ZINC ACCUMULATION AND DIFFERENT WAYS TO SEQUESTRATION OF INTRACELLULAR ZINC IN FRUIT-BODIES OF ECTOMYCORRHIZAL FUNGI *RUSSULA* SPP. AND *HEBELOMA* SPP.

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ABSTRACT

Mycorrhizal fungi, including ectomycorrhizal (EM) species, play an important role in the environmental cycling of elements and protection of their host plants against various stress factors such as toxic levels of heavy metals. The Zn content of *Hebeloma* spp. sporocarps grown in pristine environments was found within the range of 50 to 150 mg kg^{-1}, which is typical for most EM fungi, including the majority of *Russula* spp. We identified four Zn-accumulating species, *R. atropurpurea*, *R. ochraleuca*, *R. pumila* and *R. viscida*, which clade together and show common fruit-body Zn concentrations of 300 to 1100 mg kg^{-1}. Three analyzed *Hebeloma* species showed virtually all Zn of cell-free extract complexed with glutathione (GSH). In contrast, 70 to 80% of extracted cellular Zn of Zn-accumulating species of *Russula* spp. was sequestered by 6-kDa MT-like peptides, while the remaining Zn was associated with GSH. We detected Zn sequestration contributed by MT-like peptides also in 25 species of poorer Zn accumulators of *Russula* spp., thereby indicating that the capacity to produce Zn-MT is independent of phylogenetic relation and Zn-accumulation phenotype.

Keywords: Zinc uptake; Metal ligands; Metallothionein; Glutathione; Metal tolerance

INTRODUCTION

Fungi are ubiquitous components of soil communities with a substantial role in biogeochemical cycling of the elements, including heavy metal species [1]. While the capacity of macrofungi to accumulate heavy metals in large quantities has been reported since the middle of the last century, deciphering of the molecular mechanisms underlying this phenotype is being challenged only recently. Especially interesting are fungi forming the mutualistic partnerships with land plants (mycorrhizas), which appear to be of functional importance for plant nutrition and healthy growth [2]. Several studies have shown that metal-resistant arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi can contribute to the metal tolerance of associated higher plants [3-8] which might be also exploited for biotechnological application such as phytoremediation [1, 9]. The fungal mechanisms of toxic metal tolerance the plants may benefit from include metal immobilization on the soils (e.g. by biosorption on hyphae cell-walls, extracellular precipitation of secondary mycogenic minerals, binding by extracellular metabolites) and intracellular sequestration (e.g. by specialized peptidaceous cytosolic ligands, compartmentalization in vacuoles) [10-14].

Zinc is an essential divalent metal ion in all life forms, where it serves catalytic and structural roles. As a borderline Lewis acid, Zn^{2+} can accept electrons from residues of several

amino acids including cysteine, histidine, aspartate, glutamate, serine, threonine, and tyrosine, which form stable coordination spheres in binding centers of Zn-metalloproteins [15]. Like with the other essential metals, the healthy growth is impaired if an organism is unable to acquire sufficient Zn; however, Zn can be detrimental to organisms when present in excess [16]. Therefore, the Zn homeostasis must be maintained by uptake and efflux of Zn transporters and the coordinated management of the Zn pool involving compartmentalization within the cell, intracellular complex formation and deposition of Zn^{2+} at target metalloproteins via specialized metallochaperone proteins [17].

