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Different cadmium tolerance of two isolates of *Hebeloma mesophaeum* showing different basal expression levels of metallothionein (Hm*MT3*) gene

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ABSTRACT

Hebeloma mesophaeum is an ectomycorrhizal fungus frequently associated with metal disturbed environments. In this work, we examined Ag, Cd, and Zn tolerance of *H. mesophaeum* isolates from heavy metal-polluted (isolate Prib) and clean (isolate Rez) sites. Both mycelia showed essentially the same level of Ag and Zn tolerance, but Prib was more Cd tolerant. In short-term exposures, Prib accumulated slightly less Cd than Rez. Size exclusion chromatography of cell-free extracts and fluorescence microscopy of hyphae with a Cd-specific fluorescent tracer revealed that substantial proportion of Cd was contained in the vacuoles in both isolates. Considering that the proportion of Cd associated with fractions attributable to Cd complexes with cytosolic, metallothionein (MT) peptides was higher in Prib, we examined the copy number and basal levels of Hm*MTs* genes in Rez and Prib. While no difference between the isolates was observed in the gene copy numbers and basal levels of Hm*MT1* transcripts, the basal transcription of Hm*MT3* was 3-fold higher in Prib. These observations suggest that MTs provide in Prib better protection against Cd. Furthermore, the higher Cd tolerance in Prib can be to some extent also supported by the efflux or reduced uptake of Cd in the hyphae.

1. Introduction

The natural capacity of saprotrophic and ectomycorrhizal (ECM) macrofungi to effectively accumulate a wide range of heavy metals (HM) in their sporocarps has been observed for decades. Concentrations of Cd and Ag in sporocarps commonly reach up to units or even lower tens of mg kg^{-1} dry weight (dw) (Kalač and Svoboda, 2000; Kalač, 2010; Borovička et al., 2010). There are several notable exceptions: in sporocarps of *Cystoderma carcharias* concentrations of Cd up to 604 mg kg^{-1} dw were reported (Borovička et al., 2019) and significant Cd and Ag concentrations were also observed in the genera *Agaricus* and *Amanita*, respectively (Meisch et al., 1977; Borovička et al., 2007; Gryndler et al., 2012). Above-average contents of Cd (up to 40 mg kg^{-1} dw), Ag (up to 111 mg kg^{-1} dw), and Zn (and 574 mg kg^{-1} dw) were also noted in *Hebeloma mesophaeum* (Sácký et al., 2014). This ECM fungus is widespread in the northern hemisphere and reported from both pristine and disturbed or metal-polluted environments where it often occurs as pioneer species, establishing ECM connections with *Betula*, *Picea*, and *Pinus* (Hrynkiewicz et al., 2008; Krpata et al., 2008; Beker et al., 2016).

The intracellular mechanisms of HM sequestration in ECM fungi include binding with cytosolic peptides such as metallothioneins (MTs) (Osobová et al., 2011; Reddy et al., 2016), metallothionein-like peptides (Leonhardt et al., 2014), phytochelatins (PCs) (Collin-Hansen et al., 2007), glutathione (GSH) complexes (Bellion et al., 2006), or efficient transport to cellular compartments, or the efflux of the excess metal out of the cells (Beneš et al., 2018; Blaudez et al., 2000; Blaudez and Chalot, 2011; Ruytinx et al., 2013; Gube, 2016; Sácký et al., 2016). There is some evidence that HM pollution can lead to evolutionary adaptations in ECM fungi, adjusting the aforementioned mechanisms to better suit the HM-polluted environment. For example, the ECM populations of *Suillus luteus* colonizing polluted soils endured higher *in vitro* concentrations of Cd than

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populations from non-polluted soils (Colpaert et al., 2000). A potential adaptive metal tolerance trait has been described for other higher fungi including *Suillus* species (Colpaert et al., 2004; Ruytinx et al., 2013), *Cenococcum geophilum* (Goncalves et al., 2009), and *Pisolithus albus* (Jourand et al., 2010); however, the exact mechanisms of these adaptations remain unknown.

In the previous study on *Hebeloma mesophaeum* isolate from a clean area (Sácký et al., 2014) we have reported on the potential role of *H. mesophaeum* MTs (HmMTs) in the cytosolic binding of Ag, Cd, and Zn, and simultaneous deposition of Cd and Zn in cellular compartments for toxicity alleviation. Considering that the level of metal pollution may affect the metal tolerance in a particular ecotype (Goncalves et al., 2009; Colpaert et al., 2011; Ruytinx et al., 2013), we aimed to compare HM-related traits in two *H. mesophaeum* isolates originating from clean and heavily metal-polluted sites. We show that the former lead smelter area in which we document highly elevated Ag, Cd, and Zn concentrations in topsoils is inhabited by *H. mesophaeum* that showed substantial increase in Cd, but not Zn and Ag, tolerance. To learn the differences related to the handling of excess cellular HM between the isolates, we compared the metal forms, MT genes, and transcript levels between the isolates from clean and polluted sites.

