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Zinc export results in adaptive zinc tolerance in the ectomycorrhizal basidiomycete *Suillus bovinus*

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Joske Ruytinx,[a] Hoai Nguyen,[a] May Van Hees,[b] Michiel Op De Beeck,[a] Jaco Vangronsveld,[a] Robert Carleer,[c] Jan V. Colpaert*[a] and Kristin Adriaensen[a]

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On Zn-polluted soils, populations of the ectomycorrhizal basidiomycete *Suillus bovinus* exhibit an elevated Zn tolerance when compared to populations on non-polluted sites. To elucidate the mechanism of Zn tolerance, the time-course of Zn uptake was studied in isolates with contrasting Zn tolerance. Unidirectional fluxes and subcellular compartmentation of Zn were investigated through radiotracer flux analyses. Fluorescence imaging was used to support the subcellular Zn compartmentation. After 2 h of exposure to 200 μM Zn, significantly more Zn was accumulated in Zn-sensitive isolates compared to tolerant isolates, despite similar short-term uptake kinetics and similar extracellular Zn sequestration in cell walls. In Zn-sensitive isolates twice as much Zn accumulated in the cytoplasm and 12 times more Zn in the vacuole. ^{65}Zn efflux analyses revealed a considerably faster Zn export in the Zn-tolerant isolate. The adaptive Zn tolerance in *S. bovinus* is therefore achieved by a preferential removal of Zn out of the cytoplasm, back into the apoplast, instead of the usual transfer of Zn into the vacuole. Zn exclusion in the fungal symbiont eventually contributes to a lower Zn influx in host plants.

Introduction

Ectomycorrhizal (ECM) fungi are ubiquitous root symbionts of woody plants. They play a pivotal role in nutrient and metal transfer from soil solution into their host trees. This is not different when soils are naturally enriched or anthropogenically polluted with heavy metals.[1,2] Absorption roots of trees on metalliferous soils can be colonised by well-adapted fungal communities.[3] The principal benefit conferred upon plants by the ECM symbiosis is fungus-mediated access to nutrients such as nitrogen and phosphorus. This is particularly important since these metal-polluted soils are often nutrient deficient, have very poor soil structure and are freely drained.[4] In addition to an improved nutrient acquisition, ECM fungi can also alleviate toxic metal stress in their host trees.[5–8] They can act as a specific barrier by reducing toxic metal transfer to a host and thus enable trees to thrive in environments, where high concentrations of harmful metals are present. Nevertheless, this protective effect is variable and partly depends on interspecific[9] and intraspecific variation in metal tolerance amongst the fungal symbionts.[6,8,10,11]

Heavy metals such as Zn and Cu are essential micronutrients, but their ions become toxic when present in excess. Therefore, the intracellular concentration and compartmentation of metal ions have to be tightly controlled. Fungi have a range of potential mechanisms at the cellular level that are involved in homeostasis and detoxification of heavy metals. However, the mechanisms by which ECM fungi provide protection against metal toxicity, to themselves and their host plants, are not yet fully understood. Most research on mechanisms by which mycorrhizal fungi physiologically adapt to metals has focused on metal solubilization and sequestration both outside or inside the cell.[12–15] There is growing evidence that the fine-tuning of available micronutrients in eukaryotic cells mainly relies on transporters.[16–20] However, very little work has been done on metal transport in ECM fungi, apart from a Cd study in *Paxillus involutus* and the characterisation of an ER-located Zn transporter in *Hebeloma cylindrosporum*.[21,22] Modification of efflux pumps, suppression or the absence of uptake systems, highly specific influx systems or increased expression of metal efflux transporters have been demonstrated as determinants of metal tolerance in a range of organisms including bacteria,[23] yeast[16,24,25] and plants.[26–28] Insight into the different mechanisms underlying metal homeostasis in ECM fungi will contribute to our global understanding of metal management at the whole organism level, and it will reveal how

[a] *Hasselt University, Centre for Environmental Sciences, Environmental Biology Group, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium. E-mail: jan.colpaert@uhasselt.be*
[b] *Belgian Nuclear Research Centre (SCKCEN), Biosphere Impact Studies, Boeretang 200, 2400 Mol, Belgium*
[c] *Hasselt University, Centre for Environmental Sciences, Research Group of Applied and Analytical Chemistry, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium*

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mycorrhizal fungi combine their function as a supplier of limiting nutrients (including trace metals) and as a barrier for excess metals.

