non-specific and active at higher metal concentrations (Ross 1993). This is illustrated by ericoid mycorrhizal fungi for iron and siderophore production (Haselwandter 1995). At low iron concentrations, ericoid mycorrhizal fungi release siderophores such as ferricrocin or fusigen (Haselwandter et al. 1992). At higher iron availability, siderophore biosynthesis is inhibited (Dobernigg and Haselwandter 1992), but the plant still seems to be protected against iron toxicity by the mycorrhizal fungus (Shaw et al. 1990).

AM plants

AM fungal contribution to the metal tolerance of host plants is poorly documented. Loth and Höfner (1995) attributed most of the higher uptake of Cu, Zn and Cd in mycorrhizal oat roots to a larger absorbing surface of the root, since AM colonization increased specific and total root length. The results of Joner and Leyval (1997a) discussed above suggested fungal immobilization of Cd within the root system. This is in accordance with element localization, using EELS and ESI, in mycorrhizal roots of *Pteridium aquilinum* from experimental forest plots treated with high doses of heavy metals, showing accumulation of heavy metals within intracellular hyphae mainly in phosphate-rich material of vacuoles (Turnau et al. 1993b).

However, the precise mechanism of immobilization by the root itself or by the fungus within the root or at the interface has not been investigated.

Metal-tolerant mycorrhizal fungi can grow readily in heavily polluted soil, but to what extent they contribute to plant metal tolerance via accumulation of heavy metals in roots preventing translocation to shoots is uncertain. In a pot experiment with maize in sand culture, where root colonization with the metal-tolerant *G. mosseae* P2 (BEG 69) isolate was higher than with the metal-sensitive *G. mosseae* Gm isolate, there was no significant difference in Cd uptake between plants colonized by either of the two fungi (Weissenhorn and Leyval 1995). In another experiment with metal-contaminated soil, the P2 isolate enhanced Cu absorption by maize and translocation (Weissenhorn et al. 1995c). But, as noted above, maize has been reported by Inouhe et al. (1992, 1994) as a tolerant plant in comparison to dicotyledons like cucumber, tomato or lettuce. *F. rubra* and *C. epigejo*s were used by Dueck et al. (1986) to show that AM colonization alleviated the negative effect of Zn on plant growth, but in this experiment, AM infection had no effect on Zn concentration in shoots and roots. The authors stated that both plants were isolated from a site downwind from a blast furnace, while the mycorrhizal fungus came from an apparently non-contaminated dune area. It is possible that the plants and not the fungus were tolerant to metals, and that the benefit could have been due to mycorrhizal effects other than alleviation of metal toxicity. However, differences in efficiency of mycorrhizal fungi on plant metal uptake have been reported (Medeiros et al. 1994) suggesting, as observed for ectomycorrhizae, that the association between the plant and the fungus is crucial to the mycorrhizal benefit in metal-polluted soils.

Ericoid mycorrizal plants

For ericoid mycorrhizae, Bradley et al. (1981) attributed the protective effect of the fungus to the fact that the ascomycete *Hymenoscyphus ericae* displays strong affinities for metallic cations, and that ericoid mycorrhizal colonization leads to a much greater concentration of fungal material in the root than does AM colonization, thus providing a more efficient exclusion mechanism. Denny and Ridge (1995) showed that the amelioration of zinc toxicity to *C. vulgaris* by certain fungal isolates was correlated with the presence of loosely adhering extrahyphal slime.

Applied aspects

Heavy metals cannot be chemically degraded. Therefore remediation of metal-polluted soils is mainly limited to immobilization or extraction/concentration techniques. Among remediation options for metal-contaminated sites, phytoremediation methods have recently attracted much attention (Anderson and Coats 1994; Salt et al. 1995). Three different methods have been described (Salt et al. 1995): phytoextraction, rhizofiltration and phytostabilization. The objective of the first approach is to use a metal-accumulating plant producing enough biomass in the field to remove metals from the soil. Most of the wild metal accumulators belong to the family Brassicaceae, such as *Thlaspi caerulescens*, which are non-mycorrhizal plants. However, since these plants produce little biomass, other plants like *Larix* have been considered (Landberg and Greger 1997). The rhizofiltration method more or less eliminates the possible use of mycorrhizal fungi since plants are grown in liquid medium.