2. Materials and methods

2.1. Isolation of mycelial strains and general procedures

The dikaryotic mycelial isolates of *H. mesophaeum* were obtained from axenic explants from freshly broken sporocarps collected in the field at two sites in the Czech Republic. One isolate was obtained from a HM-polluted *Picea* forest plantation in the vicinity of a lead smelter at Lhota near Příbram (further labeled as Prib) in Central Bohemia (Cejpková et al., 2016). The second mycelial strain was isolated from a sporocarp collected under *Picea* growing in anthropogenic environment at Řež near Prague in Central Bohemia (rural area, further labelled as Rez); this strain was previously used in the work of Sácký et al. (2014). The sporocarps from which the strains were isolated are deposited in the herbarium of the Mycological Department, National Museum, Prague (PRM 924396 and PRM 899241, respectively). To confirm the identity of the Prib mycelia, the ITS region was sequenced as described in Sácký et al. (2014) and deposited in GenBank under the accession no. MK024255. All mycelia were routinely maintained at 25 °C on 50 % potato dextrose (PD) agar containing 2 g l^{-1} potato extract and 10 g l^{-1} glucose (For Medium, UK).

2.2. Soil analyses

In order to characterize the environment where *H. mesophaeum* sporocarps were collected, samples of moderately decomposed spruce needles (the organic Oe soil horizon) and the underlying organomineral soil (Ah horizon) were collected from both sites as composite samples of 3 sub-samples taken with a steel spade. Soils were dried at room temperature and sieved through a 2 mm stainless steel mesh. A representative part of each sample was ground in an agate mill (Fritsch, Germany), and stored in a polypropylene container. For extractions, ~0.250 g portions of each soil sample were inserted into 15 ml polypropylene tubes, and 10 ml of extraction solution were added. Each sample was extracted in triplicate with three different extraction solutions: 1 M HNO_3 (≥65 % p.a., Carl Roth GmbH + Co.KG, Karlsruhe, Germany, subboiled in-house), 1 M HCl (Rotipuran® 37 %, p.a., Carl Roth GmbH + Co.KG, subboiled in-house), and 0.05 M ethylenediaminetetraacetate disodiumsalt dihydrate (EDTA, Carl Roth GmbH + Co.KG) adjusted to pH 7.0 with ammonia (25 %, p.a., Merck KGaA, Darmstadt, Germany). The mixtures were shaken top-over-bottom on a cross-shaped rotator overnight. Afterwards, they were centrifuged at 3300 *g* for 15 min and filtered through 0.2 μm polyamide syringe filters. The filtrates were diluted 100 × with ultrapure water and HNO_3 to a final acidity of 10 % v/v. For quality control, the certified reference material BCR 483 (sewage sludge amended soil, IRMM) was treated in the same way as the analysed soils (for extracted metal concentration see Borovička et al., 2019). The concentrations of Ag, Cd, and Zn were determined by ICPMS (7700x, Agilent Technologies, Waldbronn, Germany).

2.3. Metal tolerance and uptake assay

The metal tolerance assay was conducted on PD agar plates with the addition of 5–50 μM $AgNO_3$, 0.25–100 μM $CdCl_2$, or 50 μM to 2 mM $ZnCl_2$, or without metal as control. A ~3 mm^3 agar block of mycelium from a 30-day old culture was aseptically transferred onto the center of a PD agar plate covered with a sterile cellulose membrane and incubated at 25 °C for 30 d. Potential metal tolerance (residual growth) of the mycelial strain was determined according to the formula: $residual\ growth = \frac{m_t}{m_c} \times 100\%$, where m_t was the weight of metal-treated mycelium and m_c was the weight of non-treated mycelium (fresh weight).

In the cadmium uptake study, a ~3 mm^3 agar block of mycelium from a 30-day old culture was floated on top of 25 ml of liquid PD medium in a Petri dish for 42 d. After this incubation period the mycelia were transferred to a new Petri dish with 25 ml of fresh PD medium supplemented with 5 μM, 10 μM, 25 μM, or 50 μM $CdCl_2$ (or without the addition of $CdCl_2$ as a control) and grown for another 72 h. To reduce the interference of Cd adsorbed on the mycelial cell walls, the exposed mycelia were treated as described previously with *Saccharomyces cerevisiae* (Beneš et al., 2016). The mycelia were incubated twice with 50 ml of 50 mM sodium acetate (pH = 3) for 5 min and finally washed with distilled water. The treatment removes 80–85 % of the wall-bound metal. The same efficiency was observed with 5 mM EDTA (pH = 5.5; data not shown). The washed mycelia were lyophilized, weighted, and digested with 0.5 ml of 65 % HNO_3 for 24 h. After that, the sample volume was adjusted with distilled water to 5 ml and Cd content was measured by atomic absorption spectrometry (AAS, Model Spectr AA300, Varian, Inc.). Both tolerance and uptake experiments were performed in at least three biological replicates. The obtained data were analyzed using Student's T-test and in R 3.4.3 (R Core Team, 2017) using ANOVA and *post-hoc* Tukey's HSD test for metal tolerance assays or ANCOVA and dummy variable as described by Andrade and Estévez-Pérez (2014) for metal uptake assays.