Previously, Zn-tolerant populations of *Suillus* species (Boletales) were described from Zn-polluted sites. Isolates of these ECM fungi were shown to accumulate less Zn than their Zn-sensitive counterparts from non-polluted soils, when exposed to high Zn for 12 days.[29] This feature could play a key role in metal tolerance and suggests an 'avoidance' strategy, possibly through a decreased uptake and/or enhanced efflux of Zn.[30] In the present study, Zn accumulation and fluxes were analyzed in mycelia of *Suillus bovinus* during a 48 h exposure period. Membrane functioning, unidirectional fluxes of Zn and subcellular compartmentation were characterized in order to reveal the contribution of membrane transport to the Zn adaptation of *S. bovinus*.

Materials and methods

Fungal material

Ten isolates of *Suillus bovinus* (Fr.) D. Kuntze, collected in pioneer pine forests in N-Limburg (Belgium), were studied. Half of the isolates were derived from Zn-tolerant populations thriving on Zn-polluted soil, the other half were from Zn-sensitive populations on non-polluted soil (Table 1). For the radiotracer experiments, two isolates with an average EC_{50} value in each Zn sensitivity group were selected: UH-Sbo-Ls1 (LS1) and UH-Sbo-Mg2 (MG2).

The experiments presented here were all performed with liquid submerged fungal cultures. Therefore, one-week old colonies grown on cellophane covered agar medium were fragmented using a kitchen blender (Philips HR1356/0) in 150 ml Fries medium for 40 s.[10] The mycelia were subsequently incubated in Erlenmeyer flasks on a shaking incubator in a climate room at 21 °C. After 1 week, Fries medium was replaced by modified Ingestad medium.[31] The final basic solution contained (in μM): K_2SO_4 70, KNO_3 96, KH_2PO_4 63, $K_2HPO_4{\cdot}4H_2O$ 58, NH_4NO_3 733, $Ca(NO_3)_2{\cdot}4H_2O$ 36, $Mg(NO_3)_2{\cdot}6H_2O$ 62, H_3BO_3 5, $Mn(NO_3)_2{\cdot}4H_2O$ 2, $FeCl_3{\cdot}2H_2O$ 3, $Zn(NO_3)_2{\cdot}4H_2O$ 0.1, $CuCl_2{\cdot}2H_2O$ 0.1, $Na_2MoO_4{\cdot}2H_2O$ 0.02 and 28 mM glucose. The final pH of the medium was adjusted to 4.5. The Ingestad medium, originally developed for cultivating pine seedlings, was chosen for the experiments. This medium has a mineral composition and concentration that are more representative for soil solution than the nutrient rich Fries medium. In addition the Ingestad medium contains lower concentration of potentially complexing or precipitating compounds, in particular phosphate, which readily forms precipitates with Zn and thus affects Zn availability.

Table 1 The *Suillus bovinus* isolates: collection site, distance to a Zn smelter, *in vitro* EC_{50} value for Zn toxicity. The EC_{50} value is the Zn concentration (mM) that inhibited fungal biomass production on solid Fries medium by 50% after 12 days

Isolate code	Collection site	Distance to Zn smelter (km)	EC_{50} (mM)
UH-Sbo-A19	Neerpelt	1.3	11.7
UH-Sbo-LS1	Lommel Sahara	1.4	9.0
UH-Sbo-LS4	Lommel Sahara	1.4	5.8
UH-Sbo-N5	Neerpelt	1.2	7.9
UH-Sbo-N7	Neerpelt	1.2	13.3
UH-Sbo-MG2	Meeuwen-Gruitrode	21.4	1.8
UH-Sbo-MG6	Meeuwen-Gruitrode	21.4	1.9
UH-Sbo-P3	Paal	15.6	1.9
UH-Sbo-P4	Paal	15.6	1.7
UH-Sbo-Z3	Zolder	22.6	1.8

Zn accumulation and mineral nutrient uptake in Zn-exposed *S. bovinus* mycelia

In a first experiment the 10 isolates were exposed to 200 μM or 1 mM Zn, supplemented as zinc sulfate to the Ingestad nutrient solution. Mycelial cultures from individual Erlenmeyer flasks were combined to obtain a single homogenous culture for each isolate. One gram of fresh weight mycelial pellets was transferred aseptically into 30 ml nutrient solution in Petri dishes. Dishes were incubated on a shaking incubator in a climate room at 21 °C for 0, 15, 60, 120, 240, 360 min, 24 and 48 h. At each time point 5 replicate samples of mycelia were harvested, frozen at −80 °C, and freeze-dried for Zn determinations. In order to check for toxicity and normal functioning of nutrient acquisition in hyphal cells, the depletion of P_i, NH_4^+, K^+ and Zn^{2+} was followed in the incubation solution. P_i and NH_4^+ were determined colorimetrically using a Flow Injection Analyser (Lachat, QuickChem®Method 10-115-01-1-A, 10-107-06-1-C). K^+ and Zn^{2+} were determined using ICP-OES. Chemical analyses were performed on five replicates and certified reference material was included as an external standard for elemental analyses.