In animals and plants the majority of the Zn cytoplasmic storage pool is sequestered by metallothioneins (MTs) [18, 19]. MTs are a gene-encoded group of cysteine-rich peptides of distinct lengths (e.g. 61 to 65 aminoacids in mammals, 42 to 84 in plants), capable of high affinity coordination of heavy metal ions via cysteine residues shared along the peptide sequence in Cys-X-Cys or Cys-Cys motifs. While the role of plant MTs is generally attributed to the homeostasis of essential heavy metals such as Zn and Cu [19], in mammals MTs are also associated with protection against heavy metals (especially Cd) and oxidant damage [18]. Differential transcription of MT genes during mycorrhiza development in the EM *Pisolithus tinctorius* [20] and the AM *Gigaspora margarita* [21] suggests that the respective MT peptides might be involved in regulation of the metal homeostasis. Efforts have been made towards understanding the role which MTs of AM and EM fungi play in enhanced tolerance against heavy metal stress. Unlike mammalian and plant MTs, showing sequence conservancy, fungal MTs produced in a response to stress conditions encompass a quite diverse group of peptides. Exposure to Cu (and oxidative stress) induced transcription of the *GintMT1* gene encoding 71-amino acid residue (AA) GintMT1 in the AM fungus *Glomus intraradices* [22]. A gene encoding 34-AA PiMT involved in sequestration of Cd in *P. involutus* was isolated and shown to confer higher Cu-tolerance to transgenic EM fungus *Hebeloma cylindrosporum* [23, 24]. We recently described 34-AA AsMT1a, AsMT1b, and AsMT1c (unrelated to PiMT) from Ag-hyperaccumulating *Amanita strobiliformis* [25, 26], which share 82% identity, providing the first evidence of the presence of MT isoforms in EM fungi. Only the Ag-AsMT1a complex was detected in a *A. strobiliformis* fruit body in which As*MT1a* was the prevailing transcript. In *H. cylindrosporum*, Cu induces expression of indigenous Hc*MT1* and Hc*MT2* encoding 59-AA and 57-AA MTs, which share only 40% identity [27]. While transcription of Hc*MT2* is also induced by Cd, neither Hc*MT1* nor Hc*MT2* were induced by Zn.

Besides MTs, glutathione (GSH) appears an indispensable component of heavy metal and redox homeostasis. As a fundamental antioxidant molecule, GSH directly eliminates reactive oxygen radicals induced by heavy metal ions in cells [28], and provides reducing equivalents in the ascorbate-glutathione antioxidation cycle [29]. It also appears likely that in some EM fungi, including *P. involutus*, GSH is involved in cellular detoxication of Cd^{2+} dependent upon exclusion of the metal into vacuoles [13]. The vacuolar sequestration of Cd was detailed in the yeast *Saccharomyces cerevisiae*, in which the uptake to vacuoles is accomplished by the ATP-dependent ABC-type YCF1 transporter effective on the bis(glutathionato)Cd complex [30]. Studies in the same yeast have revealed that vacuolar sequestration of Zn depends on the cation diffusion facilitator (CDF) family transporters ZRC1, mediating transport via a Zn/H^+ antiport mechanism, and COT1 [17]. Both ZRC1 and COT1 appear to be required for homeostasis sequestration of zinc for later use under zinc-limiting conditions as well as for detoxication of excess Zn. Recently Blaudez & Chalot [31] described the CDF transporter ZnT1 of *H. cylindrosporum* capable to complement *zrc1*Δ mutation in *S. cerevisiae*.

In the present paper we described the analysis of intracellular speciation of Zn in fruit-bodies of two EM genera, *Hebeloma* spp. and *Russula* spp. We showed that while the sequestration of excess Zn in *Russula* spp. is dominated by MTs, these were not detected as Zn-

ligands in analyzed *Hebeloma* spp. Our data suggested that the whole Zn storage pool of *Hebeloma* spp. is bound to GSH and/or compartmentalized.

MATERIALS AND METHODS

Collection and characterization of fruit-bodies. The young fruit-bodies of *Hebeloma* spp. and *Russula* spp. (Fig. 1) were collected from their natural habitat in pristine areas of the Czech Republic. The fruit-bodies were cleared of substrate debris, washed with sterile distilled water and stored at -80 °C. The total metal content was determined by INAA as described previously [33]. The molecular phylogeny was inspected by sequencing of ITS regions of rDNA amplified from the total chromosomal DNA in polymerase chain reaction. Total DNA was extracted from frozen tissue using the Power Soil DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions.

