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Heavy metal pollution and genetic adaptations in ectomycorrhizal fungi.

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Abstract

Heavy metal toxicity is a strong trigger for evolutionary adaptation in terrestrial biota that colonise metalliferous soils. Here, I will focus on the occurrence of metal tolerance in ectomycorrhizal fungi, the predominant group of root symbionts of pioneer trees that try to colonise severely polluted sites. A considerable amount of literature exists on metal-tolerant plants, which is in sharp contrast to what we know about the tolerance in the fungal symbiotic partners that associate with these plants on metal-polluted soils. I will deal with the ecological and evolutionary processes that drive plant and fungal communities and populations on metal-contaminated sites. The few examples of true metal tolerance in ectomycorrhizal fungi are described and mechanisms possibly involved in this tolerance are briefly summarised. How true metal tolerance in ectomycorrhizal fungi can affect a host plant is discussed in a final paragraph.

Metal toxicity triggers evolutionary processes in plants and their associated microorganisms

An unfortunate effect of many industrial activities has been the contamination of ecosystems with heavy metals. At its worst, heavy metal pollution can have deleterious effects on communities and populations of all kinds of biota. It progressively wipes out the more sensitive species and finally can lead to an almost complete elimination of organisms. The most visible effect of metal toxicity in terrestrial ecosystems can be observed on vegetation. Along transects towards metal smelters a progressive reduction in floristic diversity is obvious and in extreme cases metal deserts may develop in the immediate vicinity of emission sources (Ernst, 1990). Such metal-contaminated soils strongly differ from unpolluted ones with respect not only to heavy metal concentrations but also to numerous additional physico-chemical factors. For most plant taxa, these constraints are too strong to be overcome. However, ecosystems and species exhibit considerable resilience and even extremely metal-toxic soils eventually become colonised by vegetation. Extreme toxicity induces a strong selection pressure for adaptation and a small number of organisms eventually manage to build up metal-tolerant populations. The phenomenon is well-described for prokaryotes (Mergeay *et al.*, 2003) and plants in terrestrial environments (Bradshaw and McNeilly, 1981; Al-Hiyaly *et al.*, 1990). In aquatic ecosystems metal adaptation has been observed in benthic organisms such as worms and algae and in prokaryotes surviving in contaminated sediments.

Once an ecosystem has been exposed to severe metal contamination, the initial decrease in species diversity may again slowly increase because of the evolution of metal adaptation in a number of local species, most often grasses, and because of immigration of metal-tolerant organisms from other sites (Pauwels *et al.*, 2005). Soils that are naturally enriched with heavy metals typically harbour unique plant communities that are well-adapted and more species-rich than recently polluted sites (Ernst, 1990). The potential for the development of metal tolerance – the ability to survive on metalliferous soils – is not widespread in the plant kingdom (Antonovics *et al.*, 1971) and it is remarkable that within wide geographic ranges the same plant species seem to develop pioneer metallophytes, even when different metals are responsible for the toxicity. This is best known for some monocotyledonous plants, in particular grasses (Schat *et al.*, 2000). Evolution for metal tolerance in grasses can be very

rapid because normal, non-adapted populations often contain a low frequency (0.1 to 0.5%) of metal-tolerant individuals (Bradshaw and McNeilly, 1981; Al-Hiyaly *et al.*, 1993). Rapid evolution towards metal tolerance is probably more likely in *r*-strategists with a high reproductive rate and the production of a large number of offspring. A rapid establishment of metal-tolerant grass ecotypes has been observed on many polluted sites and against many different metals (Schat *et al.*, 2000). It is remarkable that the tolerance is usually quite specific for the particular metal that is present in toxic concentrations; suggesting that different genes must be involved and selected for. Relatively few dicots have been found to develop metal-tolerant ecotypes. Most metal-tolerant dicots are confined to metalliferous soils and only a few species, for example *Silene vulgaris*, *Thlaspi caerulescens* and *Arabidopsis halleri*, have both metallicolous and non-metallicolous populations (Ernst, 1990; Assunção *et al.*, 2003; Pauwels *et al.*, 2005).

