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Silver release from decomposed hyperaccumulating *Amanita solitaria* fruit-body biomass strongly affects soil microbial community

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Abstract Interaction of Ag with communities of soil saprotrophic organisms was studied in two different soils using a metagenomic approach. Three levels of Ag were applied to the soil samples: 0, 0.008 and 0.505 μg Ag/g soil. Silver was applied in mineral form as well as naturally bound in dry fruit-body biomass of the Ag-hyperaccumulating ectomycorrhizal fungus *Amanita solitaria*. Contrasting behavior of fungi and bacteria in reaction to Ag dosages was observed. The majority of bacterial ribotypes tended to prefer the soil with low doses of Ag, the ribotypes of fungi were more abundant in untreated soils and soils treated with the highest Ag concentration. Organically bound and mineral forms of Ag did not differ substantially in their effects on microbes in samples. The results indicate that decomposing Ag-rich fungal biomass can significantly alter the soil microbiota. This can contribute to formation of spot-like non-homogeneities in soil microbial distribution.

Keywords Soil fungi · Bacteria · Microbiota · Heavy metals · Toxicity · T-RFLP

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Introduction

Silver is a rare but naturally occurring metal with common soil concentrations ranging from <0.01 to 5 mg kg^{-1}, with most reported values below 0.1 mg kg^{-1} (Evans and Barabash 2010). Among the eukaryotic organisms, the most effective accumulators of Ag are macrofungi. Silver concentrations in macrofungal fruit-bodies harvested from pristine sites commonly reach units to tens of mg kg^{-1} (in dry matter); in Ag-polluted sites, macrofungi may accumulate even hundreds of mg kg^{-1} Ag (Falandysz and Danisiewicz 1995; Borovička et al. 2010).

An extraordinarily high ability to accumulate Ag has been reported for two European ectomycorrhizal macrofungal species *Amanita strobiliformis* (Paulet ex Vittad.) Bertill. and *Amanita solitaria* (Bull.) Fr. [syn. *Amanita echinocephala* (Vittad.) Quél.] which are classified Ag-hyperaccumulators with concentrations ranging approximately from 100 to 1,200 mg kg^{-1}; these high Ag levels were reported from unpolluted sites with natural Ag levels in soils (Borovička et al. 2007).

The extremely high Ag concentrations in the hyperaccumulating *Amanita* species suggest a possible biological importance since Ag compounds (including Ag ions and Ag nanoparticles) are known for their toxic effect on microbiota (Navarro et al. 2008; Hänsch and Emmerling 2010; Kumar et al. 2011). Furthermore, the toxic effect of silver on activity of soil enzymes has also been demonstrated (Murata et al. 2005; Chaperon and Sauvé 2007). However, the subject of the biological role of metal hyperaccumulation in macrofungi has neither been investigated, nor even considered.

Interestingly, Martens and Boyd (1994) suggested that elevated metal concentration in vascular plants be considered a new category of plant chemical defense: elemental defense ("defense hypothesis"). Most research on the ecological consequences of plant elemental composition has targeted hyperaccumulators since—as extreme examples of elemental accumulation—they are a good starting point for testing hypotheses regarding the function of accumulated elements.

Ripe fruit-bodies of the Ag-hyperaccumulating species decompose in situ and release the accumulated metal into the environment. If fungal-derived Ag significantly affects the microflora of the surrounding topsoil, this effect should be considered an additional feature of the fungal species biology—it may result in local modification of the richness, composition and spatial homogeneity of the soil microbial communities. To evaluate the effect on soil organisms of Ag contained in the fungal biomass, we established an experiment simulating the decomposition of a Ag-containing fruit-body of *A. solitaria*. We investigated shifts in terminal restriction fragment length polymorphism (T-RFLP) signals of ribotypes of fungi and bacteria caused by decomposition of Ag-rich fungal biomass. To separate the effects of Ag from the added organic matter itself, we also tested the effects of Ag-poor biomass of *A. solitaria* and also the effects of Ag ions applied at the same concentrations as were introduced by means of fungal biomass.