^{65}Zn flux analyses

For the Zn tracer experiments, 1 g of fresh weight mycelial pellets, about 1.5 mm in diameter, were immersed in 20 ml of the test solution. The standard assay medium was the Ingestad medium (see above) either enriched with cold Zn^{2+} (used as preloading solution and desorption solution) or with a mixture of cold and isotopic $^{65}Zn^{2+}$ (used as loading solution). In the assay, mycelia were transferred to fresh preloading solution for 5 min prior to immersion in the labeled uptake solution. This protocol minimized perturbation and allowed mycelia to equilibrate to the exact solution temperature and to the solution composition used during the loading. Standard assays were performed at pH 4.5 and room temperature. Each treatment had four replicates.

^{65}Zn efflux experiment

For the determination of Zn efflux rates, mycelial pellets were incubated for 24 h in loading solution with a final concentration of 200 μM Zn (containing 1.1 MBq l^{-1} $^{65}Zn^{2+}$). This concentration was selected because the previous experiment had shown that a 24–48 h exposure period resulted in a differential Zn accumulation pattern between the tolerant and sensitive isolate without disturbing nutrient assimilation or triggering K^+ leakage.

After 24 h the radioisotope-loading was terminated by rinsing the mycelia in 20 ml of desorption solution that was identical to the loading solution except that ^{65}Zn was replaced by cold Zn. Four mycelial samples were immediately harvested, four other samples were used for the desorption test. With increasing time

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intervals (5 s–100 min) the desorption solution was replaced with fresh desorption solution. After 24 h of desorption the four replicate mycelia were also harvested, oven-dried at 80 °C, weighed and heat destructed at 600 °C (muffle furnace) for further analysis. ^{65}Zn content of the mycelium was determined on the fungal pellets harvested at the start and at the end of the efflux period. ^{65}Zn was measured using a Perkin Elmer gamma counter, Wallac 1480 WIZARD 3''. The experiment was repeated twice on independent cultures.

Effect of a metabolic inhibitor on Zn accumulation and efflux

In a third experiment, the same protocol as described above was performed, except for the addition of 10 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) to the loading solution. CCCP is a protonophore, which in fungi is more efficient in dissipating the proton electrochemical gradient across vacuolar and mitochondrial membranes than across the plasma membrane.[32,33] CCCP inhibits oxidative phosphorylation, leads to low ATP levels and to a collapse of the vacuolar membrane ΔpH.

Concentration-dependent kinetics of ^{65}Zn influx

A fourth experiment was performed to determine the concentration-dependent Zn uptake kinetics. Here, the standard assay medium was enriched with cold Zn and isotopic Zn (0.48 MBq l^{-1}) to cover a Zn gradient from 0.5 to 1000 μM, a range typical for soil solution from non-polluted to severely polluted soils.[34] After the prewash, mycelia were transferred to the labeled solution for 30 min. After this 30 min uptake period, radioactive solutions were withdrawn and mycelia were first briefly rinsed with desorption solution to remove the surface film of labeled solution. Hereafter mycelial pellets were washed 3 times (in total 10 min) with fresh desorption solution to remove the cell wall bound label. Mycelia were then harvested, oven-dried at 80 °C, weighed and ^{65}Zn was quantified by gamma detection. The duration of the washing steps was based on the determination of half-times of exchange for ^{65}Zn for the cell wall and cytoplasm in the efflux experiments (see the Results section). By loading the mycelial pellets in labeled solution for less than 1.5 half-times of exchange for the cytoplasmic compartment, and by subsequently desorbing the labeled mycelium for 4 to 5 half-times of exchange of the cell wall, any errors introduced by efflux during the loading period or counts remaining in the cell wall fraction at the end of the experiment have been minimized.[35]

Intracellular Zn localization

Subcellular labeling of Zn was performed with a fluorescent marker for free Zn^{2+}, FluoZin3 (Molecular Probes, Invitrogen), which is able to detect free Zn in the 1–100 nM range. Both *S. bovinus* isolates were grown on cellophane-covered solid Fries medium and subsequently mixed using a kitchen blender in 150 ml of liquid Fries medium containing 0 or 20 μM Zn added as $ZnSO_4$. After one week pellets were mixed again in fresh medium of the same composition and grown for two additional days. Five mg fresh weight mycelium was transferred to a 2 ml Eppendorf tube with 1.5 ml TBS (Tris Buffered Saline: 137 mM NaCl, 3 mM KCl, 25 mM Tris; pH 7) containing 5 μM FluoZin3. Following an incubation of 30 min (shaking), mycelia were washed twice in TBS for 5 min. FluoZin3 fluorescence was visualized using a Zeiss LSM 510 META laser scanning confocal microscope, using a Zeiss 40× NA1.3 oil immersion objective and 2× scanning zoom. The 488 nm excitation line of the laser and a BP 500–550 nm emission filter were used. Image processing was carried out using ImageJ (NIH, Bethesda, MD, USA) software.