The principle of the phytostabilization method is to promote plant growth to reduce or eliminate the bioavailability of metals, minimize wind and water erosion, improve soil quality (organic matter content in particular) and to reduce leaching of metals. Treatments include appropriate fertilization, either a reduction of metal availability using different amendments and/or using metal-tolerant plant species. Various grasses such as *Agrostis tenuis* and *F. rubra* have been used commercially (Salt et al. 1995; Van Tichelen et al. 1996). Hetrick et al. (1994) studied the influence of mycorrhizal fungi on revegetation of chat piles from mine spoils, where plants had failed to establish naturally. They showed that mycorrhizal colonization by a mixed AM fungal inoculum improved the growth of the obligate mycotrophic plant *Andropogon gerardii,* but *Festuca arundinacea,* a facultative mycotroph which grows well without mycorrhiza in non-contaminated soil, also benefited from mycorrhiza in chat piles. The results show that mycorrhizae in combination with fertilizers will improve plant establishment on chat piles, and also on other severely disturbed sites such as mine spoils or overburdens (Hetrick et al. 1994). The benefit from mycorrhizas could be associated with increased tolerance to heavy metals, but also with better plant nutrition, since chat piles are poor in nutrients and have a very low water-holding capacity.

Phytostabilization is, however, a temporary solution, since the metals are not eliminated and there is a risk, increasing with time, of metal mobilization in the rhizosphere and of metal transfer from plants to animals. For these reasons, phytostabilizing plants should also immobilize metals in the roots and have low shoot accumulation. Mycorrhizal plants are therefore of great interest since mycorrhizae can bind metals and limit their translocation to shoots. Further development of phytoremediation requires an integrated multidisciplinary research effort that combines plant biology, soil chemistry, soil microbiology and agricultural and environmental engineering (Kumar et al. 1995). For utilization of mycorrhizal fungi in phytoremediation, additional research is required to understand the mechanism of protection against metal toxicity, host specificity and competitiveness in soil.

Ectomycorrhizal fungi have been proposed as bioindicators of air pollution (Fellner 1989). Fungal fruitbodies are potentially useful as bioindicators of radioactive contamination of the environment (Berreck et al. 1992). The suitability of wild-growing mushrooms, including ectomycorrhizal species, as bioindicators for heavy metal pollution has been investigated (Gast et al. 1988). The authors conclude that a large number of samples should be taken, since metal accumulation depends on the fungal species. In the case of radiocaesium accumulation by basidiomycetes, the variability even within one species may be large, although the accumulation pattern per se is species specific (Haselwandter 1978; Haselwandter et al. 1988; Haselwandter and Berreck 1994). A study carried out in the Ukraine including the Chernobyl area revealed a close correlation between the radiocaesium content of fungal fruitbodies and the deposition of radiocaesium in the area where the fruitbodies were collected (Grodzinskaya et al. 1995). This underlines the potential use of fungal fruitbodies as bioindicators for heavy metal pollution of the biosphere, as long as the number of samples per species and the species collected are representative.

Like ectomycorrhizal fungi, AM species have a potential which can be employed in biomonitoring programmes. The decline of AM fungal occurrence (propagule density) and infectivity in metal-polluted soils can be used as bioindicator*s* of soil contamination (Leyval et al. 1995). Mycorrhizal colonization of plant roots after soil remediation can be a sign that the metal concentration or bioavailability has decreased. Since metal tolerance evolves in some fungi from metal-contaminated soils, a sensitive AM fungus could be used and tested for its ability to colonize roots in any metal-polluted soils, providing useful information about their metal toxicity. In a current investigation on the long-term effect of metals on AM fungi in an arable field where metal-contaminated sludge has been applied for 10 years, preliminary results show that the diversity of AM fungi changes even at metal concentrations lower than the European limit for agricultural soils (P. Vandenkoornhuyse and C. Leyval, unpublished results).

The use of mycorrhizal fungi as bioremediation agents has been reviewed by Donnelly and Fletcher (1994). Mycorrhizal fungi appear to partially protect plants against the toxicity of heavy metals. On the other hand, the host plant may give the fungus a selective survival advantage at a contaminated site. This mutual benefit would make the mycorrhizal association superior to the application of single organisms, either non-mycorrhizal plants or free-living micro-organisms, for bioremediation purposes.

Concluding remarks

How the effects of metals on micro-organisms and plants are studied has a profound effect on the outcome of the experiments (McGrath 1997). To improve understanding of the interactions between heavy metals, mycorrhizal fungi and roots in contaminated soils, parameters like the tolerance of the mycorrhizal fungi but also of the host plant, the nutritional status of both organisms, soil properties, the level and duration of exposure, the availability of the metals and their specific behaviour should be considered.