Materials and methods

Characterization of selected sites and sampling

We selected two localities of *A. solitaria* for sampling: Prague-Velká Chuchle (hereinafter referred to as Chuchle) and Kalešov near Roudnice nad Labem (hereinafter referred to as Kalešov), both in Bohemia, Czech Republic. Mature fruit-bodies of *A. solitaria* were collected in August 2010, cleaned from substrate debris, refrigerated, lyophilized and pulverized in a blender. Soil samples taken from both sites were collected in mid-October 2010 (under *Tilia* in both cases), and represented the top layer ($\sim$5 cm depth) of the organo-mineral surface Ahk horizon. Before the experiment, the soil samples were homogenized and sieved through a sieve with 2 mm mesh. The soils were not sterilized.

Silver concentrations in fungal fruit-bodies were determined by INAA (Řanda and Kučera 2004). Soil silver concentrations (reported as average values) were determined in triplicate from both sites. A portion of 250 mg pulverized soil sample was digested by mixture of Teflon-distilled HNO_3 (J.T. Baker, 4 ml) and HF (Merck, 5 ml) in the Microwave Accelerated Reaction System MARS5 (CEM). After digestion, active HF was neutralized using concentrated H_3BO_3 (Merck, 30 ml), the solution was diluted to 100 ml and analyzed by HR-ICP-MS (Element 2, Thermo Scientific).

The site at Velká Chuchle (GPS: N50 01.057, E14 22.826; elevation 280 m) is a mixed forest plantation composed predominantly of *Tilia* and *Carpinus*. The soil type was identified as Rendzic Leptosol (Skeletic) (IUSS Working Group 2007) above Silurian limestones. The fruit-bodies of *A. solitaria* previously analyzed from this site repeatedly showed high Ag concentration (400–800 mg kg^{-1}). The specimen used for this study contained 505 mg kg^{-1} Ag (high Ag biomass). Soil Ag concentration in the Ahk soil horizon was 1.48 $\pm$ 0.09 mg kg^{-1}.

The site at Kalešov (GPS: N50 25.380, E14 18.424 323; elevation 220 m) is a forest plantation composed of *Tilia*. The soil type was identified as Haplic Leptosol (Calcaric) (IUSS Working Group 2007) above Jurassic marls. The fruit-bodies of *A. solitaria* previously analyzed from this site repeatedly showed low Ag concentration (5–15 mg kg^{-1}). The specimen used for this study contained 8 mg kg^{-1} Ag (low Ag biomass). Soil Ag concentration in the Ahk soil horizon was 0.83 $\pm$ 0.09 mg kg^{-1}.

Experimental design

Five treatments were established per each soil: (i) control without any modification, (ii) soil amended

with 1 % (w/w) high Ag biomass, (iii) soil amended with 1 % low Ag biomass, (iv) soil supplied with mineral Ag equivalent to high Ag biomass and (v) soil supplied with mineral Ag equivalent to low Ag biomass. Five replicates (25 ml glass vessels containing 10 g naturally wet soil of 25.4 and 16.6 % water content in Chuchle and Kalešov soil, respectively) were established per treatment.

Mineral Ag equivalent was supplied as $AgNO_3$ dissolved in distilled water. Per replicate, 100 µl of the Ag solutions (treatments iv and v) or water (treatments i, ii and iii) were added. An amount of 5.05 or 0.08 µg mineral Ag was applied per vessel as equivalent of high or low Ag biomass, respectively. The vessels were incubated for 24 days at 25 °C and periodically watered to keep the humidity at natural level.

DNA extraction and T-RFLP analysis

After the incubation period ended, the vessel content was homogenized and DNA was extracted from a soil subsample (~300 mg) using Macherey–Nagel NucleSpin Soil DNA extraction kit with lysis buffer SL1 as recommended by the supplier. Undiluted DNA extract (~120 ng/µl, measured by NanoDrop spectrophotometer) was further used as template in PCR. PCR mixture (25 µl) was composed of 25 µl of 2× Combi-PPP mix (Top-Bio Ltd., Prague, Czech Republic, contains hot start-*Taq* DNA polymerase, 5 mM $MgCl_2$, buffer, deoxyribonucleotides and loader), 1 µl 10 µM 5′-HEX-labeled forward primer, 1 µl 10 µM reverse primer, 4 µl DNA template and 19 µl water.