Data analysis

The datasets of the first experiment (time course of Zn^{2+} accumulation in *S. bovinus*) were analyzed using the TTEST procedure in SAS. Transformations were applied when necessary to approximate normality.

Efflux plots representing a first-order kinetic transformation of Zn efflux (log ^{65}Zn remaining in the mycelium as a function of time) were constructed and subsequently dissected into three different linear phases, corresponding to fast, medium, and slow exchange compartments. These three compartments correspond to three cellular compartments in series: the cell wall, cytoplasm and vacuole, as previously described for Zn efflux from *Thlaspi* (=*Noccaea*) roots[36] and Cd efflux from *Paxillus involutus* mycelial disks.[21] The customary methodology to analyze these efflux data is to perform 'curve-peeling' by calculating a linear regression upon the straight line portion of the semilog plots of the elution data. The extrapolation of this line to the *Y*-axis provides an estimate of the apparent isotopic content at the beginning of the washout period (A_v) of this exchanging compartment as well as the rate constant (k_v) for isotope exchange from that particular compartment. After subtraction of the slowest exchanging (vacuolar) component from the total isotope content of the mycelium at each interval, the remaining isotopic contents are replotted to give estimates of A_c (apparent isotopic content of the intermediate, cytoplasmic compartment) and the rate constant k_c. After subtraction of the cytoplasmic component, A_w and k_w for the cell wall are obtained.[37]

Statistical data analysis of the influx data was performed using SAS 9.2. Statistical differences were compared using multiway ANOVA assuming equality of variance and normality (Kolmogorov–Smirnov) and Tukey–Kramer's *post hoc* test for pairwise comparison. Results with $P \leq 0.05$ were considered to be statistically significant.

Results and discussion

Mineral nutrient uptake and Zn accumulation in Zn-exposed *S. bovinus* mycelia

Nutrient acquisition and Zn accumulation were followed at 200 μM and 1 mM Zn. Phosphate acquisition was very fast; within four hours most phosphate was taken up by the cells (Fig. 1), except for Zn-sensitive isolates which leaked significant quantities of P_i back into the 1 mM Zn incubation medium after 24 and 48 h. The uptake of ammonium, the preferred N source of *S. bovinus*, was severely reduced in all sensitive isolates in the 1 mM Zn treatment and at the same time the incubation medium was enriched with K^+ (Fig. 1). This impaired nutrient

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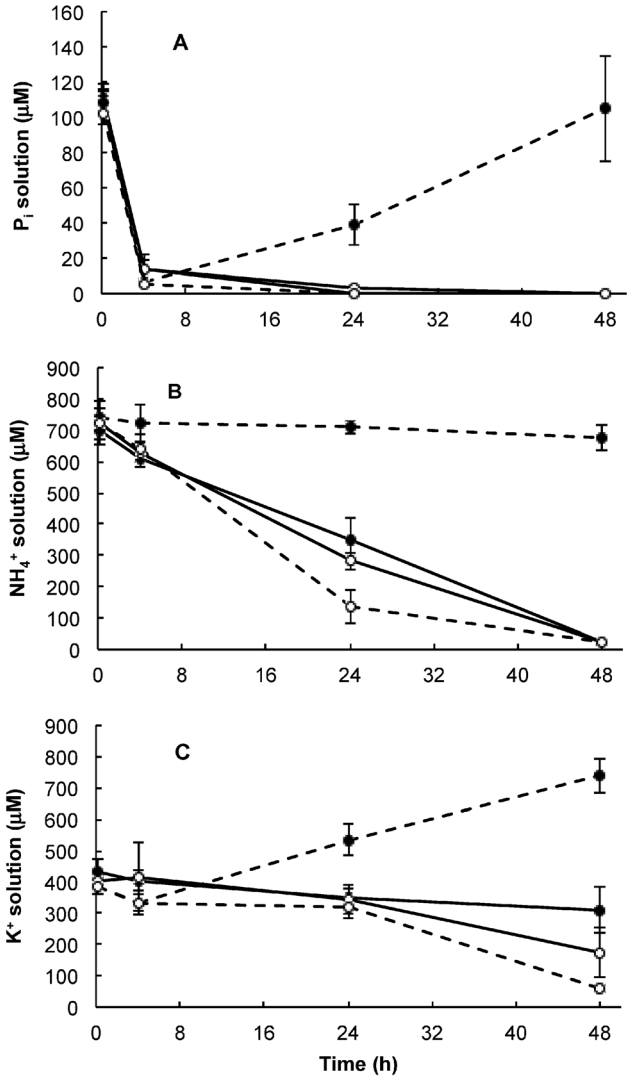

Fig. 1 Depletion of (A) inorganic phosphate (P_i), (B) ammonium and (C) potassium from the incubation medium supplemented with 200 µM Zn (solid line) or 1 mM Zn (dashed line). Averages of five Zn-tolerant (○) and five Zn-sensitive (●) *Suillus bovinus* isolates are plotted.