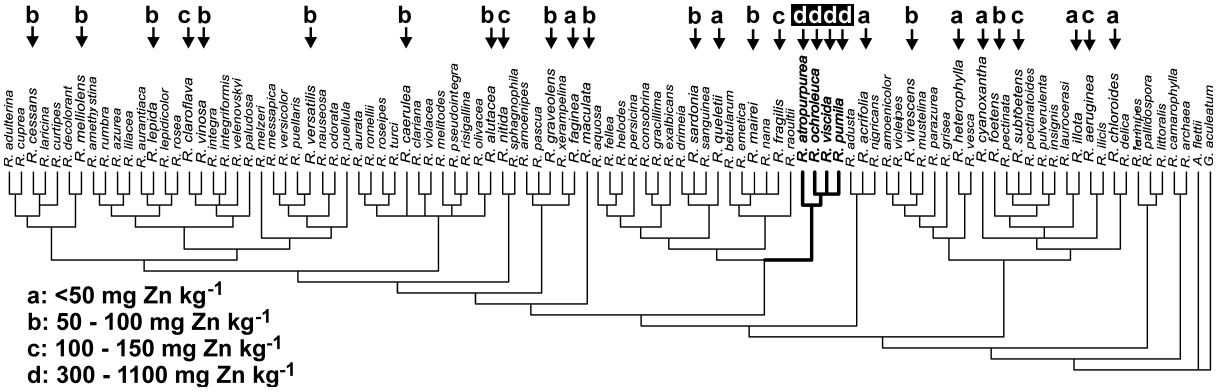

Figure 1: *Russula* species used in this study (indicated by arrows) and their phylogenetic position according to Miller & Buyck [32]. Concentration of Zn in fruit-bodies was determined by INAA and the range particular fruit-body fell in is indicated.

Isolation of metal complexes. The fungal tissue extracts were obtained from 3 g of fruit-bodies (stipes and caps in natural proportion). These were ground in liquid N_2 with mortar and pestle and the disintegrated tissue was extracted with 2.5 ml of 25 mM HEPES (pH 7.0). Cell debris was removed by centrifugation at 20 000×*g* for 10 min at 4 °C. To fractionate the extract by using size exclusion chromatography (SEC), 2 mL of the extract was loaded onto a Superdex Peptide 10/300 GL column (GE Healthcare). Separation was performed with a BioLogic DuoFlow FPLC system (BioRad) and 50 mM HEPES, 25 mM KNO_3 (pH 7.0) as a mobile phase at a flow rate of 0.5 mL min^{-1}. Ribonuclease A (GE Healthcare), ubiquitin (Sigma), synthetic 2.1-kDa peptide and glutathione (Merck) were used as molecular weight (MW) standards. Metal contents in aliquots of 0.5 ml fractions from SEC were determined by atomic absorption spectrometry (AAS; model Spectr AA300, Varian, Inc.).

Reverse phase chromatography and electrophoresis of the ligands. SEC fractions containing complexes of MW ≥5-kDa were pooled and brought to a final volume of 50 to 80 µL by ultrafiltration with Microcon YM-3 (Millipore). Pooled fractions containing the low-molecular weight Zn-complexes were freeze-dried and the lyophilizate was dissolved in 50 µL of distilled water. The sulfhydryl containing molecules of isolated complexes were fluorescent labeled in reaction with 7-fluorobenzofurazan-4-sulfonic acid (SBD-F) and resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) or sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [26, 34]. The RP-HPLC analyses were performed with an Agilent 1200 HPLC with PEEK capillaries, an Agilent G1321A

spectrofluorimetric detector (Agilent Technologies, Inc.; excitation at 385 nm, emission at 515 nm) and an analytical 250 mm column (4 mm id) packed with Separon SGX C8 (5 μm; Tessek, Ltd.).

