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Element profiles and growth in Zn-sensitive and Zn-resistant Suilloid fungi

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Abstract Zn pollution has triggered evolution for adaptive Zn tolerance in populations of Suilloid ectomycorrhizal fungi. The objectives of this study were to determine differential physiological responses that are linked to the Zn tolerance trait and to obtain more insight in the general mechanism responsible for the differential growth in Zn-enriched medium. Therefore, we identified intrinsic growth rates and element profiles in Zn-sensitive and Zn-tolerant genotypes. Isolates from Zn-polluted and unpolluted sites were exposed in vitro to increasing Zn^{2+} stress. The Zn concentration which inhibits growth by 50% (EC_{50}) was determined, and element (Zn, Fe, Mn, Cu, Mg, Ca and P) profiles in the mycelia were analysed. The intraspecific variation in growth rate and nutrient content of the in vitro grown mycelia is great and was not reduced in Zn-tolerant populations. The Zn resistance was not correlated to the intrinsic mycelial growth rate of the isolates or to the concentrations of the elements analysed, except for Zn. At low external Zn, Zn-resistant genotypes had lower Zn concentrations than sensitive isolates. At high external Zn, the differential Zn accumulation pattern between resistant and sensitive isolates became very prominent. Zn-exclusion mechanisms are most likely involved in the naturally selected adaptive Zn resistance. Other mechanisms of Zn detoxification such as sequestration of Zn on cell wall compounds or intracellular chelation and/or compartmentation are probably active but cannot explain the differential Zn sensitivity of the isolates.

Keywords Ectomycorrhiza · Zinc resistance · *Suillus luteus* · *Suillus bovinus* · *Rhizopogon luteolus*

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Introduction

Zinc is a trace nutrient indispensable in life. It is a constituent in more than 300 metalloenzymes and other proteins (Kambe et al. 2004). However, elevated concentrations of Zn in soil solution can lead to toxicity in plants and associated micro-organisms. The exact mechanisms of Zn toxicity are not completely elucidated, but toxicity is probably due to a displacement of other essential cationic cofactors (e.g. Fe, Mn, Mg) of proteins resulting in loss of function (Marschner 1995). Excess Zn also interferes with the uptake of other micronutrients such as Fe and Mn (Hall 2002). Although zinc cannot directly cause oxidative injury, it seems to disturb the anti-oxidative defence mechanisms of cells through interactions with enzymes from the glutathione cycle (Cuypers et al. 2001; Schützendübel and Polle 2002). Nevertheless, basic Zn tolerance is ubiquitous, and numerous cellular mechanisms (and genes) have been described playing a role in Zn homeostasis in cells (Clemens 2001; Hall 2002; Hall and Williams 2003).

Some plant species and micro-organisms have evolved Zn-tolerant ecotypes that can survive on Zn-toxic soils, presumably by adapting mechanisms that are involved in the general homeostasis of Zn (Hall 2002). Metal-tolerant ecotypes often show enhanced avoidance and/or homeostatic mechanisms to prevent the onset of metal stress. In plants, elevated, naturally selected Zn tolerance seems to be governed by a small number of major genes with small contributions from some minor modifier genes (Schat et al. 1996; Macnair et al. 1999). In Zn-tolerant plants, there is also little evidence for co-tolerance against other metals,

suggesting the involvement of specific mechanisms for the Zn tolerance (Hall 2002). The cellular mechanisms of the adaptive Zn tolerance in accumulator and non-accumulator plants are intensively investigated but are not yet fully understood (Chardonnens et al. 1999; Assunção et al. 2003a,b; Dräger et al. 2004).

Naturally selected Zn tolerance also exists in some Suilloid ectomycorrhizal (ECM) fungi that thrive in pine forests subject to Zn pollution, and in these fungi, there is no co-tolerance for other metals (Colpaert et al. 2000, 2004). The constitutive mechanisms utilised by ECM fungi at the cellular level to resist heavy metals are probably similar to the strategies that exist in other eukaryotes. However, few constitutive or adaptive tolerance mechanisms have been studied in detail in ECM fungi (Meharg 2003).