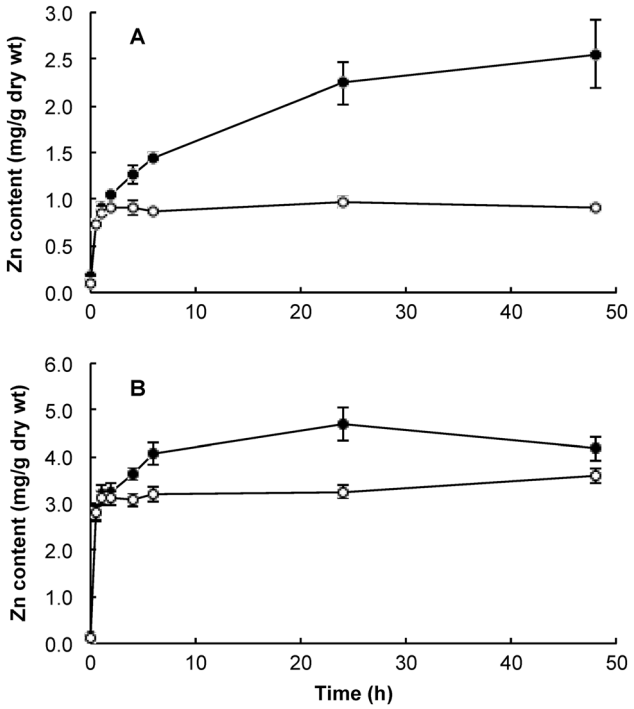

Fig. 2 The 48 h time course of the total Zn content of *Suillus bovinus* mycelial pellets when transferred to a medium supplemented with (A) 200 µM Zn or (B) 1 mM Zn. Averages of five Zn-tolerant (○) and five Zn-sensitive (●) *Suillus bovinus* isolates are plotted.

acquisition in the five Zn-sensitive isolates illustrates that the 1 mM Zn treatment becomes very toxic for these isolates, most likely because of a gradual loss of membrane functionality during the exposure period.

The accumulation of Zn in the mycelia proceeded very quickly during the first 15 minutes of Zn treatment. From then on, the increase in Zn content slowed down until the end of the experiment (Fig. 2). After 1 h, the Zn content of all five Zn-tolerant isolates reached an equilibrium, suggesting that Zn influx and efflux rates soon become similar. After 2 h of exposure to 200 µM Zn, a significant differentiation in Zn accumulation was observed between the two Zn-sensitivity groups, a differentiation that became more pronounced at later time points. In the 1 mM Zn exposure, Zn contents of mycelia raised approximately two-times and three-times in, respectively, sensitive and tolerant isolates, when compared to the 200 µM condition. The Zn accumulation pattern was similar within each sensitivity group up to 24 h. However, at 48 h of exposure to 1 mM Zn, mycelia of Zn-sensitive isolates started to lose Zn, possibly as a result of loss of membrane integrity.

The comparison of the two Zn-sensitivity groups of *S. bovinus* shows that Zn-tolerant isolates, collected from different sites, use a similar strategy to overcome excess Zn. They could maintain a lower Zn level in their mycelia than sensitive isolates, confirming previous results obtained from a population screening with 54 *S. bovinus* isolates exposed for 12 days to a gradient of Zn in solid media.[29] Because the 1 mM Zn exposure was very harmful for the sensitive isolates, we decided to investigate Zn fluxes using the 200 µM Zn concentration that had no deleterious effects on membrane functioning after 48 h of exposure.