2.4. Speciation of intracellular cadmium in the mycelia of H. mesophaeum

The mycelial blocks were grown in a 250 ml flask with 25 ml PD medium for 40 d. After the initial growth period, the mycelia were transferred to 25 ml of fresh PD supplemented with 20 μM $AgNO_3$, 50 μM $CdCl_2$, or 1000 μM $ZnCl_2$ and further incubated for 3 d. The metal-exposed mycelia (0.18–0.49 g) were disintegrated by using One Shot Cell Disrupter (Constant Systems Ltd.) and the disrupted cells were extracted with 2 ml of 50 mM HEPES (pH 7.3) supplemented with EDTA-free cOmplete protease inhibitors (Roche) according to manufacturer's recommendation. Tissue debris was removed by centrifugation at 30,000 g and 4 °C for 15 min. To determine the content of the monitored metals that failed to extract, the tissue debris was digested with 1 ml 65 % HNO_3 for 24 h. After that, the volume was adjusted to 5 ml with distilled water and the sample metal contents were measured by AAS. To perform size exclusion chromatography (SEC) and fractionate the mycelial

extracts, a Superdex Peptide 10/300 GL column (GE-Healthcare) was used as described previously (Sácký et al., 2014). Ribonuclease A, ubiquitin (Sigma–Aldrich) and glutathione (GSH; Merck) were used as molecular mass standards. The element contents in each fraction and in the digested debris were determined by AAS.

2.5. Fluorescent microscopy

The mycelia for microscopy were grown on solid PD medium in the presence of 2.5 μM $CdCl_2$ for 28 d. The conditions of the fluorescence microscopy and staining, as well as the used fluorescent probes, were the same as described by Hickey et al. (2004). Briefly, an approximately 10 mm^2, 3 mm thick block of PD agar bearing the mycelium was cut from the edge of the colony and submerged for 1 h in PD medium containing 30 μM dye FM4-64 (Molecular Probes, Invitrogen, Carlsbad, CA) for vacuole staining. The mycelium block was then chased for 6 h in fresh PD medium and afterwards stained for 1 h with 100 μg ml^{-1} Leadmium™ Green AM Dye (Cd-specific dye, Molecular Probes; product information MP1024). Finally, the mycelium was washed with fresh PD medium and observed with a U-DM-Cy5 filter (excitation band: 590–650 nm, emission band: 665–740 nm; Olympus) and a U-DM-DA-FI-Tx2 FITC filter (excitation band: 495/15 nm, emission band: 530/30 nm; Olympus) on a BioSystems Imaging station Cell^R with an MT20 illumination and a DSU semi-confocal unit on an IX-81 microscope (Olympus BioSystems, Planegg, Germany) with the model C9100 EM-CCD camera (Hamamatsu Photonix, Hamamatsu, Japan). Resulting images were processed with the help of the ImageJ software (Schneider et al., 2012).

2.6. Relative quantification of HmMTs

The mycelia were grown from agar blocks in a 250 ml flask with 25 ml of PD medium for 40 d, and then incubated in the same volume of fresh PD for 24 h. Isolation of genomic DNA and total RNA was performed by using the NucleoSpin Plant II kit (Macherey–Nagel) and RNeasy Plant Mini Kit (Qiagen) from 20 mg lyophilized mycelium tissue according to the manufacturer's protocols. To remove residual DNA from total RNA samples, the RNase-Free DNase Set (Qiagen) was used. cDNA from mycelia was obtained from 1 μg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

To assess the relative abundance of Hm*MT*s in the genome and the relative basal levels of corresponding transcripts, 12 μl qPCR reaction mixtures were prepared. The reaction contained iTaqUniversal SYBR Green Supermix (Bio-Rad) as recommended by the manufacturer, 0.35 μM specific primers (Table 1), 10 ng of genomic DNA or 1.5 μl of cDNA. The qPCR analyses were performed on the MiniOpticon Real-Time PCR system (Bio-Rad). The PCR conditions were the same as described by Sácký et al. (2014). The E values for genomic Hm*MT1* and Hm*MT3* were within the range of 98 % and 103 %, respectively. The E values for transcriptomic Hm*MT1* and Hm*MT3* were within the range of 99.8 %–112 % as calculated previously Sácký et al. (2014). The relative amount of Hm*MT*s was calculated according to the $2^{-\Delta ct1}$ method with the gene with the lowest expression level as a reference gene. Sequences for Hm*MT1* and Hm*MT3* are available in GenBank under the accession numbers KF278558 and KF985028, respectively. All qPCR measurements were performed on independent biological samples from five replicate experiments in two technical replicates.

Table 1
Primers used in this study. Sequences for Hm*MT1* and Hm*MT3* are available in GenBank under the accession numbers KF278558 and KF985028, respectively.

Target	Primer	Sequence (5′→3′)
genomic Hm*MT1*	gHmMT1F	TTCGCTCCATTAGACTTCCACC
	gHmMT1R	CGAGCAGGTGCAGCTGGTGATG
genomic Hm*MT3*	gHmMT3F	TTCAAAAGTCTTCTGAATGCAC
	gHmMT3R	AGTACAGGTGCAGGAAGAGCTG
coding Hm*MT1*	HmMT1F	ATGCAATTCACTTCCACCCTCGTC
	HmMT1R	TCAAGAGCAGTTGCAGTTGTTGG
coding Hm*MT3*	HmMT3F	GATCGTTCAAAAGTCTTCTGAATGC
	HmMT3R	CATTTGCACTCGCCAGGCTTGCAG

3. Results

3.1. Metal mobility in soils

Data on HM extractability in the soils from the smelter-polluted site at Lhota near Příbram and clean site at Řež near Prague are presented in Table 2. Generally, the metal concentrations were considerably higher in the soils from the polluted area. The Cd extractability was more or less similar for all extractants. On average, Cd was ~30× higher in the Oe horizon and ~130× higher in the Ah horizon at Lhota near Příbram. Zinc was enriched in the organic Oe soil horizon at both sites and only ~2× higher at Lhota (but ~20× higher in the Ah horizon at Lhota). Silver was effectively extracted only by HCl and approximately 100× higher at Lhota in both investigated soil horizons.