The acetonitrile proportion in water (both with 0.1 % [v/v] trifluoroacetic acid) during elution was 5 to 25% (v/v) linear gradient from 0 to 20 min, 25 to 70% from 20 to 25 min, 70% from 25 to 33 min and 70 to 5% from 33 to 35 min. The electrophoretic separation of ligands was conducted in the presence of SDS and under reducing conditions in 16% polyacrylamide gels (SDS-PAGE) in a Tris-Tricine buffer system with 6M urea as described previously [26, 34]. The fluorescent electrophoreograms were recorded with a ≥605 nm filter after suboptimal excitation at 312 nm. Glutathione (Merck), γ-glutamylcysteine and cysteine (Sigma), (γ-glutamylcysteine)$_n$glycine (phytochelatin; n=2 and 3; Vidia, Ltd.) were used as standards.

RESULTS AND DISCUSSION

The fruit-bodies of *H. mesophaemum*, *H. sacchariolens*, *H.* cf. *crustiliniforme* analyzed here were obtained from pristine soil with common Zn background levels and contained 164, 121 and 61 mg Zn kg^{-1} of dry weight. To inspect the cellular Zn-deposition form, the disintegrated fruit-body tissues were extracted under mild conditions of neutral pH. The disintegration allowed extraction of nearly 80% of the total accumulated Zn. The size exclusion chromatography (SEC) of extracts from all three species showed that the majority of Zn (81 to 83%) was contained in low-MW fractions of apparent MW of ≤1 kDa (Fig. 2A shows speciation of Zn in extracts from *H. sacchariolens* as an example). Remaining Zn portions associated mainly with proteins of MW ≥ 20kDa (column size exclusion limit) could be attributed to physiological Zn-containing metalloproteins of the fungus. The size of the major Zn-complex suggested that GSH or short phytochelatin (PC) molecules could be considered likely ligands. PCs are small peptides of general structure (γ-Glu-Cys)$_n$X (PCn; n = 2-11; X represents Gly, Ser, β-Ala, Glu, Gln or no residue) enzymatically synthesized from GSH or its analogues in a metal-dependent manner. They have pivotal role in the detoxication of various heavy metals (especially Cd) in plants and certain yeasts [35, 36] and PC2 and PC3 were also reported as dominant Cd-binding ligands in the EM *Boletus edulis* [37]. Although PCs have the capacity to bind Zn and PC synthesis is seemingly induced in plant cultures by Zn present in media [38], it is unlikely that they play a role in the Zn homeostasis and detoxication in plants and yeasts [35, 36].

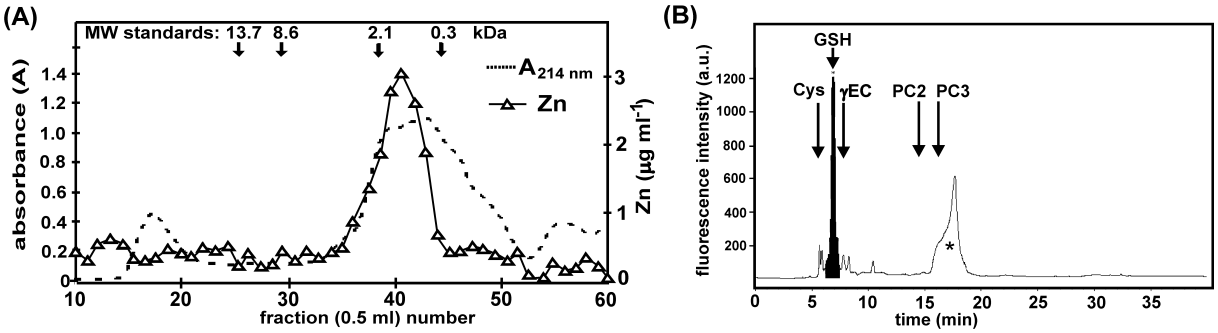

Figure 2: Speciation of Zn in fruit-body of *H. sacchariolens* and characterization of thiol-containing peptides from ≤1 kDa Zn-complex. **(A)** SEC fractionation of the fruit-body extract. The elution maxima of the MW standards are indicated by arrows. **(B)** RP-HPLC of peptides from *H. sacchariolens* labeled with SBD-F. The retention times of GSH, γ-glytamylcysteine (γEC), cysteine (Cys) and phytochelatins PC2 and PC3 are indicated by arrows. Asterisk indicates a peak observed also in a blank sample prepared with water instead of the SEC fraction aliquot.