^{65}Zn efflux study

Compartmental efflux analyses have been widely used in higher plants and fungi to investigate cellular compartmentation and unidirectional fluxes of several monovalent and divalent cations including K, Cu, Zn and Cd.[21,36,38–41] The present study is one of the first using this approach to investigate the unidirectional fluxes and compartmentation of Zn in an ECM fungus. Representative efflux plots for the LS1 and MG2 isolates are shown in Fig. 3. After 24 h loading, MG2 had accumulated 3.6 times more ^{65}Zn than LS1. The estimated half-times for Zn in the different compartments are presented in Table 2. The half-times of exchange from the cytoplasmic compartment were 37 min and 58 min for LS1 and MG2, respectively. This means that the 24 h loading period represents more than five times the

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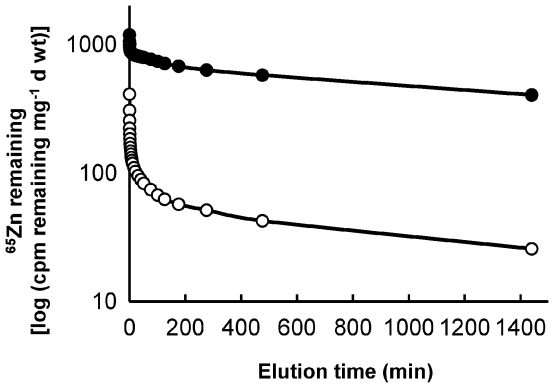

Fig. 3 Desorption plots of ^{65}Zn from mycelial pellets of a Zn-tolerant (○) and a Zn-sensitive (●) *Suillus bovinus* isolate after 24 h incubation in an uptake solution containing 200 μM Zn. Data points represent means (n = 4) and error bars do not extend outside symbols.

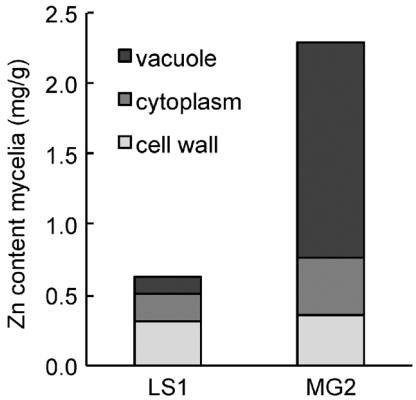

Fig. 4 Compartmentation of Zn in mycelia of a Zn-tolerant (LS1) and a Zn-sensitive (MG2) *Suillus bovinus* isolate after 24 h of incubation in an uptake solution containing 200 μM Zn. Data were calculated from desorption plots of ^{65}Zn.

half-time of exchange for the cytoplasmic compartment, after which the specific activity of the tracer in this compartment theoretically should have reached 96.86% of its value in bulk solution. Hence, the assumption of steady state with regard to specific activity of the tracer, a prerequisite for using compartmental analysis, can be made with reasonable justification.[37]

The half-times of Zn exchange from the cell wall compartment were similar for both isolates (Table 2); a significant amount of Zn was bound to cell walls (Fig. 4) and was easily exchangeable. The physiological advantage of metal sorption on fungal cell walls is strong metabolic inactivation with a low cost. Such a sequestration of metals on cell walls may be part of the constitutive mechanism of metal tolerance in soil fungi.[21,42] Nevertheless, the compartmentation study does not support differential extracellular Zn sequestration in the isolates studied and, therefore, cannot explain the differential Zn tolerance in *S. bovinus*.

Zn efflux is significantly faster in LS1 compared to MG2 (Table 2, Fig. 3). Eventually after the 24 h desorption period 39% of ^{65}Zn remained in the mycelium of MG2 while only 9% remained in that of LS1. In absolute numbers, almost 16 times more ^{65}Zn remained in the mycelia of MG2 compared to LS1 after the 24 h elution. A similar differential Zn efflux pattern was previously found for root cells of *Thlaspi arvense* and *Noccaea* (previously *Thlaspi*) *caerulescens*.[36] Zn-tolerant *N. caerulescens* accumulated less Zn in root cell vacuoles which had a nearly 50% shorter half-time for Zn release compared with Zn-sensitive *T. arvense*. In *N. caerulescens* the enhanced Zn efflux from root cells contributes to the high transfer of Zn to the shoots and thus to the Zn hyperaccumulation in this plant. Variability in Cd tolerance and Cd accumulation in *N. caerulescens* populations is also clearly linked to gene expression, copy number and activity of an efflux transporter.[28] In the ericoid mycorrhizal fungus, *Hymenoscyphus ericae*, an enhanced As efflux system was operating in arsenate resistant isolates from As-rich mine soils.[43]

Most metal efflux systems involved in toxic ion tolerance are known and described from prokaryotes,[23,44,45] but knowledge of eukaryotic metal selective transporters that remove transition metals from cytoplasm is increasing. The metal transporting P_{1B}-ATPases (HMAs) translocate cations out of the cytoplasm across biological membranes, including the plasma membrane, using energy from the hydrolysis of ATP. This family of metal efflux transporters is becoming better characterized in plants and fungi.[16,18,25,28] Metal efflux may be driven directly by plasma membrane transporters or indirectly by the secretory pathway. The latter was proven to be indispensable for Mn detoxification in plants.[46] Further research is needed to functionally characterize and localize specific Zn transporter proteins in Zn-tolerant *Suillus* species.