3.2. Metal tolerance and Cd uptake assay

Metal tolerance of the two investigated *H. mesophaeum* isolates (Prib from the polluted site and Rez from the clean site) was calculated as a weight percentage ratio of metal-treated mycelium to control mycelium. In the case of Ag and Zn exposure, slightly stunted growth of the Rez isolate can be observed (Fig. 1A–B), which, however, did not prove statistically significant when Prib and Rez groups were compared by ANOVA. In contrast, isolate Prib was remarkably more tolerant to Cd than the isolate Rez (Fig. 1C; $P = 5.25 \cdot 10^{-4}$ by ANOVA). The growth of the Rez isolate was significantly diminished already at 5 μM Cd, while the Prib isolate was still quite viable until 50 μM Cd.

The difference in total Cd accumulation of the mycelial isolates was assessed by measuring the total Cd contents in pre-grown mycelia exposed to 5 μM, 10 μM, 25 μM, and 50 μM $CdCl_2$ for 3 d. Interestingly, the Cd concentrations in less tolerant Rez isolate were slightly higher than the Prib isolate in the presence of all Cd concentrations (Fig. 2; the linear models of Prib and Rez groups vary significantly with $P = 0.0046$).

Table 2
Metal extractability in soils from the polluted site at Lhota near Příbram and clean site at Řež.

Extractant	Soil	Metal concentration (mg kg^{-1})[a]		
		Cd	Zn	Ag
1M HNO_3	Rez Oe	1.41 ± 0.09	282 ± 16	<0.004
	Rez Ah	0.23 ± 0.02	32.8 ± 1.1	0.06 ± 0.01
	Prib Oe	39.5 ± 0.5	558 ± 7	0.20 ± 0.01
	Prib Ah	29.4 ± 0.1	470 ± 13	2.21 ± 1.02
1M HCl	Rez Oe	1.36 ± 0.02	285 ± 5	0.36 ± 0.01
	Rez Ah	0.24 ± 0.01	35.5 ± 0.60	0.08 ± 0.01
	Prib Oe	37.6 ± 0.3	642 ± 14	33.3 ± 0.4
	Prib Ah	27.3 ± 2.2	454 ± 51	9.43 ± 0.8
0.05M EDTA	Rez Oe	1.27 ± 0.08	226 ± 22	0.03 ± 0.002
	Rez Ah	0.18 ± 0.02	11.5 ± 0.8	<0.004
	Prib Oe	38.7 ± 1.6	430 ± 29	3.30 ± 0.15
	Prib Ah	27.7 ± 2.0	300 ± 18	0.60 ± 0.05

[a] Data represent mean ± standard deviation of three analytical replicates.

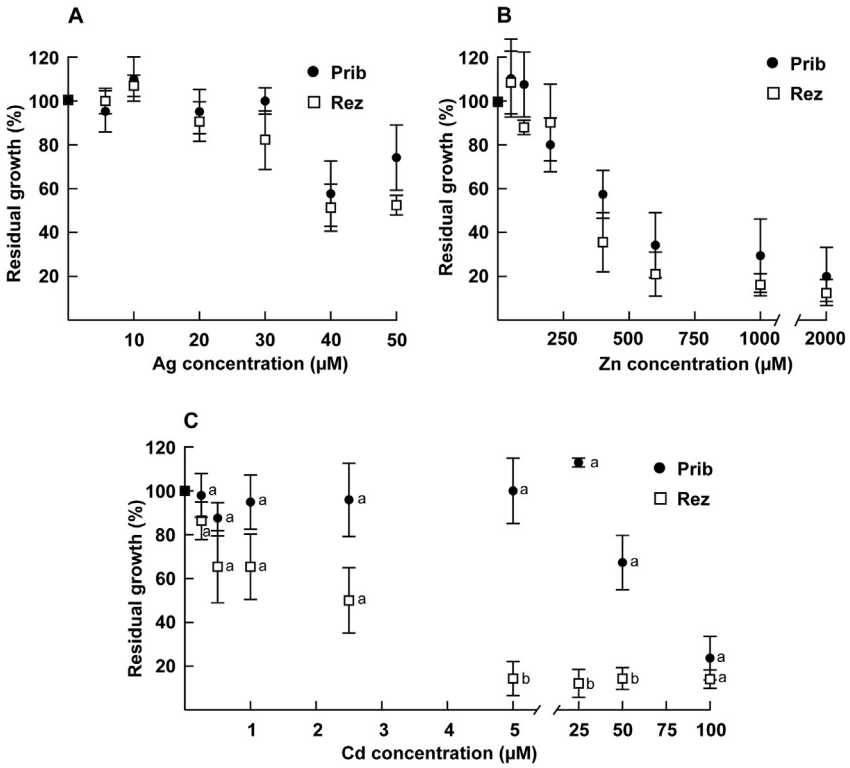

Fig. 1. Metal tolerance assay of *H. mesophaeum* isolates Rez (metal-sensitive) and Prib (metal-tolerant) in the presence of various concentrations of Ag (A), Zn (B), and Cd (C). At least three replicate experiments were carried out at each concentration; the bars represent standard deviations and significant differences observed only in Cd tolerance ($p < 0.1$, Student's T-test) are indicated by different letters.