To characterize Zn-ligands of the low-MW Zn complexes of *Hebeloma* spp. cell-free extracts, the corresponding SEC fractions were pooled, concentrated by lyophilization and thiol-containing compounds were labeled with a sulfhydryl-specific SBD-F probe. Separation of

labeled compounds by RP-HPLC revealed the presence of a high intensity GSH peak in fractions from all analyzed species (Fig. 2B shows *H. sacchariolens* as an example), thereby suggesting the presence of a glutathionatoZn complex in the cell-free extracts. It also showed that PC2 and PC3 were absent from the complex (Fig. 2B). It should be noted that our approach does not allow us to conclude that GSH represents the major Zn-ligand sequestering the metal *in vivo*. As the cytosolic GSH levels lay generally within a range of 2 to 3 mM [39], the formation of a secondary glutathionatoZn complex from Zn^{2+} released from cellular compartments in the extract could not be excluded. In plants and yeasts subcellular compartmentalization of Zn depends on transporters of the CDF family, which mediate secondary active transport of Zn^{2+} ions into organelles [17, 30]. The CDF transporter ZnT1 present in the endoplasmatic reticulum membrane and the presence of a Zn pool in small cellular vesicles (but not in vacuoles) described in vegetative mycelia of *H. cylindrosporum* [31] suggest that excess Zn is organelle-sequestered in this species. If the localization of Zn in *Hebeloma* spp. fruit-bodies is the same as in the mycelia and if it is present in vesicles as a free or, e.g., carboxylate bound ion or as a glutathione-Zn complex remains to be elucidated.

In an extensive study scoring the Zn contents in 383 species of basidiomycetous fungi Vetter et al. described the average fruit-body Zn content as 119 mg kg^{-1} of dry tissue weight [40]. Our data obtained for analyzed *Hebeloma* spp. and most *Russula* spp. (Fig. 1) corroborate these results. The authors also reported Zn concentrations in fruit-bodies of *Russula atropurpurea* ranging from 763-1067 mg kg^{-1}. The pronounced capacity of *R. atropurpurea* to accumulate Zn was confirmed also in our survey of fruit-bodies of this species collected from pristine areas [41]. The genus-wide screening was performed to inspect the Zn content in fruit-bodies of species of various phylogenic groups. Most members of the genus fell in the range of 50 to 100 mg of Zn kg^{-1}, but we identified *R. ochraleuca*, *R. pumila* and *R. viscida*, close relatives of *R. atropurpurea*, that showed common fruit-body Zn concentrations of 300 to 1100 mg kg^{-1} (Fig. 1). Although the Zn accumulation potential of these species is exceptional, they could not be regarded as Zn-hyperaccumulators. According to Brooks, species are considered as hyperaccumulating if they deposit in their organs at least 100-fold higher concentrations of a particular element than other species growing over an underlying substrate with the same characteristics [42].