For the analysis of fungal communities, the primers ITS1F and ITS4 (White et al. 1990) were used in PCR to amplify the ITS1-5.8S-ITS2 region of the rRNA gene cassette. In this particular case, the thermal cycler program was 95 °C for 4 min, followed by 34 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 90 s. Final extension at 72 °C lasted for 5 min. PCR of bacterial 16S rRNA gene was performed using primers 16Seu27f and 783r (Sakai et al. 2004). After initial incubation for 5 min at 95 °C, the mixture was subjected to 34 PCR cycles involving 45 s at 94 °C, 45 s at 57 °C and 90 s at 72 °C. Final extension at 72 °C lasted for 5 min.

Amplified fungal DNA fragments were purified using UltraClean PCR Clean-up DNA Purification Kit (MoBio), evaporated under vacuum to a volume of 15 µl and combined with 2 units of *Taq*I restriction endonuclease (NEB), 0.3 µl of 1 % BSA supplied with the enzyme and 3 µl Nebuffer 3. Resulting restriction mixture was supplied with water up to the volume of 30 µl and incubated at 65 °C for 1 h, then inactivated for 15 min at 80 °C, desalted by Post-Reaction Clean-Up Column (Sigma) and analyzed using capillary electrophoresis as indicated below.

PCR amplified bacterial DNA fragments were cleaved by restriction endonuclease *Alu*I (NEB) as follows: purified PCR products were mixed with 5 µl of NEBuffer 4 and 20 units of *Alu*I enzyme, incubated 2 h at 37 °C, supplied with another 10 units of *Alu*I enzyme and incubated for another 2 h at 37 °C. Finally, the restriction enzyme was inactivated at 65 °C for 20 min and the sample was desalted by Post-Reaction Clean-Up Column (Sigma) and analyzed using capillary electrophoresis.

After restriction cleavage and desalting, 0.5 µl of GeneScan 400HD ROX size standard (Applied Biosystems) per 14 µl sample aliquot was added, the samples were transferred to the sample plate, denatured at 96 °C for 5 min, instantly cooled down to 4 °C in a thermocycler (Biometra) and analyzed using capillary electrophoresis (ABI Prism 3130 × l Genetic analyzer, Applied Biosystems) at run voltage 15 kV and oven temperature 60 °C. POP7 polymer (Applied Biosystems) was used for fragment analysis. Samples were loaded electrokinetically (15 s at 1.6 kV).

Data analysis

The data were tabulated using GeneMarker 1.85 software (Soft Genetics LLC, State College, USA) and further analyzed using distance-based redundancy analysis (dbRDA, Canoco 4.5 software package, Biometris, Wageningen, The Netherlands). Only signals stronger than 50 fluorescence arbitrary units of fragments fitting into the length range 60–420 bp and occurring in at least 10 samples were taken into account. The Bray-Curtis distance of untransformed TRF intensity generated by PrCoord Canoco tool was analyzed (Grant and Ogilvie 2003) and negative eigenvalues were corrected. Monte-Carlo test (499 permutations) was exploited to evaluate the significance of the results.

Three approaches were applied during the data evaluation. In the first one, the data from treatments where biomass was applied were excluded from the

analysis so that only the effects on soil microflora of mineral Ag and soil origin were evaluated. In the second approach, the treatments where no mineral Ag was applied were left aside and the soil origin was taken as covariable defining Monte-Carlo permutation blocks. The purpose of this approach was to compare the effects of mineral and organic Ag on soil microflora. In the third approach, the complete data were analyzed and the amount of Ag applied (either mineral or organic) was taken as covariable, evaluating the net effects of the biomass applied on the soil microflora. In this case, the soil origin was also taken as covariable defining permutation blocks. Results with $p \leq 0.05$ were considered statistically significant. The results of dbRDA are graphically presented using CanoDraw tool, a component of Canoco software, in the form of biplots containing scores of environmental data and scores representing the behavior of bacterial and fungal ribotypes.