Many of the (pseudo)metallophytes can develop in the absence of suitable mycorrhizal fungi, which is probably an advantage during the primary succession of metal wastes or deserts. In general, there is little evidence that mycorrhizal fungi are absolutely essential for the first stages of primary succession of plant communities on soils subjected to major environmental perturbations (Smith and Read, 1997; Wu *et al.*, 2004). This is not to say that these pioneer plants are never colonised by mycorrhizal fungi; many of these plants are facultative mycotrophs and most grasses and even pioneer species within the Brassicaceae and Polygonaceae family, taxa traditionally considered as being non-mycorrhizal, become colonised with arbuscular mycorrhizal (AM) fungi during the first stages of primary succession (Wu *et al.*, 2004), smoothing the path for the more obligate mycotrophs. This pattern is not different for the primary succession of vegetation on metal-contaminated soils (Vangronsveld *et al.*, 1996). There is even evidence that the AM symbiosis can increase the fitness of these host plants that usually exhibit 'low mycorrhizal response' (Hetrick *et al.*, 1994; Shetty *et al.*, 1995; Vogel-Mikus *et al.*, 2006).

Woody plants often are not considered as primary colonisers of metal-polluted soils (Schat *et al.*, 2000), but on a number of sites pioneer tree species, such as willows, poplars, birches and pines are able to build up small pioneer populations (Vrålstad *et al.*, 2002; Colpaert *et al.*, 2004; Adriaensen *et al.*, 2005). In trees with their long reproductive cycles, the adaptive potential for metal tolerance seems to be low (Meharg and Cairney, 2000) and even if there is selection for individuals with a higher tolerance, it may take many decades before a reasonable tolerant population is built up. Apart from the lack of fast evolution for metal tolerance in woody plants, there is a second reason why colonisation of polluted soils by trees may be very slow. Woody pioneers rely much more on their ectomycorrhizal (ECM) fungi than herbaceous pioneer plants rely on their AM mycobionts, irrespective of any soil pollution (Ashkannejhad and Horton, 2006). Recent investigations clearly illustrate the great importance of generalist ectomycorrhizal (ECM) fungi for the establishment of woody species during primary succession on a volcanic desert (Nara, 2006a,b). After severe disturbances, when mycorrhizal propagules are very scarce, ECM plants are slow colonisers. This is completely different in secondary successions where tree seedlings rapidly recruit ECM fungi, more often specialists, from dormant spore banks or other resistant propagules (Nara, 2006b; Izzo *et al.*, 2006). On severely metal-polluted soils, an additional constraint is toxicity. Suppose the relatively rare occasion that a germinating pioneer tree and a germinating ECM propagule come close enough to form a compatible symbiotic association. At that time both

partners must be able to withstand the metal toxicity during all stages of colonisation events. It is likely that the fungus is the most vulnerable partner in the presymbiotic phase. Despite this hostile environment, the ECM symbiosis persists on strongly metal-contaminated sites that are slowly colonized by mycotrophic tree species such as birches, pines and willows. Therefore one can only conclude that trees resist extreme metal toxicity through a large phenotypic plasticity and through their association with a small guild of well-adapted ECM fungi (Wilkinson and Dickinson, 1995). Plant adaptation to selective pressures is often considered to be regulated by the plant genome, but it is absolutely clear that also mutualistic microorganisms can alleviate heavy metal stress in plants (Schützendübel and Polle, 2002; Hall, 2002; Adriaensen *et al.*, 2004). Not only mycorrhizal fungi should be mentioned here. Numerous metal-tolerant rhizosphere and endophytic bacteria that form loose or intimate associations with plants are known to improve plant fitness on metal-contaminated soils (Vivas *et al.*, 2006).

Mycorrhizal fungi in metal-polluted soils

Plants of facultative metallophyte populations from metalliferous soil almost invariably exhibit higher levels of metal tolerance than those of normal soil populations (Schat *et al.*, 2000). Compared to the vast body of literature published on the impact of metal toxicity on plant communities and populations, relatively few information is available on the existence of metal-tolerant fungal species, populations or communities in natural habitats. Up to now, no highly adapted fungi with a distribution restricted to contaminated soils were detected. There is only some circumstantial evidence that mycorrhizal communities, both ECM and AM, are affected by heavy metal pollution. Species diversity seems to be lower on the most polluted areas (Staudenrausch *et al.*, 2005), but low ECM fungal diversity is rather typical for pioneer conditions. What we can be sure is that succession is slower on polluted soils because the invasion of new plants and microorganisms is much slower than in unpolluted conditions. In situ treatment of 3 ha Maatheide soil with a metal-immobilising substance decreased toxicity significantly and resulted in a quick increase in plant species diversity of metal-tolerant grassland (Vangronsveld *et al.*, 1996). It remains unclear to what extent the slow succession on metal-contaminated soils is really affected by a lack of suitable saprotrophic and biotrophic microorganisms. In this respect it is worth noting that at least plant species richness is higher in primary successions on disturbed sites close to metal outcrops (Ernst, 1990). A larger pool of metal-adapted plants (genes) is present in these areas and it is tempting to assume that this might be true as well for biota from the other trophic levels of the ecosystem.