To determine differential physiological responses that are linked to the Zn-tolerance trait and to better understand the general Zn-tolerance mechanism in Suilloids, we have analysed intrinsic growth rates and phosphorus and micronutrient contents in a large number of Zn-tolerant and Zn-sensitive genotypes of *Suillus luteus*, *Suillus bovinus* and *Rhizopogon luteolus*. Isolates were grown in vitro on normal medium and on medium enriched with Zn. Mineral nutrient and trace element profiling has been proven to be a useful tool to determine plant mutants with altered elemental profiles and may provide further insight into metal tolerance mechanisms (Lahner et al. 2003). Element profiles are also used to find inter-population differences in nutrient acquisition, metal tolerance and bio-accumulation of heavy metals. Metal-adapted populations of plants and fungi may exhibit different growth rates and morphological characteristics, or they may have very different nutrient requirements than normal populations, possibly as part of the detoxification mechanism (Baker and Dalby 1980; Macnair 1987; Meharg 2003; Kidd et al. 2004). Even characteristics that are unrelated to the tolerance itself may be selected in metal-adapted populations. Natural selection can lead to a rapid fixation of the alleles responsible for the tolerance trait, and many alleles, which are linked to the tolerance genes, may be taken to fixation by hitchhiking (Macnair 1987). In addition, metal-contaminated soils are often nutritionally very poor, have a low organic matter content and a low water retention capacity. Adaptation to such conditions can co-occur, a phenomenon described in metal-adapted grasses and *Mimulus gutatus* (Macnair 1987).

Materials and methods

Site descriptions and fungal material

Basidiomes of *S. luteus* (L.: Fr.) Roussel, *S. bovinus* (L.: Fr.) Roussel and *R. luteolus* Fr. emend. Tul. & Tul. were collected in pine forests along a Zn gradient in the Belgian Limburg province. The pollution sources and fungal collection sites are described in detail in Colpaert et al. (2004). All 12 sites are situated in the Campine phytogeographic district, which is characterised by base-poor, sandy soils of low fertility. In the present investigation, we included ECM Suilloids from populations in Lommel-Maatheide (Lm), Lommel-Sahara (Ls), Neerpelt (N), Overpelt-fabriek (Of), Eksel (E), Maasmechelen (Mm), Hechtelse heide (Hh), Paal (P), Houthalen (Hr), Meeuwen-Gruitrode (Mg), Zolder (Z) and Dilsen-Stokkem (Ds).

Zn treatments

Populations from these three different Suilloid fungi were tested for Zn tolerance in a large-scale screening experiment. Zn tolerance was tested on solid agar media covered with cellophane sheets. Composition of the medium is given in full detail in Colpaert et al. (2004). Ten Zn treatments were established through addition of $ZnSO_4 \cdot 7H_2O$ to the nutrient medium. Zn^{2+} was added at concentrations of 0.15, 3, 6 up to 27 mM Zn, increasing in steps of 3 mM. The pH of the final media was adjusted to 4.5. For each genotype–treatment combination, there were three replicates. The fungi were incubated at 23°C in the dark. Mycelia of *S. luteus* and *R. luteolus* were harvested after 10 days of incubation, *S. bovinus* after 12 days. Mycelia were frozen at -80°C and subsequently freeze-dried before weighing. The dry weight (d. wt.) increment during the 10- or 12-day test period was determined. Tolerance indices were calculated for each isolate as the percentage of biomass retained in the metal-enriched media as compared to the growth on the control medium (0.15 mM Zn). The tolerance index obtained at 6 mM external Zn was used to discriminate between tolerant and sensitive isolates (Colpaert et al. 2004).

Element concentrations in untreated mycelia

A large number of isolates grown in vitro at the control, non-toxic concentration of 0.15 mM Zn was analysed for element content. Genotypes from uniformly Zn-tolerant (Lm, Ls, N, Of) or Zn-sensitive populations (P, Mg, Hr, Z) were included, as well as isolates from populations with both tolerant and sensitive genotypes (E, Hh, Mm, Ds) (Colpaert et al. 2004). Eighty-four genotypes of *S. luteus*, 54 genotypes of *S. bovinus* and 11 from *R. luteolus* were analysed for their contents of Zn, Fe, Mg and P. In 22 *S. luteus* genotypes, Cu, Mn and Ca concentrations were also measured.

Element concentrations in Zn-treated *S. luteus*

In a second analysis, nutrient concentrations were determined in *S. luteus* mycelia grown over a wider Zn concentration range. Sixteen genotypes were selected for this analysis; eight Zn-tolerant isolates were from Lm, Ls, Of or Ds and eight sensitive isolates were from P, Mm or Ds.