3.3. *Deposition of Ag, Cd, and Zn in the mycelia of* H. mesophaeum

To inspect the intracellular metal species, the mycelial extracts obtained under neutral pH conditions from mycelia subjected to Ag, Cd, and Zn exposures were fractionated by SEC. This analysis revealed only very minor differences in the distribution of the examined metals between the Rez and Prib isolates (Fig. 3). Almost all intracellular Ag (82 % and 76 %), Cd (70 % and 61 %), and a small portion of Zn (20 % and 10 %) in both isolates was present in the early fractions corresponding to the molecular mass of >10 kDa (column exclusion limit). The rest of the detectable intracellular Ag (18 % and 24 %), but not all Cd (only 4 % and 22 %), and virtually no Zn, was associated with peptidaceous fractions corresponding to molecular mass of 3–8.6 kDa (Fig. 3A, B). In these peptidaceous fractions, a more distinct peak for both Ag and Cd was detected for the extracts from the Prib isolate. The remaining part of the intracellular Cd (26 % and 17 %) and Zn (60 % and 78 %) appeared in the low molecular weight fractions of <0.3 kDa, where for the Prib isolate the Zn peak is more distinct (Fig. 3B, C). It should be noted that due to the metal sensitivity of the Rez isolate, the wet weight of its mycelia was lower compared to the metal tolerant Prib isolate; however, the total concentrations (including the metals bound by biosorption) of Ag, Cd, and Zn were nearly identical for both Rez (8.34, 4.20, 30.0 μg g^{-1} respectively) and Prib (9.36, 4.40, 29.5 μg g^{-1} respectively) isolates.

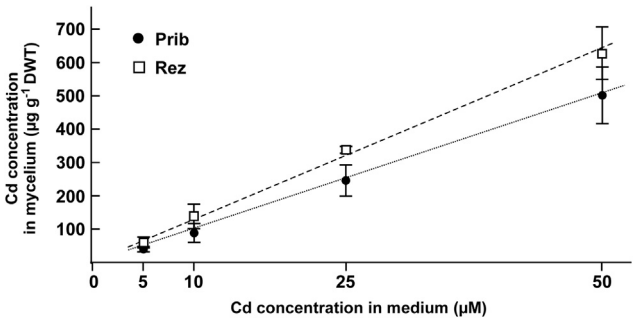

Fig. 2. Accumulation capacity of *H. mesophaeum* mycelia Prib and Rez. Three replicate experiments were carried out at each Cd concentration; the bars represent standard deviations and indicated is a linear dose-dependent Cd accumulation model used in the analysis of covariance (ANCOVA; significant variation with P = 0.0046).

3.4. *Fluorescent microscopy of intracellular deposition of Cd in mycelia*

To attest whether vacuoles can be involved in the sequestration of Cd in both Rez and Prib isolates, we investigated the intracellular deposition of Cd in the compartments of hyphal cells by using Leadmium as a Cd-staining marker and FM4-64 as a vacuole tonoplast marker. The double staining of the mycelial hyphae of both isolates with Leadmium and FM4-64 revealed a bright green fluorescence (Fig. 4B, F) inside the red vacuolar membranes (Fig. 4C, G). The relatively higher abundance of the vacuoles in the Rez isolate seen in Fig. 4 was not observed consistently through the sample and no quantification attempts were made.

3.5. *Relative quantification of Hm*MTs *in the genome and their basal levels*

To assess the relative number of copies of Hm*MT1* and Hm*MT3* in the genomes of mycelial isolates by qRT-PCR, Hm*MT3* from Prib