In our field surveys along the Zn pollution gradient in N-Limburg (Colpaert *et al.*, 2004), we focused on the occurrence of ECM fungi in pioneer pine forests. Only on the most polluted area in Maatheide a very low number of ECM morphotypes (4) was found on roots of 25 yr-old pine trees. Remarkable is the frequent occurrence of a dark ascomycete from the *Hymenoscyphus ericae* aggregate, which was rare or absent from the less polluted plots. Similar ascomycetes are also present on pioneer pine trees that colonise the Folldal Cu mine spoil in Norway (Vrålstad *et al.*, 2002) and dark ascomycetes (Sordariaceae) were even so dominant mycorrhizas on roots of young *Salix caprea* trees thriving on very toxic soil close to the Pb-smelter of Zerjav in Slovenia (Pers. com. M. Regvar). These observations are in the same line as those observed by Bradley *et al.* (1981) who demonstrated that heather (*Calluna vulgaris*) is entirely dependent on the ascomycete *Hymenoscyphus ericae* for survival on

acidic Cu, Zn and (or) Pb polluted soils in the UK. Whether this ericoid mycorrhizal fungus can improve fitness of an ECM host under metal stress remains unclear. There are indications that particular ascomycetes are more stress-resistant than basidiomycetes and show up more frequently in mycorrhizal communities facing harsh environmental conditions or after severe disturbances (Baar *et al.*, 1999; Trowbridge and Jumpponen, 2004). Apart from the dark ascomycetes, the metalliferous sites in Maatheide and in Folldal had also a basidiomycete ECM fungus in common: *Suillus luteus*, a typical mycobiont of young pine trees; a fungus that is not confined to polluted sites and that is very common on young pines in primary successions (Fig. 1). On the Belgian sites this fungus is the most prolifically fruiting species, usually on the bare soil and most distantly from the stem base of its host trees.

<Figure 1>

In general, we have only a very incomplete view on the biodiversity of mycorrhizal fungi in metal-polluted environments. Waiting for belowground community studies, I would like to refer to two community studies, based on aboveground sporocarp observations, along metal gradients in north and south Sweden (Rühling *et al.*, 1984; Rühling and Söderström, 1990). Although molecular studies regularly find a considerable lack of correspondence between the above- and belowground communities of ectomycorrhizal colonisers (Gardes and Bruns, 1996), the field studies of Rühling suggest that particular mycorrhizal species disappear with increasing metal stress. Sporocarp production in *Picea abies* forests along a Cu-Zn gradient was investigated (Rühling *et al.*, 1984). The average number of species of macrofungi per plot (1000 m²) decreased significantly along the gradient: 35 species at a Cu concentration of about 100 µg/g organic matter, 25 species at 1000 µg/g and only 15 species at 10000 µg Cu/g organic matter. Some ectomycorrhizal taxa decreasing along the gradient were: *Cantharellus cibarius*, *Cortinarius* sp., *Dermocybe* sp., *Gomphidius* sp., *Hydnum* sp., *Lactarius* sp., *Paxillus involutus*, *Russula* sp. Taxa which were not affected or that increased in frequency, include: *Albatrellus ovinus*, *Amanita* sp., *Cantharellus tubaeformis*, *Laccaria laccata*, *Leccinum* sp. In north Sweden, a similar study was performed along a more complex metal (As, Cu, Pb, Cd, Zn) gradient but very similar results were obtained as well (Rühling and Söderström, 1990). In the least polluted plots, 4.4 species of macrofungi were found per 100 m² whereas only 1.3 species were observed in the most heavily polluted plots. The number of observations of the genus *Amanita* increased in the most heavily polluted area, the genera *Cortinarius*, *Lactarius* and *Russula* showed decreasing numbers of observations. The reduced sporocarp production and the decreasing aboveground diversity do not necessarily mean that the percentage of root colonisation decreased over the same gradient. It is possible that a few fungi dominate the community or that species without aboveground sporocarps take over. It would be interesting to know whether there is an increase in mycorrhizal ascomycetes over the metal gradients studied by Rühling. Other basidiomycete taxa that have been frequently found on heavily polluted soils include *Hebeloma* sp., *Pisolithus tinctorius* (Turnau *et al.*, 1988), *Rhizopogon* sp. (Turnau *et al.*, 1996), *Scleroderma* sp. (Jones and Hutchinson, 1986), and the Cd-accumulating *Amanita muscaria* (Gast *et al.*, 1988; Kalač *et al.*, 1991).