Element analysis

Freeze-dried mycelium from cultures (3–25 mg) was wet-digested in Pyrex tubes in a heating block, two cycles with addition of 1 ml HNO_3 (65%) followed by one cycle in 1 ml HCl (37%) at 120°C for about 5 h. Samples were eventually resolved in HCl and diluted to a final volume of 5 ml (2% HCl). All analyses were performed on triplicate samples, and certified reference material was included for element analyses: Virginia tobacco leaves (CTA-VTL-2, Institute of Nuclear Chemistry and Technology, Warsaw, Poland) and spinach leaves (Standard reference material 1570a, National Institute of Standards & Technology, Gaithersburg, MD, USA). Phosphate was determined colorimetrically with a Flow Injection Analyser (FIA, Lachat), using the phosphomolybdate assay. Metals (Zn, Fe, Mg, Mn, Cu, Ca) were analysed with atomic absorption spectroscopy (AAS) in the first analysis and with inductively coupled plasma optical emission spectroscopy (ICP-OES) in the second experiment.

Interspecific differences in element concentrations were analysed with one-way ANOVA. Intraspecific relationships between biomass production, element contents and Zn tolerance were statistically analysed through the generalised estimating equations method (Liang and Zeger 1986). Genotypes were collected from 12 populations. Individuals from the same location might be more similar than isolates taken at different locations because they share the same environment. In case of a positive correlation effect (i.e. samples at the same location are more similar than at different locations), ignoring this correlation in the statistical analysis can lead to serious underestimation of the variances of parameters of interest. This might result in spuriously 'significant' effect. In order to obtain reliable estimates that account for such correlations in the data, the generalised estimating equations procedure was used (Liang and Zeger 1986).

Results

Fungal biomass

The intraspecific dry weight production on control medium was highly variable within the three species, from 25 to 60 mg in *S. luteus*, from 17 to 38 mg in *S. bovinus* and from 18 to 55 mg in *R. luteolus*. This variation in growth was overall very similar between Zn-sensitive and Zn-tolerant genotypes (Fig. 1), and only in *S. luteus* was there a small positive correlation between biomass production and Zn tolerance of the isolates.

Nutrient contents in untreated mycelia

Although the three related species were grown on the same nutrient medium, there were significant interspecific differences in the concentrations of P, Zn, Fe, Mg and Cu in the mycelia (one-way ANOVA, $P<0.01$). For most elements, intraspecific variation in concentration was quite large. However, this was not the case for the P concentration in *S. luteus*, in which it was surprisingly similar over all genotypes (4.03±0.28 mg g^{-1} d. wt.) (Fig. 2). In *S. bovinus*, the P concentration was higher and more variable (6.31±0.96 mg g^{-1} d. wt.), whereas in *R. luteolus*, the P concentration averaged at 4.75±0.58 mg g^{-1} d. wt. (Fig. 2). In the untreated *S. luteus* and *R. luteolus* isolates, the P concentration of the mycelia was not correlated to their Zn tolerance index. In *S. bovinus*, there is a small positive relationship between mycelial P concentration and Zn tolerance (Fig. 2).

Copper levels were twice as high in *S. luteus* (10 μg g^{-1} d. wt.) than in *S. bovinus* (5 μg g^{-1} d. wt.). Iron in the mycelia showed a very large intraspecific variation ranging from 180 to 840 μg g^{-1} d. wt. in *S. luteus*, from 260 to 770 μg g^{-1} d. wt. in *S. bovinus* and from 130 to 500 μg g^{-1} d. wt. in *R. luteolus*. The intraspecific Mg concentration in the mycelia varied from 430 to 970 μg g^{-1} d. wt. in *S. luteus*, from 540 to 1280 μg g^{-1} d. wt. in *S. bovinus* and from 380 to 790 μg g^{-1} d. wt. in *R. luteolus*. Within