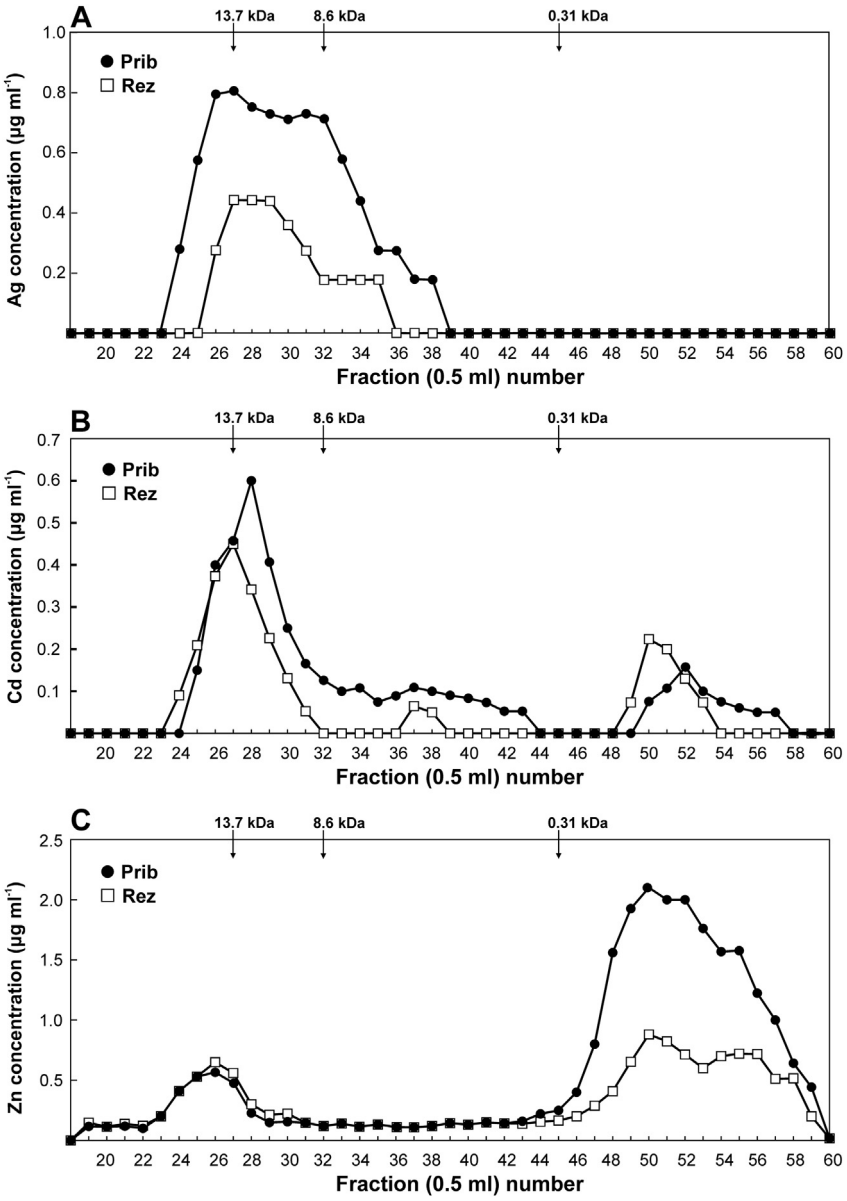

Fig. 3. Size exclusion chromatography (SEC) fractionation of cell-free extract from metal exposed *H. mesophaeum* mycelial strains Rez and Prib: Ag (A), Cd (B), and Zn (C). Ribonuclease A (13.7 kDa), ubiquitin (8.6 kDa), and glutathione (0.31 kDa) were used as molecular standards to monitor SEC elution profile. Note that the extracts originated from different amounts of biomass (panel A: Prib 0.43 g and Rez 0.18 g wet weight; panel B: Prib 0.48 g and Rez 0.32 g; panel C: Prib 0.36 g and Rez 0.23 g wet weight).

isolate was chosen as the reference gene and its value was taken as 1. Whereas the relative level of Hm*MT3* from the Rez isolate was close to the reference value, the relative levels of Hm*MT1* from both Rez and Prib isolates were elevated approximately 6–7-fold compared to the reference (Fig. 5A). To assure this observation, primers for Hm*MT1* and Hm*MT3* were used simultaneously in a qRT-PCR reaction, which resulted in the same abundance of gene copies as the sum of the individual Hm*MT*s.

The basal levels of Hm*MT*s mRNAs (as relative to Hm*MT1* transcripts in the Prib isolate) were assessed in the mycelia cultivated without any metal addition. As shown in Fig. 5B the basal level of Hm*MT1* from the Rez isolate was almost identical to that in Prib isolate, while the relative basal levels of Hm*MT3* were significantly increased in both Prib and Rez isolates. The only difference was observed for Hm*MT3* basal levels – in the Prib isolate they were elevated approximately three times compared the Rez isolate.

4. Discussion

We investigated two mycelial isolates of *Hebeloma mesophaeum* from contrasting sites: HM-polluted (Prib) and clean (Rez). Congruent with previous studies (Borovička et al., 2019; Cejpková et al., 2016), the extractable contents of Ag, Cd, and Zn at the sites from which the sporocarps used to isolate the mycelia were collected were markedly higher at the Prib site. In the previous study, we described that sporocarps of *H. mesophaeum* from polluted site accumulated significantly higher concentrations of Ag and sometimes Cd compared with sporocarps from clean sites (Sácký et al., 2014), which could be simply the result of increased HM presence at the metal-polluted location. However, long term presence of toxic levels of metals in soils can lead to evolutionary adaptations of the soil organisms. Such adaptations, especially towards metal tolerance, have been reported in several fungal species: *P. albus* (Jourand et al., 2010), *Suillus* spp. (Colpaert et al., 2000,

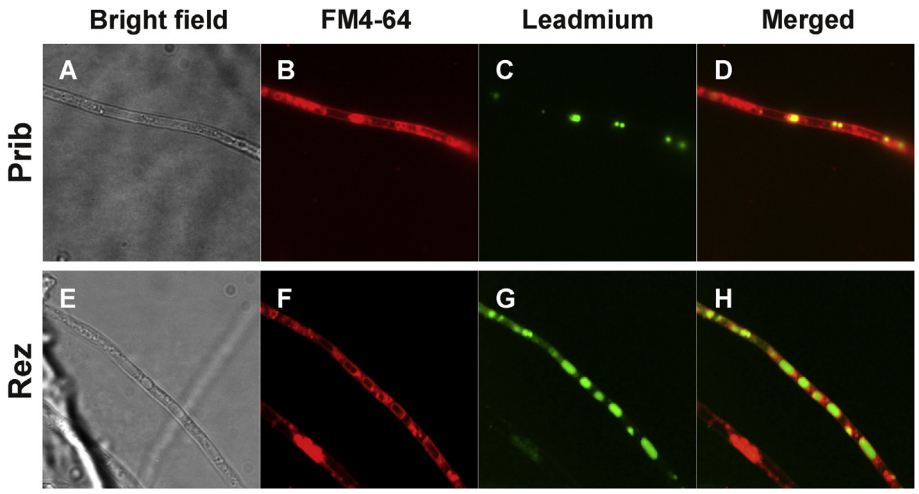

Fig. 4. Localization of Cd in *H. mesophaeum* hyphae. The mycelia were grown on solid PD media with a supplement of 2.5 μM $CdCl_2$. (A, E) Bright field of mycelia, (B, F) FM4-64 fluorescence (red) of stained vacuolar membranes, (C, G) Leadmium fluorescence (green) of Cd-exposed hyphae. (D, H) Merge (yellow) of Leadmium and FM4-64 staining. (For the interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