Detailed studies on AM fungal communities from polluted sites are even so scarce but also from these surveys a reduced number of species seems to be able to invade heavy metal-polluted sites (da Silva *et al.*, 2005), although a relatively high number of AM fungi can be present in old vegetations on metal outcrops (Pawlowska *et al.*, 1996; Tonin *et al.*, 2001)

Field studies on mycorrhizal community or population structure are much more difficult and laborious to obtain than studies on the vegetation itself. Because mycorrhizal fungi are cryptic organisms and hyphal growth is indeterminate, establishing even the most basic population features, such as number and size of individuals, net secondary production, can be difficult and can only be estimated via indirect molecular techniques. In addition, AM fungi are coenocytic clonal organisms and it is still not very well understood how genetic traits are transferred to descendants since we know little about the behaviour of the nuclei during the life cycle of these fungi. This knowledge on reproductive biology is essential to better understand the selection process for genes that confer improved fitness in fungi from metal-polluted substrates. It is of practical importance to know whether 'metal-tolerance' genes can be maintained in a population of nuclei for long periods when the selection pressure is removed.

The evolution towards heavy metal tolerance is expected to cause a strong genetic bottleneck during colonisation of polluted areas, thus leading to a reduction of gene diversity in metal-tolerant populations as compared to populations from normal soils. Many studies of pseudometallophytes exhibiting populational tolerance have therefore attempted to detect reduced genetic variation in these metallicolous populations as compared to normal ones. Surprisingly, most of them failed to detect strong founder effects (Pauwels *et al.*, 2005). We investigated the genetic structure of the *S. luteus* populations along the Zn gradient in N-Limburg (Muller *et al.*, 2004). Both AFLP and microsatellite markers revealed high levels of genetic diversity within the geographic subpopulations, but genetic differentiation between subpopulations was limited, suggesting substantial gene flow and frequent sexual reproduction. Although a priori we expected that heavy metal pollution would have a strong effect on the genetic structure of *S. luteus* populations, no evidence was found for a consistent reduction of the genetic variation of the subpopulations in polluted habitats. This is in fact a result similar to what plant population geneticists found for several pseudometallophytes (Vekemans and Lefèvre, 1997; Van Rossum *et al.*, 2004; Pauwels *et al.*, 2005). Several nonexclusive processes may explain the surprisingly high level of variation in the metal-tolerant *S. luteus* populations. Successive colonisation events - the species is a common pioneer - or a relatively high frequency of metal tolerance in natural populations could have reversed the initial genetic bottleneck. In addition recurrent migration of tolerant genotypes that originate due to admixture in nonpolluted areas may attribute to the high level of genetic diversity of populations inhabiting contaminated soils. Admixture between the tolerant *S. luteus* populations and surrounding populations inhabiting nonpolluted soils is very likely, as indicated by the low level of population differentiation, and it would explain the relatively high frequency of tolerance observed in some of the populations in the transition zone from a high to low degree of pollution (Colpaert *et al.*, 2004).

Metal-tolerant mycorrhizal fungi

From the above paragraph one can presume that mycorrhizal fungi that survive and reproduce in metal-contaminated substrates must have acquired a high resistance against metal toxicity. However, it proved to be quite difficult to demonstrate unequivocally that metal toxicity exhibited selection pressure on fungal communities and populations. Particular mycorrhizal guilds may have a high constitutive metal tolerance so that evolution for higher tolerance is simply not necessary (Meharg and Cairney, 2000). The occurrence of dark ascomycetes in the

most contaminated soils gives some support to this hypothesis. The absence of metal adaptation would be in contrast to what is known for plants and bacteria.

Mycorrhizal fungi that colonise acidic soils can be exposed to high levels of toxic metals such as aluminium, iron and manganese. These fungi must have evolved mechanisms for coping with these elements and it is possible that some of these mechanisms confer some resistance to other metals as well including Cu, Cd, Pb and Zn. However, this reasoning should also apply to the plants themselves and there is no evidence that Al- or Fe-tolerant species have a high constitutive tolerance for Cu or Zn or that plants from acidic soils are better colonisers of contaminated areas. Excess metals may cause partly similar problems in cells (for example oxidative stress) and this damage may be healed with similar remedies, but on the other hand each metal has different characteristics, different kinetics and pathways for entering and leaving cells, causes some specific molecular interactions and needs specific molecules for chelation and sequestration. Uptake and homeostasis of heavy metals, at least of the essential ones, is strictly controlled which is only possible through a specific fine tuning of the activity of highly metal-specific transporters located in the plasma membrane, tonoplast and membranes of other organelles. For example in yeast the activity of the Zn transporters is regulated at both transcriptional and posttranscriptional levels in response to Zn (Eide, 2003). A fungus that copes well with excess Cu is not necessarily well equipped to withstand excess Fe or Zn and vice versa. Metal tolerance mechanisms are usually quite specific in all kinds of organisms although low levels of co-tolerance sometimes have been found.