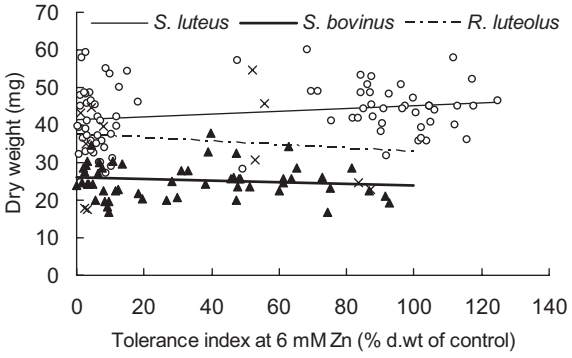

Fig. 1 Average mycelial dry weight (n=3) of genotypes of (○) *S. luteus*, (▲) *S. bovinus* and (×) *R. luteolus*, grown for 10, 12 and 10 days, respectively, on a medium without elevated Zn. Dry mass is plotted against the Zn tolerance index of the genotypes. This index was calculated from the mycelial dry weight produced at 6 mM Zn. *Regression lines* are shown. *S. luteus*, y=0.037x+41.51, P=0.09; *S. bovinus*, y=−0.024x+26.213, P=0.32; *R. luteolus*, y=−0.046x+37.412, P=0.71

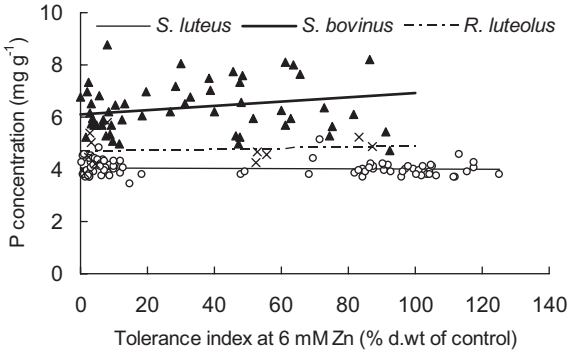

Fig. 2 Phosphorus concentration (n=3) of genotypes of (○) *S. luteus*, (▲) *S. bovinus* and (×) *R. luteolus*, plotted against their 6-mM Zn-tolerance index. *Regression lines* are shown. *S. luteus*, y=−0.0006x+4.0529, P=0.53; *S. bovinus*, y=0.0083x+6.089, P=0.008; *R. luteolus*, y=0.002x+4.671, P=0.32

each species, there was no clear correlation between Zn tolerance and Cu, Fe or Mg concentration in untreated mycelia. The mycelial concentrations of Mn and Ca were also not correlated to Zn tolerance (data not shown).

Interestingly, in all three fungi, the Zn concentration in the mycelium was negatively correlated with the Zn tolerance of the isolates. Genotypes that turned out to be Zn-sensitive have in general a higher zinc concentration in control medium. Tolerant isolates accumulate less Zn in control medium (Fig. 3). This pattern was also consistent within all populations.

Nutrient contents in Zn-treated *S. luteus*

At 6 mM $[Zn]_{ext}$, all sensitive *S. luteus* isolates showed very little growth; at 3 mM $[Zn]_{ext}$, their biomass was 8 to 80% lower than in control medium. The Zn concentration in the mycelia increased strongly with increasing exposure to Zn (Fig. 4a). The increase was much more pronounced in the Zn-sensitive isolates than in the Zn-tolerant isolates. At 0.15 mM $[Zn]_{ext}$, Zn in mycelia varied from 0.25 to 0.42 mg g^{-1} d. wt. in the eight tolerant *S. luteus* genotypes and from 0.47 to 0.92 mg g^{-1} d. wt. in the eight sensitive isolates. At 3 mM Zn, these values ranged from 1.8 to 3.4 mg g^{-1} d. wt. in tolerant isolates and from 4.5 to 6.1 mg g^{-1} d. wt. in sensitive isolates. At the highest Zn concentration applied, the *S. luteus* tolerant genotypes had a mean tolerance index of 61% and contained on average 15.6 mg Zn g^{-1} d. wt. (Fig. 4a).

The P concentration in the Zn-resistant *S. luteus* genotypes remained fairly similar over the whole Zn range (Fig. 4b). In the Zn-sensitive *S. luteus* isolates, P concentration increased about 50% at 3 and 6 mM $[Zn]_{ext}$. Zn-treated mycelia showed no changes or only small reductions in Cu, Mn, Mg and Ca concentration with increasing Zn stress (data not shown), and the patterns were similar for Zn-tolerant and Zn-sensitive isolates. Only for Fe was a differential accumulation pattern in Zn-tolerant and Zn-sensitive isolates (Fig. 4c) noticed.