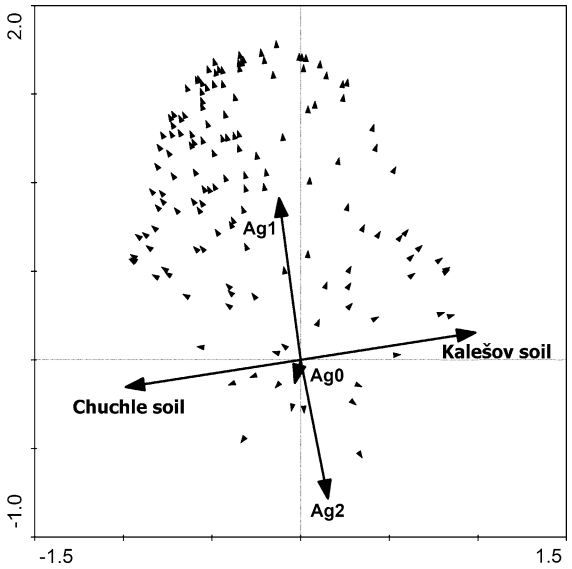

Fig. 1 Biplot of dbRDA results describing the interaction between mineral Ag at 3 levels (Ag0—control without $AgNO_3$ addition, Ag1—0.008 μg mineral Ag applied per 1 g soil, Ag2—0.505 μg mineral Ag applied per 1 g soil) with *bacterial ribotypes* in two different soils (Chuchle, Kalešov). Scores of both environmental parameters (Ag level and soil) are represented by *large arrows*. Scores of bacterial ribotypes are represented by *small triangles*

Results

As seen from Table 1, all the three combinations of environmental factors and covariables highly significantly affected bacterial and fungal communities. Larger *F* values for fungal data were noted, indicating stronger response of fungi to Ag application.

Figure 1 indicates that the majority of bacterial ribotypes tended to prefer lower level of mineral Ag applied (Ag1), the Chuchle soil being more rich in bacterial ribotypes than the Kalešov soil. Zero (Ag0) or higher (Ag2) mineral Ag levels were not preferred by bacterial ribotypes in most cases. When the soil samples supplied with lower or higher mineral Ag levels were compared with the samples supplied with equivalents of Ag bound in fungal biomass (Fig. 2), the bacterial ribotypes tended to associate with lower Ag level, the Ag form applied being of slight

Table 1 Results of Monte-Carlo test of the effects of silver and biomass on communities of soil bacteria and fungi

Organisms	Bacteria			Fungi		
Covariables	None	Soil origin	Soil origin, Ag applied	None	Soil origin	Soil origin, Ag applied
Environmental variables	Soil origin, mineral Ag	Mineral Ag, organic Ag	Biomass applied	Soil origin, mineral Ag	Mineral Ag, organic Ag	Biomass applied
Significance of 1st canonical axis						
Eigenvalue	0.178	0.188	–	0.467	0.211	–
F	5.623	9.305	–	22.804	13.504	–
p	**0.002**	**0.002**	–	**0.002**	**0.002**	–
Significance of all canonical axes						
Trace	0.252	0.274	0.144	0.51	0.246	0.188
F	2.922	5.177	9.285	9.026	5.642	16.412
p	**0.002**	**0.002**	**0.002**	**0.002**	**0.002**	**0.002**