It is remarkable that some organisms seem to be predestined to evolve specific metal-tolerance mechanisms. Such particular species are well-known among prokaryotes (Mergeay *et al.*, 2003) and in the plant kingdom (Ernst, 1990; Schat *et al.*, 2000). These organisms can acquire tolerances against many different metals. In bacteria plasmids play a major role in this multiple heavy metal tolerance. In most organisms true adaptation seems to be governed by a small number of genes. Nevertheless, such genetic modifications occur slowly, in particular in eukaryotic species with long reproductive cycles.

Organisms trying to survive in heavy metal-contaminated ecosystems have a whole battery of responses to their disposal to cope with heavy metal stress. Species with the least efficient detoxification systems will disappear from the ecosystem. In conditions where the heavy metal pollution is so severe that there are consistent detrimental effects on metabolism, organisms are subjected to selective pressures for increased resistance to toxic metals. These adaptations involve modifications in the genetic patrimony of an organism. There has been a long debate whether mycorrhizal fungi have evolved adaptive tolerance against particular heavy metals (Hartley *et al.*, 1997). A major reason was the lack of sufficient data from different populations from sites with high and low levels of pollution. Measuring and comparing metal tolerance can only be achieved by screening a significant number of individuals from one or more species. It is necessary to analyse the intra- and interpopulation variation in metal tolerances. This is far easier for plants than for their fungal partners. The isolation and axenic cultivation of large numbers of mycorrhizal fungi can be quite troublesome, especially for AM fungi. Luckily some pioneer ECM fungi are easier to cultivate so that inter- and intraspecific comparisons for metal tolerance become possible. Nevertheless, inter-species comparisons *in vitro* can still be misleading because some fungi might be much more sensitive to stress *in vitro* than in symbiosis in contrast to species that

grow equally well *in vitro* as in nature. Intraspecific comparisons are probably less susceptible to such confounding factors. Ideally once tolerance identified *in vitro*, it should also be verified in a plant experiment.

Selection for adaptive metal tolerance in mycorrhizal fungi has been discovered in only a few higher fungi. *Pisolithus tinctorius* isolates from an old coal mining site had higher Al tolerance than isolates from rehabilitated and forest sites (Egerton-Warburton and Griffin, 1995). In the pioneer forests around several Zn smelters in Belgium adaptive Zn tolerance was found in *Suillus luteus*, *S. bovinus* and *Rhizopogon luteolus*, but not in *Paxillus involutus* (Colpaert *et al.*, 2004). Other ECM fungi could not be tested because of a lack of sufficient isolates. Monokaryons from Zn-tolerant *S. luteus*, obtained through basidiospore germination, are also more Zn-tolerant than monokaryons from sensitive genotypes (unpublished), confirming the heritability of the tolerance trait. Screening the dikaryotic *Suillus* isolates against elevated Cd revealed that isolates from the most polluted soil in Maatheide, had acquired adaptive Cd tolerance (Colpaert *et al.*, 2000). However, the Cd tolerance was less pronounced than the Zn tolerance and it was far less widespread, which might be due to a lower selection pressure exerted by Cd. Grasses in the same area also developed a high Zn tolerance and some additional Cd tolerance.

The occurrence of *S. luteus* associated with poorly growing pine seedlings invading a Cu mine spoil prompted us to check this mine population for Cu tolerance. Indeed this particular population showed a significantly higher Cu tolerance than populations from uncontaminated or Zn-contaminated sites. The Cu-tolerant isolates were not tolerant to elevated Zn. This indicates that also in fungi the evolution of metal-specific tolerance mechanisms is strongly triggered by the pollution of the local environment (Adriaensen *et al.*, 2005). Co-tolerance was so far not detected in the *S. luteus* isolates. The evolution towards adaptive Cu-tolerance is a rare phenomenon even in the plant kingdom relatively few plants seem to be able to evolve Cu-tolerant ecotypes (Wu and Lin, 1990; Schat and Vooijs, 1997). Cu is a well-known fungicide but there are few papers that report on the evolution towards additional Cu resistance in fungi that are frequently treated on Cu-containing fungicides.

Arbuscular mycorrhizal fungi also can develop metal tolerance, although the trait seems to be less stable than in higher fungi and plants. *Glomus* cultures with a high tolerance to Mn lose the specific tolerance when cultivated in metal-free substrate in contrast to cultivation in original contaminated soil (Malcová *et al.*, 2003). Metal tolerance in AM fungi has not yet been assessed directly *in vitro*, it is usually based on tests for spore germination, germ tube growth and root colonisation. Relatively few genotypes were investigated. Shetty *et al.* (1995) also showed that the AM fungi isolated from Zn-contaminated soil surrounding mine tailing were more effective in increasing the plant biomass at high levels of Zn, whereas AM fungi from a non-contaminated site promoted plant growth only in the soil containing lower Zn concentrations.