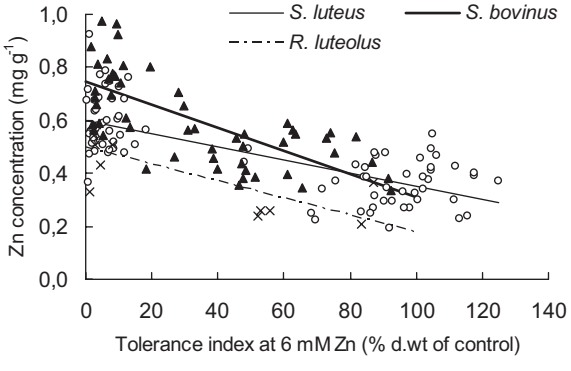

Fig. 3 Zinc concentration (n=3) of genotypes of (○) *S. luteus*, (▲) *S. bovinus* and (×) *R. luteolus*, plotted against their 6-mM Zn-tolerance index. *Regression lines* are shown. *S. luteus*, $y=-0.0025x+0.601$, $P<0.0001$; *S. bovinus*, $y=-0.0044x+0.748$, $P<0.0001$; *R. luteolus*, $y=-0.0032x+0.499$, $P<0.0001$

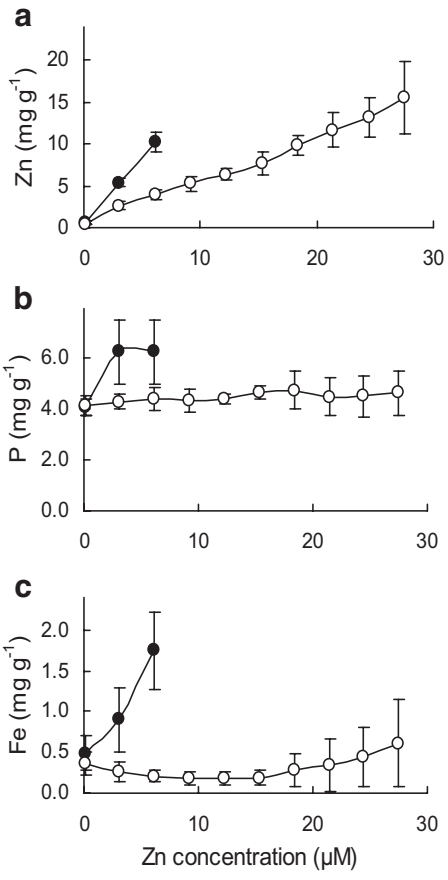

Fig. 4 Element concentrations in mycelia of Zn-tolerant (*open circles*) and Zn-sensitive (*closed circles*) isolates of *S. luteus* (n=8) exposed to increasing Zn concentrations for 10 days. Zn (**a**), P (**b**), Fe (**c**). *Bars* represent SD

Discussion

The intraspecific variation in growth rate and nutrient content of the in vitro grown mycelia is remarkable. High genetic and functional diversity within species of mycorrhizal fungi seems to be a common phenomenon (Cairney 1999; Muller et al. 2004). Only extensive screenings under controlled conditions can reveal reliable information on the intraspecific variations in physiological capabilities of particular taxa or populations (Cairney 1999; Munkvold et al. 2004). Element concentrations in the fungi are the result of the activities of a whole set of genes that regulate metal homeostasis. In model plants such as *Arabidopsis thaliana*, the determination of (trace) element profiles, ionomics, is a useful tool to determine mutants with altered elemental profiles (Lahner et al. 2003; Salt 2004). Most of the mutants with altered element profiles have mutations in membrane transporters, resulting in a differential uptake of only one or two very related elements. In the present work, a similar approach was used to compare genotypes from natural *Suillus* populations. We tried to link the Zn-tolerance trait to the element profiles in the ECM fungi. The present data do not provide information on the specific cellular compartmentation of the elements

or the tolerance mechanism itself, but the element patterns allow us to eliminate a number of physiological mechanisms that could play a role in the specific Zn adaptation.

When the three Suilloid species are grown at a low Zn concentration (0.15 mM Zn), Zn-resistant isolates cannot be distinguished from sensitive ones on the basis of morphological characteristics. Intraspecific growth rates are very heterogeneous in all three species, and there is no evidence that Zn-tolerant isolates or populations have slower or faster growth rates than normal Zn-sensitive isolates. This is in contrast to what has been observed in plant populations in which morphological differentiations evolve when there is adaptation to metal contamination. Metal-adapted plant ecotypes often have slower growth rates in control conditions than individuals from normal populations (Macnair 1987). Therefore metal-tolerant plants are thought to be in a competitive disadvantage under normal conditions. So far, we cannot find a discriminating morphological factor that distinguishes Zn-tolerant and Zn-sensitive genotypes in the Suilloid fungi. The same conclusion can be drawn from the nutrient profiles of the mycelia, except for their Zn concentration.