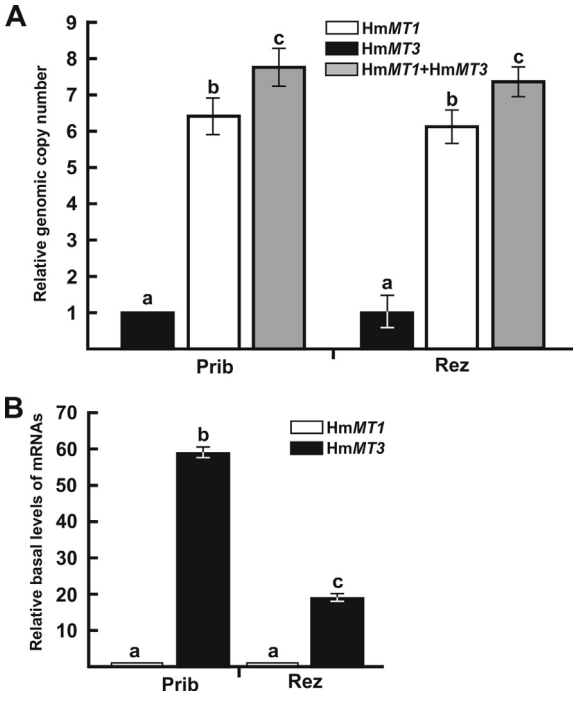

Fig. 5. The relative genomic copy numbers (A) and basal expression levels (B) of Hm*MT*s genes measured by qPCR in mycelia grown in plain PD medium. The relative amounts of Hm*MT*s were calculated according to the $2^{-\Delta ct1}$ method with Hm*MT3* (Prib) as a reference gene for the quantification of the relative genomic copy number, and Hm*MT1* (Rez) as a reference gene for the assessment of basal mRNA (reverse transcribed) levels. The plotted values represent the average of five biological replicates ± standard deviation of the mean, and significant differences ($p < 0.1$, Student's T-test) in Prib and Rez pairs are indicated by different letters above the bars.

2004; Ruytinx et al., 2013), and *C. geophilum* (Goncalves et al., 2009). In all these cases, metal-tolerant isolates of the same species have been found in metal-polluted areas. Similarly, metal-tolerant isolate of *H. mesophaeum* was found at the smelter-polluted site at Lhota near Příbram. This suggests a common trait in fungi regarding their capability of adaptation to HM-polluted environments.

The only significant difference regarding the metal tolerance of the two mycelial isolates was observed for Cd, while the same tolerance level was observed for Ag and Zn. It is worth mentioning that the strain from the polluted location accumulated *in vitro* less total Cd than that from the clean site. Similar result was observed by Colpaert et al. (2005) for ECM *S. luteus*, when Zn- and Cd-tolerant mycelium from a polluted area accumulated less metals *in vitro* compared to the isolate from a pristine area. This phenomenon was more evident with prolonged exposure: whereas within the first 2 h in the presence of sublethal Cd concentration the total accumulated Cd contents were similar in both metal sensitive and tolerant *S. luteus*, after 48 h the Cd sensitive mycelium accumulated 2 times more Cd than the tolerant strain. More importantly, it was shown that Cd efflux from the cells was faster in the tolerant isolates (Colpaert et al., 2011), analogous to faster Zn efflux in Zn tolerant *Suillus bovinus*, compared to its Zn sensitive isolate (Ruytinx et al., 2013). These observations indicate that the diminished metal accumulation observed in this study could also be a result of Cd efflux in the Cd-tolerant *H. mesophaeum* strain. However, we cannot dismiss the possibility that the reduced accumulation of Cd in the tolerant isolate could also be a result of a more efficient exclusion of the metal by extracellular chelation/precipitation or reduced uptake (Pócsi, 2011).

In order to deal with the increased metal concentration inside the cells, the fungus must employ some sequestration and/or storage mechanism in addition to the supposed export of the metal. In the previous study (Sácký et al., 2014) we concluded that *in vitro* sequestration of HM in *H. mesophaeum* (Rez) from the clean site involved compartmentalization of Cd into vacuoles and binding with MT in cytoplasm. The presented data from fluorescent microscopy did not reveal a significant shift in the deposition of Cd – it was detected predominantly in the vacuole in both isolates. Likewise, the data from SEC support the notion that there is no significant difference in the availability of mechanisms involved in the sequestration and storage of Cd (also for Ag and Zn) for the metal-tolerant isolate. Taken together, it is reasonable to assume that both isolates can employ binding with MTs for the detoxification of cytosolic Ag and Cd and compartmentalization for the detoxification of excess Cd and Zn. Considering the high molecular mass (>10 kDa) of the metal species eluting from SEC, it must be stressed that our cell disintegration and extraction approach does not allow us to distinguish between ions released from compartments and/or mobilized from cell walls, which then bind non-specifically to cellular proteins; hence, we believe that high

molecular mass species could represent an extraction artefact rather than the physiological metalation of (a specific) metal detoxification protein (Borovička et al., 2019).