Mechanisms of metal tolerance

The mechanisms that are involved in metal homeostasis and detoxification of essential and non-essential metals in ECM fungi were recently reviewed by Bellion *et al.* (2006). Extracellular mechanisms such as chelation and cell-wall binding as well as cellular

mechanisms such as binding to (non)-protein thiols and transport into intracellular compartments play a role in the metal housekeeping of ECM fungi. These mechanisms are not different from those that are present in other eukaryotes. Some of these mechanisms are constitutively present, whereas others come into full operation when excess metals show up in the cytoplasm. Additional antioxidative detoxification systems, which allow the fungus to counteract the accumulation of reactive-oxygen species directly or indirectly are part of the detoxification response. Whether one or more of these mechanisms are modified in the evolution towards adaptive true tolerance in ECM fungi remains unclear.

Reduced uptake of metals into the cytosol might be achieved by extracellular chelation or precipitation of metals with organic compounds, mostly acids such as citrate and oxalate. Nevertheless it is unlikely that metal detoxification is the primary function of organic acid excretion. It is probably more involved in nutrient (cation and phosphate) mobilisation from minerals and in maintaining charge balances over membranes. In many fungi organic acid production largely depends on the N source in the medium, on the phosphate limitation and on the carbon status of the culture. It remains to be demonstrated that there is sufficient organic acid excretion in the myco(rhizo)sphere of mycorrhizal fungi that colonise metal-polluted soils in the absence of a readily available C source. Organic acids also have a high turnover in a soil as a consequence of microbial degradation. Eventually the organic acid exudation should reduce metal uptake into the fungal cells. This is not obvious. Martino *et al.* (2003) studied ZnO solubilisation in isolates from the ericoid mycorrhizal fungus *Oidiodendron maius*. Strains from polluted sites had a lower ZnO solubilisation activity and a lower organic acid production than isolates from control sites. Such a reduction in acid exudation could be an adaptation to Zn pollution in this ericoid mycorrhizal fungus and may reflect specific strategies to maintain homeostasis of essential metals under different soil conditions (Martino *et al.*, 2003). This pattern of reduced Zn solubilisation was not observed in Zn-tolerant *S. luteus* and *S. bovinus* isolates exposed to poorly soluble $\text{Zn}_3(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ (hopeite), neither was found a precipitation of an organic Zn compound in the culture medium (Fomina *et al.*, 2004, 2005). The Zn-tolerant *Suillus* strains yielded more biomass, acidified the MMN medium more and dissolved more of the Zn phosphate than less tolerant strains.

Fungal cell walls have high capacities for metal binding (biosorption), but the trait is depending on fungal species and metal. Both AM and ECM fungi can bind substantial amounts of metals (Blaudez *et al.*, 2000; Joner *et al.*, 2000), although there is no strong evidence that this binding is an efficient strategy to keep excess metals out of cells. It is likely that binding sites of growing hyphal tips are almost immediately saturated with metals in strongly contaminated soils. Once the equilibrium with the surrounding soil solution is reached, the role of the cell wall in metal immobilisation is played. Biosorption is a mechanism not depending on the metabolic activity of the fungus whereas excretion of organic substances such as acids or metal-binding polyphenolics relies on the activity of the hyphae. Hydrophobins covering hyphae may affect the transfer rate of nutrients and cations from soil solution towards the plasma membrane but it is evident that also the deposition of a hydrophobic layer around hyphae is not a metal-specific barrier and has more general functions such as maintaining hydraulic balances in fungi. It cannot be excluded that metal toxicity in fungi will lead to changes in cell wall composition and hyphal growth in an effort to avoid or reduce metal exposure. Lanfranco *et al.* (2002) showed that changes in hyphal

morphology occur when an ericoid mycorrhiza-forming ascomycete is treated with millimolar concentrations of Zn. This led to apical swellings and increased branching in the subapical parts as well as a significant increase in the amount of chitin in metal-treated hyphae.