The Zn concentrations in the mycelia of all treatments were very high and, at the highest Zn application, even reaches concentrations (15.57 mg g^{-1}) representative of plant hyper-accumulators (Frérot et al. 2003). Turnau et al. (2001) used micro-proton-induced X-ray emission (PIXE) true elemental maps to identify tissue patterns of metals in cryo-fixed *S. luteus* mycorrhizas collected from Zn wastes. They found that the Zn concentration in *Suillus* rhizomorphs averaged 12.83 mg g^{-1}. Zinc ions were concentrated in tissues that make direct contact with the contaminated substrate, such as rhizomorphs and the outer fungal mantle. *Suillus* mycorrhizas grown in Zn-spiked substrate also accumulate very high concentrations of Zn, while transfer to the host plant remains low (Colpaert and Van Assche 1992).

Nevertheless, Zn-resistant isolates of the three species studied—irrespective of their site of origin—accumulated on average less Zn than their sensitive counterparts when cultured at low external Zn. The fact that the concentrations of the other micronutrients did not correlate to the Zn tolerance points to a specific adaptation of a specific Zn-tolerance mechanism and explains the absence of co-tolerance in these fungi. In the second set of analyses with Zn-exposed *S. luteus*, it became evident that the differential Zn uptake is even more pronounced at 3 and 6 mM $[Zn]_{ext}$ than at 0.15 mM $[Zn]_{ext}$. These observations suggest that the adaptive Zn-resistance mechanism in all three Suilloids is at least partly based on a specific zinc-exclusion mechanism. In the ECM fungus *Pisolithus tinctorius*, differential Al tolerance could also be attributed to a reduced net Al influx in the mycelia of Al-tolerant isolates from a mine site (Egerton-Warburton and Griffin 1995).

Evolutionary adaptation to Zn-enriched soils can be achieved in several ways. In plants, Zn tolerance seems to be functionally related to transport processes which 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 and 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 (Meharg 2003). Substantial extracellular cell wall binding of Zn is important in mycorrhizal fungi. Extracellular binding to negative charges present in cell walls is probably part of the constitutive mechanisms intercepting metal cations (Colpaert and Van Assche 1992; Frey et al. 2000; Joner et al. 2000). However, the current data of a reduced Zn content in Zn-tolerant genotypes indicate that the adaptive resistance mechanism in Suilloids cannot be attributed to a higher Zn-sequestration capacity of the cell walls of Zn-tolerant genotypes. An improved compartmentation of Zn in the vacuole or other cell organelles and an improved complexation of Zn in the cytosol are also not compatible with the current observations. This is not to say that these mechanisms are not important for zinc detoxification in these fungi, but it is unlikely that they are involved in the genetic modifications triggered by the Zn contamination.

A reduced Zn influx, an increased efflux of Zn and/or an increased exudation of organic acids that chelate or precipitate Zn ions in the medium are the most likely mechanisms that can explain the lower accumulation of Zn in the Zn-resistant Suilloids. These avoidance mechanisms can stop metals from entering cells. Restrictions in metal uptake were discovered previously in other eukaryotes adapted to heavy metals (Nielsen et al. 2003). Reduced uptake of Zn in Zn-adapted plant genotypes thriving on calamine soils has been described several times, even in hyper-accumulators such as *Thlaspi caerulescens* (Assunção et al. 2003a,b; Frérot et al. 2003). Also, in bacteria, Zn resistance is mostly achieved by exclusion systems. Several Zn efflux systems are known (Nies 2003). In plants and fungi, a range of gene families are likely to be involved in homeostasis of transition metals (Hall and Williams 2003). The Zn-transporting members within the family of cation diffusion facilitator (CDF) proteins have been found in bacteria, mammalian cells (Kambe et al. 2004), plants (Dräger et al. 2004) and mycorrhizal fungi (Gonzalez-Guerrero et al. 2005). These transport proteins facilitate the efflux of Zn from the cytosol to the outside of cells or into intracellular organelles. Zn efflux transporters may also be present in other gene families of metal transporters (Hall and Williams 2003). New experiments have been set up to investigate the influx and efflux kinetics of Zn in selected *Suillus* genotypes to reveal the role of membrane transport in the Zn adaptation.