Values in bold indicate statistical significance at $p \leq 0.05$ level

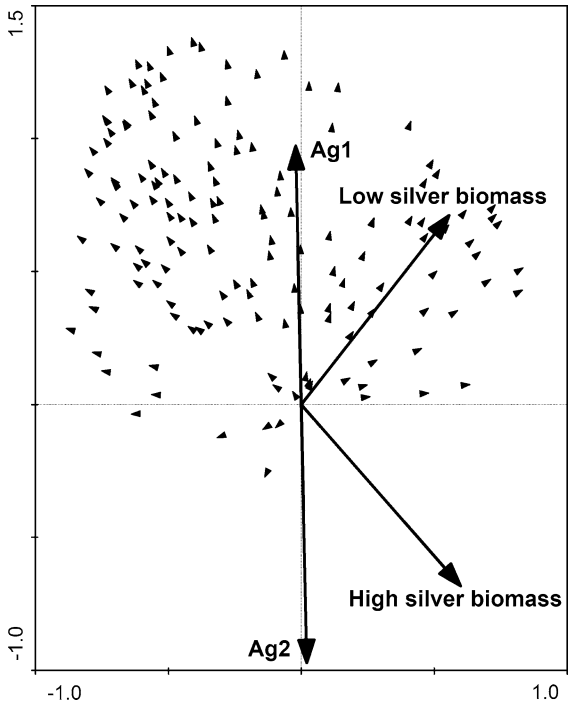

Fig. 2 Biplot of dbRDA results describing the interaction between two levels of mineral Ag (Ag1—0.008 μg mineral Ag applied per 1 g soil, Ag2—0.505 μg mineral Ag applied per 1 g soil) and two levels of silver bound in fungal biomass (low silver biomass—equivalent to Ag1 level of mineral Ag, high silver biomass—equivalent to Ag2 level of mineral Ag) with *bacterial ribotypes* in two different soils as covariables. Scores of both environmental parameters (mineral Ag levels and silver in biomass) are represented by *large arrows*. Scores of bacterial ribotypes are represented by *small triangles*

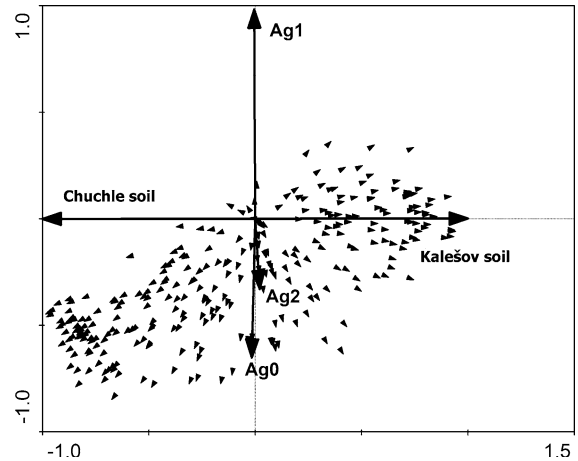

Fig. 3 Biplot of dbRDA results describing the interaction between mineral Ag at 3 levels (Ag0—control without $AgNO_3$ addition, Ag1—0.008 μg mineral Ag applied per 1 g soil, Ag2—0.505 μg mineral Ag applied per 1 g soil) with *fungal ribotypes* in two different soils (Chuchle, Kalešov). Scores of both environmental parameters (Ag level and soil) are represented by *large arrows*. Scores of fungal ribotypes are represented by *small triangles*

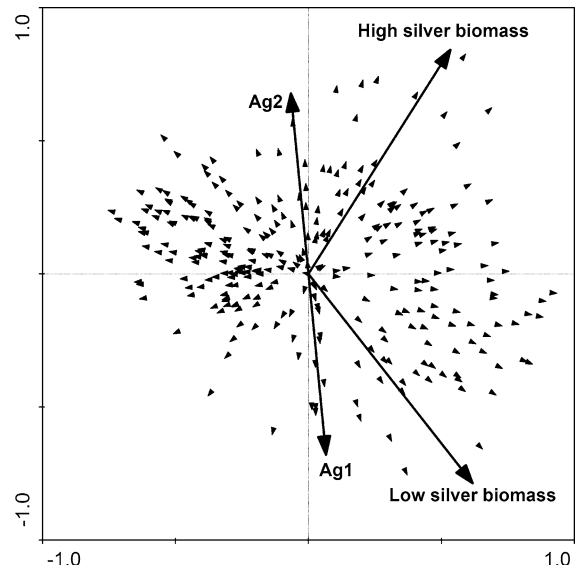

Fig. 4 Biplot of dbRDA results describing the interaction between two levels of mineral Ag (Ag1—0.008 μg mineral Ag applied per 1 g soil, Ag2—0.505 μg mineral Ag applied per 1 g soil) and two levels of silver bound in fungal biomass (low silver biomass—equivalent to Ag1 level of mineral Ag, high silver biomass—equivalent to Ag2 level of mineral Ag) with *fungal ribotypes* in two different soils as covariables. Scores of both environmental parameters (mineral Ag levels and silver in biomass) are represented by *large arrows*. Scores of fungal ribotypes are represented by *small triangles*

importance only (the scores of mineral and organically bound Ag have similar direction).