Despite extracellular chelation and cell-wall binding, large amounts of metal eventually enter into hyphae that are exposed to excess metals. At low external concentrations essential metals enter cells through metal-specific high affinity transporters. It is less clear how non-essential metals penetrate cells. Blaudez *et al.* (2000) have quantified the proportion of Cd in the cytosol and the vacuole of *Paxillus involutus* and estimated it to be 20% and 30%, respectively. At high external Zn concentrations Zn influx in *S. luteus* is high irrespective of its Zn tolerance or metabolic activity (25 or 0°C). Although downregulation of metal transporters plays a role in metal homeostasis in fungi (Eide, 2003) and plants (Clemens, 2001; Hall, 2002), it cannot explain the adaptive Zn tolerance in *S. luteus*. Evolutionary adaptation to Zn-enriched soils can be achieved in several ways. In some plants, Zn tolerance seems to be functionally related to transport processes that permit compartmentation of Zn ions and prevent the accumulation of toxic levels in the cytoplasm (Chardonnens *et al.*, 1999; Clemens, 2001; Hall, 2002). Improved accumulation of Zn in vacuoles, reduced uptake or increased extracellular efflux of Zn are all possible mechanisms that may reduce Zn toxicity in adapted plant ecotypes (Chardonnens *et al.*, 1999; Hall, 2002; Dräger *et al.*, 2004). In mycorrhizal fungi, similar mechanisms may operate, but little detailed information is available.

To avoid cellular damage metals that are in excess in the cytosol must be efficiently removed. Efflux back into the apoplast or compartmentalisation into organelles is also an option in fungi. The vacuole is probably the best candidate for mass storage of excess metals such as Cd and Zn (Blaudez *et al.*, 2000; Eide, 2003). For more details on transporters possibly involved in metal homeostasis in mycorrhizal fungi, I refer to Eide (2003) and Bellion *et al.* (2006). In *S. luteus* there is evidence that Zn efflux over the plasma membrane may explain the adaptive Zn tolerance (Adriaensen unpublished). A Zn exclusion mechanism explains the lower Zn uptake in Zn-tolerant Suilloids (Colpaert *et al.*, 2005). In the ECM fungus *Pisolithus tinctorius*, differential Al tolerance could also be attributed to an Al exclusion mechanism in the mycelia of Al-tolerant isolates from a mine site (Egerton-Warburton and Griffin, 1995).

The reactivity and limited solubility of most metal ions require constant chelation once they are taken up into the cell. Free metal concentrations are very low so that most metals are strongly complexed by protein and non-protein ligands, often compounds with a relatively high specificity for only one or few metal species. Synthesis and activity of these compounds is probably strictly controlled. Possible metal chelators in mycorrhizal fungi include phytochelatins, metallothioneins and organic acids. Courbot *et al.* (2004) found that glutathione increased under Cd exposure in *Paxillus involutus*, as well as γ -glutamylcysteine and a Cd-binding metallothionein (Courbot *et al.*, 2004). Phytochelatins were not detected in *P. involutus*. The expression of the metallothionein was studied at the transcriptional level in *P. involutus* exposed to different metal stress, and the results indicated a correlation between metal exposure and expression level (Bellion *et al.*, 2006). Whether intracellular complexation of metals plays a role in adaptive metal tolerance is not known. In plants these metal chelators are important for basic tolerance and homeostasis but there is little

unequivocal evidence that they are involved in the generation of naturally selected tolerance (Clemens, 2001).

In general our knowledge on metal fluxes, compartmentation and intracellular complexation in mycorrhizal fungi is still very limited. Different detoxification mechanisms may also have an indirect impact on host plants. Fungi that compartmentalise metals into vacuoles, may increase transfer of these metals to their host. Motile tubular vacuoles are an important vector in the transport chain of mineral nutrients from the site of uptake at hyphal tips to the exchange region in the mycorrhizal root (Ashford and Allaway, 2002). The Zn exclusion observed in Zn-tolerant Suilloid fungi will not only help to prevent the onset of metal stress in these fungi, but it will also contribute to the protection of a host plant. The assumption that adapted isolates do not store extra Zn in their vacuoles is an attractive prospect for a host plant. Some ECM fungi are known to accumulate specific metals in their sporocarps, even on soils that are nonpolluted; a well-known example being the Cd accumulation in *Amanita muscaria* (Lepp *et al.*, 1987; Lepp, 1992). In this respect it could be interesting to investigate whether this accumulation also leads to an increased Cd transfer to a host plant. Accumulation of Zn in vacuoles could greatly increase the transfer of zinc to the host plant and this exactly did not happen in pines colonised with a Zn-tolerant *S. bovinus* isolate (Adriaensen *et al.*, 2004).

Does adaptive metal tolerance in the mycorrhizal fungus increase host plant fitness?