In Zn-sensitive MG2, two times more ^{65}Zn accumulated into the cytoplasm after 24 h and even twelve-fold more in the vacuole compared to LS1. In the arbuscular mycorrhizal fungus *Glomus intraradices*, and in yeast, excess Zn accumulates in the vacuole, which is a hot spot for metal detoxification.[17,47] The vacuolar Zn concentration in *Saccharomyces cerevisiae* can rise to 100 mM.[48] Vacuolar sequestration is thought to be an important pathway for detoxification of metals in both plants and fungi.[17,18,21,49] In Zn-sensitive *S. bovinus* the largest fraction of Zn, 67%, was accumulated in the vacuolar compartment, compared to only 21% in LS1 (Fig. 4).

Table 2 Half-times ($t_{1/2}$) for Zn desorption from different fungal compartments in mycelia of a Zn-tolerant (LS1) and a Zn-sensitive (MG2) *Suillus bovinus* isolate. Data are expressed as means ± SE of four replicates

	Isolate	Cell wall	Cytoplasm	Vacuole
$t_{1/2}$ (min)	LS1	1.1 ± 0.2	37.4 ± 1.4	1752 ± 13
	MG2	0.8 ± 0.2	58.3 ± 3.3	2650 ± 57

Subcellular Zn localization

Accumulation of Zn was further investigated by using fluorescence detection microscopy. In fungi, Zn can be compartmentalized in the vacuole or in small punctuate cytoplasmic vesicles.[22,48,50] The intracellular probe for Zn illustrates that in washed *S. bovinus* mycelia free Zn can easily be detected in the vacuoles of both isolates when sufficient Zn is present in the culture medium (Fig. 5). Mycelia grown without Zn showed

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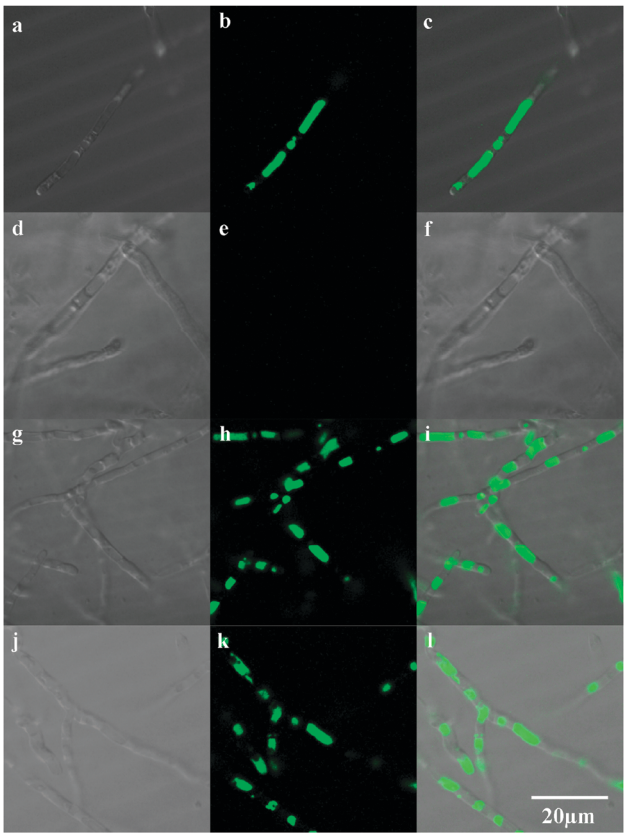

Fig. 5 Fluorescence of free Zn, marked with FluoZin3, in *Suillus bovinus*. Top panels (a–f): 0 µM Zn, bottom panels (g–l): 20 µM Zn. a–c and g–i are images from the Zn-sensitive isolate MG2; d–f and j–l are from the Zn-tolerant isolate (LS1). Left column: differential interference contrast image, middle column: fluorescence image, right column: overlay of both images, showing fluorescence in vacuoles.

few vacuoles with some fluorescence in MG2, no FluoZin3 fluorescence was detected in vacuoles of LS1. This may indicate a smaller Zn storage pool in the Zn-tolerant isolate, a possible trade-off of the adaptive tolerance. The fluorescence was on average higher in MG2 than in LS1. Nevertheless lack of fluorescence in vacuoles has to be interpreted with caution because vacuolar pH and the concentration of Zn-complexing ligands may be different among the isolates. *S. bovinus* hyphal cells in different developmental stages were viewed. A fluorescence pattern comparable to the one detected in *Hebeloma cylindrosporum*,[22] indicating Zn-accumulating cytoplasmic vesicles, was never detected in *S. bovinus*.