In contrast to the bacterial ribotypes seen in Fig. 1, the fungal ribotypes tended to avoid the samples with lower level of mineral Ag (Fig. 3). The ribotypes preferring Kalešov soil seemed to be more tolerant to low Ag dosis than ribotypes preferring Chuchle soil (the left-targeting scores tend to avoid the Ag1 silver level). Figure 4 shows that the fungal ribotype preferences are almost uniformly distributed with respect to the level of Ag applied, some ribotypes preferring the low dose and some other the high Ag dose. Similarly as in the case of biplot of bacterial ribotypes, the scores of mineral and organically bound Ag of the same dose have similar direction which indicated that both Ag forms affected the fungal community in a similar manner.

When the effects of the applied Ag and soil origin were subtracted as covariables, the slight effect of fungal biomass visible in Figs. 2 and 4 as differences in direction of scores of mineral and organically bound Ag was significant by Monte-Carlo test (Table 1). This indicates that the addition of organic matter itself slightly affects the communities of soil fungi and bacteria.

Discussion

The results of our experiments indicate that soil microbial communities are sensitive to the addition of small amounts of Ag-rich fruit-body biomass of *A. solitaria*. As the effects of Ag-rich fruit-body biomass was very similar to the effects of mineral Ag alone, we attribute the main changes of the microbial communities to Ag released from the decomposing fruit-body biomass.

High or zero doses of mineral Ag resulted in a depauperate bacterial community and an enriched community of soil fungi. The bacterial ribotypes thus tended to be more abundant in the treatment receiving low doses of mineral Ag. This is the exactly opposite trend than that observed in the case of soil fungi, suggesting that fungi are replaced by the bacteria if traces (sublethal concentrations) of mineral Ag are applied.

However, high Ag concentration was preferred by the fungal community, suggesting that fungi are mostly more tolerant to Ag than bacteria. This preference is independent of the form (mineral or bound) of silver applied. Whereas the bacterial ribotypes tended to be less abundant in both treatments with high Ag doses, fungal ribotypes were positively correlated not only with low but also with high Ag doses. This may reflect different sensitivity of eukaryotic and prokaryotic microorganisms to Ag as demonstrated by Pshennikova et al. (2011) and may be due to the release of Ag ions and/or possibly Ag nanoparticles from fungal biomass, with more distinct antibacterial activity compared to antifungal activity (Kathiresan et al. 2010).

Similar behavior of organically bound Ag and mineral Ag in our experiment is interesting. Biologically bound Ag may be present in fungal biomass in the form of a complex with an organic molecule such as metallothionein as demonstrated in *A. strobiliformis* (Osobová et al. 2011) but possible occurrence of Ag nanoparticles cannot be omitted (Gade et al. 2010); their significant effects on the leaf litter decomposing aquatic microbial community was observed (Pradhan et al. 2011). In this case, the strong inhibitory effects of silver nanoparticles on growth of bacterial biomass was observed and sporulation ability of the fungal community was severely reduced.

The fate of applied mineral Ag is questionable but some might precipitate chloride ion as AgCl, leaving low concentrations of free Ag cations in the soil solution. Interestingly, the Ag nanoparticles could be formed in soil directly from the Ag cations under the reductive effects of humic substances and degraded under oxidative conditions (Akaighe et al. 2011). This indicates that different forms of soil Ag are interconvertible, which may explain similar effects of mineral and organically bound Ag in our experiment, as any soluble form of the metal can be converted to any other form.

Even though our experiment was performed under laboratory conditions in vitro, the results suggest that fruit-bodies of fungi hyperaccumulating Ag may strongly affect the soil microbial communities under natural conditions. The effect of the heavy element itself need not necessarily consist in direct toxicity to particular components of the community. Its sublethal concentrations may affect the fitness of the community components, their competitiveness and consequently also their relative abundance (Kumar et al. 2011).

As the fruit-bodies of many macrofungal species (Ag-hyperaccumulating *Amanita* and *Agaricus* spp.) concentrate considerable levels of Ag, they represent Ag hot-spots where significant amounts of Ag are concentrated by the transport activity of mycelium from large soil volumes. Primarily in common soils with low Ag concentration, silver released from rotting fruit-bodies might significantly affect the soil biota and increase non-homogeneity in spatial distribution of the microbial communities, as well as their species richness.

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