Alleviation of metal toxicity in plants through ectomycorrhizas has been demonstrated in a number of experiments (for a review: Jentschke and Godbold, 2000). In most of these experiments it was shown that mycorrhizal plants were better off with a suitable ECM fungus than without; and it was also shown that some fungi were more efficient in plant protection than others. Only a few researchers made intraspecific comparisons with fungi from different origins. For protection of a host plant that has the potential to grow on a particular contaminated soil, three major aspects are particularly important for a successful mycorrhizal association: 1. survival of the fungus in the toxic substrate, 2. efficient nutrient transfer to the host, 3. low transfer of the toxic metal to the host.

The survival of the fungus is essential to establish the symbiosis. The same is of course true for the roots of the germinating trees. There is good evidence that a number of fungi cannot withstand metal concentrations in soil solutions of polluted soils. The intraspecific comparisons with Zn- and Cu-tolerant *Suillus* species demonstrate that normal (sensitive) isolates suffer severely from Zn and Cu concentrations that do not affect growth of tolerant isolates (Adriaensen *et al.*, 2004, 2005, 2006). In particular the external mycelium that colonises the soil is most sensitive to excess metals. However, close to active roots even sensitive isolates can survive for several weeks.

Improved plant nutrition is a major function of the mycorrhizal symbioses. This is not different for ECM plants on metal-polluted soils. Most of these soils are poor in essential nutrients and it is clear that pioneer ECM fungi can alleviate this low nutrient stress. Their external mycelia contribute largely to the nutrient uptake and transfer to the host. This can only be achieved when the fungus can maintain the growth of its mycelium. Eventually this improved nutrition should lead to a better health and growth of trees associated with the most

tolerant isolates. In comparisons between mycorrhizal and nonmycorrhizal plants this was demonstrated in a number of experiments (Jentschke and Godbold, 2000). Also the metal-tolerant *Suillus* isolates can outperform their sensitive counterparts at least in a long-term experiment (Adriaensen *et al.*, 2006).

<table 1>

Fungal metal uptake, translocation and transfer to the host are a complex process. It is usually analysed indirectly by measuring metal contents in plants. Mycorrhizal fungi can improve uptake of essential elements in their host when present at low concentrations. However, there is probably a lot of inter- and intraspecific variation among fungi in nutrient uptake and transfer (Munkvold *et al.*, 2004). In a recent experiment we detected a differential Zn transfer to pines colonised with *S. luteus* exposed to a low (not deficient) Zn concentration (Table 1). Nonmycorrhizal plants had the lowest Zn concentration, plants colonised with a tolerant isolate had a slightly higher concentration, but the highest Zn concentration was observed in plants colonised by a normal *S. luteus* isolate. Pine growth rate was similar in all plants. The differential transfer of Zn in *S. luteus* can probably be explained by the adaptation that occurred in the Zn-tolerant Suilloids. A reduced transfer of metals at high external concentrations was also demonstrated in the dose-response experiments by Adriaensen *et al.* (2004, 2005), although interpretation of these data becomes more difficult as a result of the decreasing transpiration stream with increasing toxicity (Jentschke and Godbold, 2000).

Conclusions

Despite the scepticism about the need to evolve true tolerance in mycorrhizal fungi, there is more and more evidence that heavy metal adaptation in some ECM fungi has occurred and that it is a prerequisite for the survival of both trees and fungi on the most toxic soils. However, compared to what is known about metal tolerance in plants, we still have a long way to go. There is a definite need for studies on mycorrhizal population and community dynamics in polluted soils and the search for more species with true tolerance should continue. Among metal-tolerant ECM fungi, there are metal excluders and accumulators; we have no idea whether this behaviour does affect the metal transfer to a host plant. Furthermore, we are still at the dawn of the elucidation of the molecular mechanisms involved in metal homeostasis, detoxification and tolerance in filamentous fungi in general. A better understanding of metal transport mechanisms including their regulation and in the underlying biochemical and physiological mechanisms of tolerance are of key interest.

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Table 1. Differential zinc uptake in shoots and roots of *Pinus sylvestris* seedlings inoculated with *Suillus luteus* or not. Plants were exposed to a low Zn concentration.

Fungal treatment	Shoot	Root
	$\mu\text{g g}^{-1}$ d. wt	$\mu\text{g g}^{-1}$ d. wt
Nonmycorrhizal	20.7 ± 1.9	49.0 ± 2.7
Zn-sensitive <i>S. luteus</i>	54.3 ± 2.9	79.9 ± 7.0
Zn-tolerant <i>S. luteus</i>	30.5 ± 3.0	57.8 ± 3.5

Values are means from 10 plants \pm SE.

Fig. 1. *Suillus luteus*, a pioneer ectomycorrhizal fungus that evolved metal-tolerant ecotypes on metal-polluted soils.