To inspect the Zn-containing complexes in accumulators *R. atropurpurea*, *R. ochraleuca*, *R. pumila* and *R. viscida*, the disintegrated tissues were extracted and the extracts were subjected to SEC. Zn ligands were further characterized by using RP-HPLC and SDS-PAGE as described under Materials and Methods. The SEC chromatograms and isolated Zn-ligands showed the same pattern and characteristics among all four species. Fig. 3 shows data obtained from cell-free extracts of *R. pumila*. The majority of extracted Zn (70 to 85 %) was found associated with a MW fraction of 6 to 9 kDa (Fig. 3A). The size of the complex suggested possible involvement of MTs in sequestration of intracellular Zn. The RP-HPLC showed a multiple-peak cluster pattern (Fig. 3B) similar to that we previously observed with purified 3.4-kDa AsMT1 of *A. strobiliformis* [26]. The higher elution volumes, compared to those of AsMT1, suggested higher MW of the Zn-compex ligands. Considering their proteinaceous nature, the labeled thiol compounds were also resolved using SDS-PAGE. As shown in Fig. 3C, the 9-kDa complex from *R. pumila* contained the labeled MT-like peptide of MW similar to that of the 6.1-kDa rabbit MT1a plus minor larger peptide. Such MT-like peptides were detected also in Zn-complexes from *R. atropurpurea*, *R. ochraleuca* and *R. viscida*. A significant portion of extracted Zn was eluted from the SEC column with the fraction of apparent MW of ≤1 kDa (Fig. 3A), which resembled Zn-complexes of *Hebeloma* spp. extracts (Fig. 1A). Indeed, the RP-HPLC analysis of these fractions revealed the presence of GSH accompanied by uncharacterized minor thiols (Fig. 3D).

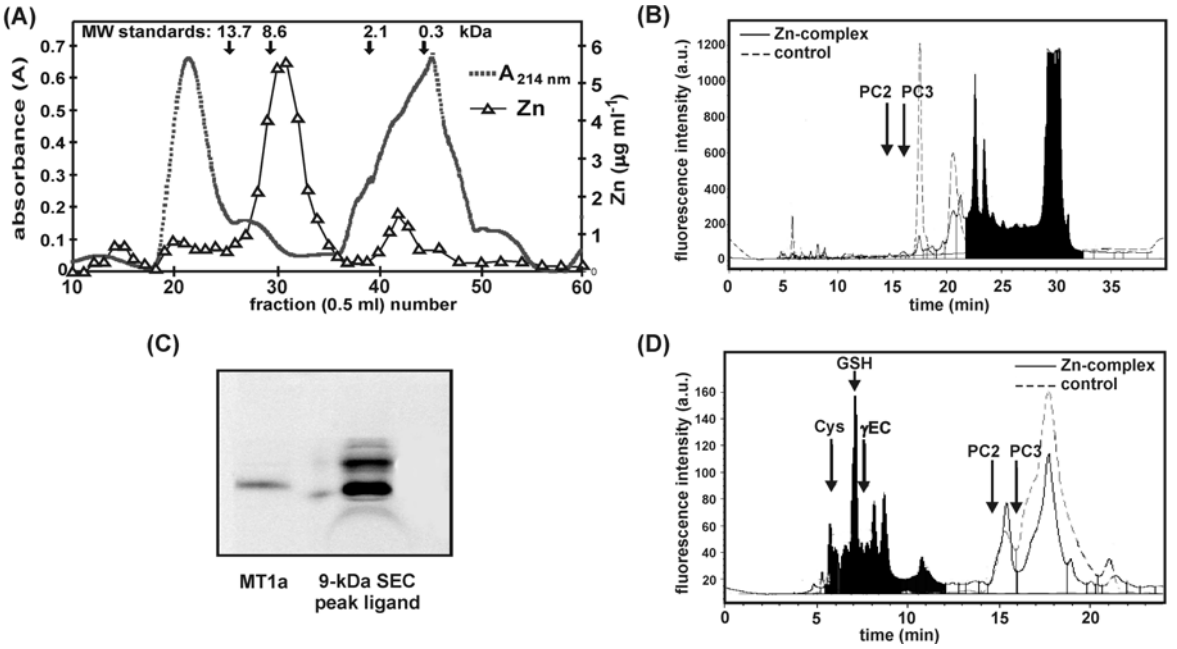

Figure 3: Speciation of Zn in fruit-body of *R. pumila* and characterization of thiol-containing peptides of Zn-complexes. **A)** SEC fractionation of the fruit-body extract. **(B)** RP-HPLC of SBD-labeled peptides from 9 kDa Zn-complex. **(C)** Electrophoretic analysis of SBD-labeled peptides contained in 9 kDa Zn-complex. MT1a denotes the SBD-labeled 6.1-kDa rabbit metallothionen 1a. **(D)** RP-HPLC of SBD-labeled peptides from ≤1 kDa Zn-complex. The elution maxima of the MW standards used in SEC, retention times of GSH, γ-glytamylcysteine (γEC), cysteine (Cys) and phytochelatins PC2 and PC3 on reverse-phase column are indicated by arrows.