Under natural conditions, the benefit to plants of mycorrhizal colonization in terms of reduced metal translocation is difficult to demonstrate (Ietswaart et al. 1992). The extent to which mycorrhizal fungi can alleviate metal toxicity to plants under field conditions remains to be established, as does the importance of the mycorrhizal benefit relative to other plant defence mechanisms.

In a recent review, Haselwandter et al. (1994) pointed out that for conclusive informations on the effect of AM fungi on metal uptake by plants, it is necessary to consider and estimate (1) the uptake rates per unit of root biomass or length, (2) the root colonization intensity by the AM fungi and their activity, (3) the interactions between metals and other cations, (4) the level of availability of the metals and (5) the differences in mycorrhizal and non-mycorrhizal plant size. George et al. (1994) stated that plants differ in so many aspects in the presence and absence of mycorrhiza that differences in micronutrient content between mycorrhizal and non-mycorrhizal plants do not necessarily reflect micronutrient uptake and transport via mycorrhizal hyphae. This can be extrapolated to heavy metals.

Haselwandter et al. (1994) further stated that studies on mycorrhizal fungi in metal-polluted soils have not employed any kind of vital staining to measure root colonization, hence there is uncertainty about the viability of the fungi inside and outside the roots. This limits the ecological relevance of such data for an assessment of the role mycorrhizal fungi play in metal-contaminated habitats.

Extraradical mycelium of mycorrhizal fungi is of paramount importance not only for metabolism-independent binding of heavy metals to cell walls, but also, and probably even more so, for metabolism-dependent intracellular uptake of heavy metals and transport to the associated host plant. Unfortunately, data on the amount of extraradical mycelium produced by mycorrhizal fungi are lacking in most of the studies on mycorrhiza-mediated effects of heavy metals.

In most of the reported experiments on heavy metals and mycorrhiza, only one or a few isolates have been considered and the origin of these isolates is not always given. And yet, differences in metal tolerance between genera, species and within species of mycorrhizal fungi have been shown in polluted soils and for ectomycorrhizal fungi in unpolluted soils as well. To understand the effect of heavy metals on mycorrhizal fungi, it would be necessary to study and compare the diversity within populations of mycorrhizal fungi in heavy-metal-polluted and unpolluted soils. Preliminary results (C. Leyval and P. Vandenkoornhuyse, unpublished results) show that the intraspecific diversity of AM fungi decreases in a metal-polluted soil. A key point to understanding the interactions between heavy metals and mycorrhizae is to take into account the functional diversity of mycorrhizal fungi and functional compatibility with plants (see Ravnskov and Jakobsen 1995).

The protective effect of ectomycorrhizal and ericoid mycorrhizal fungi against metal toxicity in plants is quite clear, and various mechanisms have been demonstrated, the main one being fungal structures acting as a barrier to metal uptake in plants. For AM fungi, more work should be done to understand the mechanisms involved in metal immobilization in the hyphae inside and/or outside the roots. The compartment system described above is a promising approach for such studies. Relatively large amounts of extraradical hyphae can be produced and separated from the roots, which should allow study of metal adsorption and uptake by AM hyphae, transfer to the root and translocation to the shoot. The functioning of different fungi could be compared (comparison between P and metal uptake) in such systems. Element localization using EELS and ESI can be done when only hyphae but not roots have taken up metals. Other experimental devices, including normal or transformed roots (Bécard and Fortin 1988; Giovannetti et al. 1993), or excised hyphae (Johansen et al. 1996) might also be useful to study metal tolerance of AM fungi.

Results with ectomycorrhizal fungi show metal-specific tolerance mechanisms and competition between metals for uptake. This should also be further investigated for all kinds of mycorrhizas, and especially for the possible use of mycorrhizal fungi in phytoremediation.

There is no doubt that genetic engineering aimed at producing plants which are more tolerant or resistant to heavy metals may play an important role in the future. But such attempts must not overlook possible changes in the susceptibility of the plants to mycorrhizal infection. A metal-resistant plant breed should still be susceptible to mycorrhizal symbiosis and when colonized by a metal-resistant mycorrhizal fungus (derived from a natural population or made metal resistant through genetic engineering) would be of great value for the rehabilitation of metal-contaminated soils. The management of soil micro-organisms including mycorrhizal fungi is a prerequisite for the success of future restoration programmes (Haselwandter and Bowen 1996; Haselwandter 1997).

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