Considering that the proportion of Cd associated with SEC fractions corresponding to Cd-MT complexes was higher in Prib isolate, we resorted to investigate whether the difference in tolerance of the isolates may stem from a difference on the molecular level, more specifically in the capacity of MTs, low molecular weight cysteinyl-rich peptides, to provide swift protection against Cd entering the cytoplasm. There is ample evidence that MTs are generally responsible for the sequestration or handling of HM in eukaryotes (Capdevila and Atrian, 2011; Palacios et al., 2011). Several studies revealed that multiple MT genes can be responsible for the housekeeping, storage, and detoxification of HM (Palacios et al., 2011; Vašák and Meloni, 2011; Leszczyszyn et al., 2013; Krężel and Maret, 2017). Furthermore, gene duplication can be considered an important mechanism related to adaptation to adverse environmental conditions (Wapinski et al., 2007). Focusing on metal tolerance, the duplication of MT gene *Mtn* was observed in *Drosophila melanogaster*, which resulted in the production of more MT transcripts and increased tolerance to Cd and Cu (Maroni et al., 1987). In the model yeast *S. cerevisiae*, the copy number of CUP1 gene can be within the range of 1–18 (Zhao et al., 2014) and the multiplication of CUP1 gene through tandem duplication or aneuploidy was observed in 27 of the 34 Cu-adapted *S. cerevisiae* lines (Gerstein et al., 2015). In the ECM fungal Ag-hyperaccumulator *Amanita strobiliformis*, three isoforms of AsMT1 were described as responsible for the sequestration of Ag (Osobová et al., 2011). Regarding *H. mesophaeum* PRM 899242 (a different sporocarp isolate from Příbram locality), our previous data show that except for the two standard MTs (Hm*MT1* and Hm*MT3*), one more MT (Hm*MT2*, accession number KF278559) which shares a high degree of identity on the DNA level with Hm*MT3* occurs in the genome of the isolate (Sácký et al., 2014). Therefore, it was reasonable to investigate MT-gene variations in the studied isolates. However, no variability in the copy number of MT genes that would suggest an adaptation via increased gene dosage was observed in the genome of Rez and Prib mycelia. In both mycelial genomes, only the two standard MTs (Hm*MT1* and Hm*MT3*) were detected, while Hm*MT2* observed in the PRM 899242 sporocarp (Sácký et al., 2014) was not detected (data not shown) in either of the genomes, suggesting that the Prib mycelium was isolated from a different sporocarp population. Indeed, the ITS sequences obtained from the Prib mycelium (PRM 924396, GenBank accession no. MK024255) and PRM 899242 sporocarp (accession no. HF678208) are not identical (99 % identity). While the relative genomic copy numbers of the Hm*MT1* and Hm*MT3* genes were the same in both mycelia (Hm*MT1* was 6-fold higher than Hm*MT3*), the basal level of the Hm*MT3* transcript from the Cd-tolerant isolate was increased approximately 3-fold compared to the Cd-sensitive isolate. Considering this, it is tempting to hypothesize that the higher expression of Hm*MT3* in the Prib isolate can contribute to its higher tolerance against Cd. It appears reasonable to assume that the higher constitutive level of Hm*MT3* in the Prib isolate may provide a faster and thus better protection against Cd entering the cytoplasm before the metal is eventually transmitted into vacuoles or out of the cells. It is worth mentioning that the basal levels of Hm*MT1* transcripts were substantially lower compared to Hm*MT3* in both isolates. Similar roles of MTs in the shuttling of HM is not without precedent and is involved in the passage of excess cytosolic Zn into vesicular Zn stores in mammalian cells (Colvin et al., 2010; Krężel and Maret, 2017). Noteworthy, 2 to 4 times higher basal expression of *MT2b* and *MT3* genes in Zn/Cd-hyperaccumulating *Arabidopsis halleri* compared to non-accumulating *Arabidopsis thaliana* is thought to be one the factors contributing to a higher metal tolerance in the hyperaccumulating species (Chiang et al., 2006). Considering that the exposure to Cd leads to oxidative stress generating high levels of reactive oxygen species (ROS; Schützendübel and Polle, 2002) and that MTs have ROS scavenger capability (Hassinen et al., 2010; Krężel and Maret, 2017), the possibility that higher Hm*MT3* levels result in more efficient ROS-detoxification, and not merely HM binding, cannot be excluded.

5. Conclusions

The Prib isolate of *H. mesophaeum* obtained from the metal-polluted site withstands much higher Cd concentrations *in vitro*, but presumably uses the same mechanisms to detoxify it as the Rez isolate obtained from the clean area. The only perceived differences between these two *H. mesophaeum* isolates were slightly reduced Cd accumulation and elevated basal level of Hm*MT3* transcripts in the Prib strain. Together with a possible more efficient metal exclusion system or reduced Cd uptake, it is reasonable to assume that Cd tolerance in the Prib isolate may involve HmMT3, acting via direct binding of Cd imported into the cells or ROS scavenging and thus reducing the Cd toxicity in cells. Future research in Prib and Rez isolates should thus involve mainly Cd influx and compartmental efflux analyses with metabolic inhibitors to obtain information about transport kinetics and involved transporter families as targets for molecular characterization, and proteomic analyses of Cd-peptide complexes to attest whether HmMT3 alone or with HmMT1 contribute to the sequestration of cytosolic Cd.

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