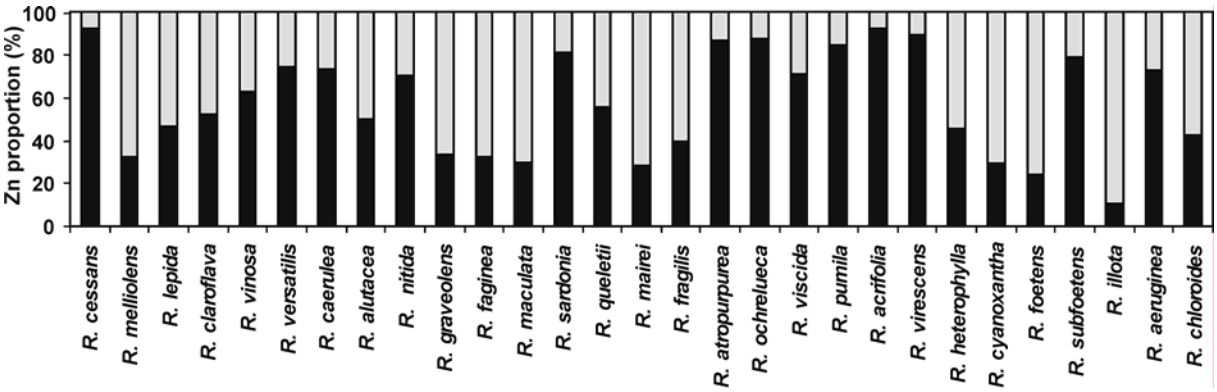

Figure 4: Distribution of Zn between MT-bound (black) and GSH-bound (grey) fractions in fruit-body extracts of *Russula* spp. Zn contents were determined in pooled SEC fractions corresponding to Zn-MT peak and Zn-GSH peak and the results are expressed as % of Zn recovered in Zn-MT plus Zn-GSH fractions (data from the 2010 season collection).

In order to get insights concerning the intracellular Zn in the genus *Russula*, we inspected the speciation of Zn in species of different phylogeny as indicated in Fig. 1. To this end, the individual cell-free extracts were fractionated by SEC and Zn contents were determined in pooled fractions corresponding to Zn-MT peak and Zn-GSH peak. The data summarized in Fig. 4 show that the capacity to deposit Zn in complexes with MT peptides and ≤1 kDa complexes was common and independent of their phylogenetic relation or Zn-accumulation phenotype. These results signify that Zn homeostasis in the genus *Russula* involves both Zn-MTs and formation of

a glutathionatoZn complex (or sequestration of Zn in subcellular compartments?). It should be noted that most eukaryotic cells localize metal-MT complexes in the cytoplasm [18, 19, 36]. It also appears that *Russula* species accumulating high Zn levels preferentially employ MTs to sequestrate the excess metal, a trait that was not detected in *Hebeloma* spp.

CONCLUSIONS

Our data show that *Russula* spp. and *Hebeloma* spp. employ distinct strategies for intracellular sequestration of excess Zn in their sporocarps, indicating that the tolerance against excess Zn would be dominated by different mechanisms. The inspection of the subcellular localization of the metal complexes is under way. Well aware of the fact that the mechanisms underlying extreme Zn accumulation must involve also efficient metal uptake, we are currently challenged with identifying the plasma membrane Zn transporters of *Russula* spp. accumulators by the expression library screening. Better understanding of molecular basis of metal uptake and metallotolerance in EM fungi would lead to improved applications in bioremediation and forestry.

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