Effect of a metabolic inhibitor on Zn accumulation and efflux

Addition of CCCP to the loading solution resulted in a very reduced uptake of ^{65}Zn in MG2, resulting in a total Zn accumulation quite similar to that of LS1. The most striking result compared to the control (–CCCP) treatment was noticed for the Zn compartmentation. In the presence of CCCP, Zn was no longer efficiently evacuated to the vacuoles, in none of the isolates, most likely because of the collapse of the vacuolar membrane ΔpH.[32] In fungi, CCCP is known to preferentially dissipate the proton

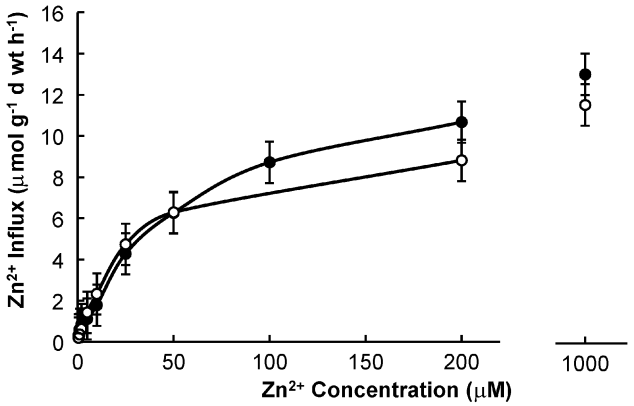

Fig. 6 Concentration-dependent kinetics of Zn uptake into mycelia of Zn-tolerant (○) and Zn-sensitive (●) *Suillus bovinus*. Cell wall bound tracer was washed away. Data points and error bars represent means (n = 4) and SEM, respectively.

electrochemical gradient across the tonoplast whereas the effect on the gradient across other membranes such as the plasma membrane can be limited.[32,33] The Zn content of vacuoles of LS1 dropped from 127 to 30 µg g^{-1} dry weight and in MG2 it fell from 1500 to 45 µg g^{-1}.

Concentration-dependent kinetics of $^{65}Zn^{2+}$-influx

Zn uptake within a 0.5 to 1000 µM concentration range was characterized by a smooth, non-saturating curve (Fig. 6). Because ^{65}Zn uptake was measured over a short time period (30 min), the results mainly represent unidirectional influxes. Both isolates showed similar influx kinetics, suggesting that similar Zn uptake systems are operating in both sensitive and tolerant isolates. Only at the lowest external Zn concentrations (0.5 and 1 µM) a slightly lower influx rate was measured in the tolerant isolate compared to the sensitive isolate.

Conclusion

The results point to a different Zn management in Zn-tolerant and Zn-sensitive *Suillus bovinus* isolates when they are exposed to supra-optimal concentrations of Zn. The Zn tracer data as well as the fluorescence imaging pictures indicate the vacuole as a subcellular storage site for Zn in *S. bovinus*. As in most eukaryotes, surplus Zn ions are stockpiled in the vacuole for detoxification and/or storage purposes. This transfer of Zn into vacuoles is an energy-dependent process, mediated by CDF transporters that make use of the proton electrochemical gradient across the tonoplast.[51]

In Zn-sensitive isolates the efficient transfer of Zn into the vacuoles leads to a very high accumulation of Zn in this organelle and eventually its Zn storage capacity is exceeded. A failing vacuolar functioning could be due to a shortage of Zn-complexing ligands, an increasing backflow of Zn into the cytoplasm, damage to the tonoplast or a combination of these. Any backflow, together with the high influx over the plasma membrane will lead to increasing Zn concentrations in the cytoplasm, which are no longer manageable. Only a sufficient efflux of Zn from the cytoplasm back into the environment as

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observed in Zn-tolerant isolates can avoid an overloading of vacuole and cytoplasm at high external Zn. The preferential evacuation of Zn over the plasma membrane results in a lower net Zn influx in the mycelia of Zn-tolerant *S. bovinus*. The export of Zn back into the culture solution (or soil) has only a minor effect on the external Zn concentration when steady state is reached.

The Zn exclusion mechanism in Zn-tolerant Suilloid fungi will not only help to prevent metal stress in the fungal hyphae, but it will also contribute to the protection of the host plants by these mycorrhizal fungi. The observation that Zn-tolerant *Suillus* isolates do not store extra Zn in their vacuoles can explain the reduced transfer of Zn to pine shoots in pine seedlings colonized by these Zn-adapted isolates.[8,52] Motile tubular vacuoles are an important vector in the transport chain of mineral nutrients from the site of uptake at hyphal tips to the plant–fungus interface in the mycorrhizal roots.[53]

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