The exudation of organic acids by fungi is a process that can affect metal toxicity. Precipitation of cations with oxalates is well described, also in ECM fungi, and there is some evidence that the production of oxalic acid is stimulated in *Suillus variegatus* when exposed to elevated

Cu (Ahonen-Jonnarth et al. 2000). Organic acid and proton excretion in the myco(rhizo)sphere, however, will also increase metal solubilisation from poorly soluble metal compounds in polluted soils. Martino et al. (2003) studied ZnO solubilisation in strains 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 modification in acid exudation could be an adaptation to Zn pollution in this ericoid mycorrhizal fungus. This pattern of reduced Zn solubilisation was not observed in Zn-tolerant *S. luteus* and *S. bovinus* isolates exposed to poorly soluble $Zn_3(PO_4)_2 \cdot 2H_2O$ (hopeite), neither was a precipitation of an organic Zn compound in the culture medium found (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.

The presence of Zn-P deposits, often observed in electron microscopic micrographs of Zn-treated ECM fungi, has led to the suggestion that vacuolar polyphosphates act as chelating compounds that detoxify excess Zn ions (Leyval et al. 1997). Nevertheless, the results of micro-analytical studies on the subcellular compartmentation of Zn in ECM fungi and mycorrhizas are sometimes confusing and difficult to interpret. The reliability of such studies can be problematic because of migration of ions during specimen preparation (Bücking et al. 1998; Ashford et al. 1999; Turnau et al. 2001). Cryo-preparation of mycelial tissues is a good option to avoid redistribution of elements. Bücking et al. (1998) showed that the negative charges of polyphosphates in vacuoles of *S. bovinus* are balanced by K, Mg and basic amino acids. Frey et al. (2000) using similar techniques determined the compartmentation of Zn in spruce-*Hebeloma crustuliniforme* mycorrhizas. In their root samples, Zn was both complexed extracellularly in fungal cell walls and sequestered in the fungal cytosol. The fungal vacuoles contained relatively high P concentrations but were not a significant storage pool for Zn, at least not in this fungus. Also in the present study, we do not find a strong case for the involvement of P in the detoxification of excess Zn. *S. bovinus* is generally more sensitive to excess Zn than *S. luteus* (Colpaert et al. 2004), despite the higher P acquisition in the former species. The analyses show that the adaptive Zn tolerance is not related to a better P nutrition of Zn-resistant *S. luteus* and *R. luteolus*; although in *S. bovinus* there was a weak positive trend between Zn tolerance and P content. Both in control medium and under Zn exposure, the P concentration remains fairly constant in most isolates. Only the sensitive *S. luteus* isolates accumulated more P than the resistant genotypes when Zn stress increased. It is possible that excessive accumulation of Zn in sensitive isolates reduces the free phosphate concentration in the cytosol. Under such conditions, a stimulation of the P uptake capacity seems reasonable, but is not able to prevent Zn toxicity.

With increasing Zn concentrations in the medium, no significant changes were observed in the mycelial concentrations of Cu, Mn, Ca or Mg. Fe uptake seemed to be more affected by Zn toxicity. Although there could be an initial decrease in uptake—probably also through extracellular displacements from cell walls—mycelia suffering from Zn stress increased the uptake of this micro-element. Zn-induced intracellular displacements of Fe from proteins are probably very harmful and may trigger an increased influx of Fe (Marschner 1995).

Apart from Zn, we did not find any inter-population differences in element profiles. In addition, the nutrient analyses do not indicate that the genotypes from the Zn-resistant *Suillus* populations show a reduced variation in physiological responses, which is in agreement with the lack of any reduction of genetic diversity in these Zn-adapted populations (Muller et al. 2004). The occurrence of high levels of morphological, enzymatic or genetic variation within metal-adapted plant populations inhabiting polluted soils is not unusual (Baker and Dalby 1980; Vekemans and Lefèbvre 1997; Mengoni et al. 2000). It suggests that founder effects due to a strong selection pressure for heavy metal tolerance do not always occur or rapidly disappear from these adapted populations.

In conclusion, the element profiles suggest the existence of a Zn exclusion mechanism in Zn-tolerant Suilloid fungi. Such a mechanism will not only help prevent the onset of metal stress in the fungal hyphae, 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 tempting because it is very likely that 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). 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).

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