Biol Fertil Soils (2005) 41: 439–446
DOI 10.1007/s00374-005-0849-4

ORIGINAL PAPER

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Diversity of the ectomycorrhiza community at a uranium mining heap

Received: 30 September 2004 / Revised: 7 February 2005 / Accepted: 12 February 2005 / Published online: 24 March 2005

Abstract Ectomycorrhiza (EM) community structure was analyzed at one bare heap site (BHS), one heap site with organic cover (HS-OH) and one reference site (RS) in the former uranium mining area near Ronneburg (Thuringia, Germany). Twenty-three EM morphotypes were distinguished, and 14 of them were additionally characterized by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and internal transcribed spacer (ITS) sequence analysis. Colonization of birch by the different morphotypes was quantified, and the EM diversity at the different sites was investigated. Compared to RS, total EM colonization was reduced by 6% (P=0.851) at HS-OH and by 58% (P<0.001) at BHS. Likewise, EM diversity was reduced by 16% (P=0.229) at HS-OH and 52% (P<0.001) at BHS. The Sørensen similarity between EM samples from RS was nearly independent from the sampling date, whereas at HS-OH and especially BHS, the Sørensen similarity decreased with increasing time between the samplings. All EM fungal species dominating at the two heap sites were also present at RS. Thus, fungi with high tolerance against uranium and other stress factors at the heap sites (e.g. heavy metals, nutrient limitation, drought) were selected among all EM fungi of the area. Highly adapted fungi with a distribution restricted to the contaminated soils were not detected.

Keywords *Betula pendula* · Ectomycorrhiza · ITS analysis · Morphotyping · Uranium mining

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00374-005-0849-4.

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Introduction

One of the largest uranium ore mining areas worldwide was located in the former German Democratic Republic near Ronneburg (Thuringia, Germany) where 220,000 t of uranium was produced between 1946 and 1990. The mining was stopped in 1990, leaving a total plant area of ~37 km^2 with a multitude of shafts, sludge ponds, mine waste heaps and a big hole left over from opencast mining (Hambeck et al. 1996).

The mining heaps at Ronneburg are extreme sites for plant growth. The deposited low-grade black schist ore contains up to 0.05% uranium (Schippers et al. 1995), but also elevated concentrations of heavy metals like arsenic, copper, molybdenum, nickel and vanadium (Lange and Freyhoff 1991). Dryness, high temperatures at the sun-exposed slopes and limited access to mineral nutrients are additional stress factors. Under such conditions, mycorrhizal symbioses play an important role for plant establishment, growth and nutrition (Smith and Read 1997). Enhanced tolerance of mycorrhizal plants of heavy metal stress has been demonstrated for both arbuscular mycorrhiza (AM) (Gildon and Tinker 1983; Hildebrandt et al. 1999) and ectomycorrhiza (EM) (Jentschke and Godbold 2000). Arbuscular mycorrhizal fungi can take up and transport uranium under root-organ culture conditions (Rufyikiri et al. 2002), but the role of mycorrhizal symbioses in uranium-contaminated soils is unclear.

Whereas first steps have been taken to characterize AM in heavy-metal-contaminated soils (Turnau et al. 2001), studies on the influence of heavy metals, including uranium or other radionuclides, on the below-ground EM diversity are missing, although techniques for the analysis of EM communities have been developed (Buscot et al. 2000).

The objective of this case study was the characterization of birch EM at a uranium mining heap of the Ronneburg region in order to elucidate the influence of the extreme environmental factors on EM diversity. Three hypothetical EM communities might be expected: (1) a community with fungal species adapted and restricted to the heap conditions, comparable to the typical plant communities of central European heavy metal soils; (2) a community with reduced EM diversity due to selection of tolerant species among the EM fungi of the area; and (3) a community without significant differences to reference sites.

Material and methods

Experimental sites and soil analysis

Three sites in the Gessenbachtal (50°51′N, 12°09′E) within the former uranium mining area Ronneburg (Thuringia, Germany) were investigated. Two sites were parts of a heap deposited 1958 to 1970 and revegetated by planting birches (*Betula pendula* Roth) and some aspens (*Populus tremula* L.). Additionally, a few *Quercus* sp. and *Salix* sp. had established spontaneously. Towards the top of the heap, a bare heap site (BHS) was selected, characterized by the absence of an organic horizon. A heap site with organic horizon (HS-OH), 1–5 cm thick, was located about 50 m apart. A natural site at a distance of 500 m from the heap, covered by an organic horizon comparable to HS-OH, was selected as reference site (RS), which was dominated by birch in a mixed stand with oaks and a few other trees. For a further description of the Ronneburg area, including mining history and current vegetation processes, see Sänger and Jetschke (2004).

Three soil samples from each heap site and two samples from RS were collected from a depth of 0–10 cm (~1 kg each) and air dried. Fresh litter above the rhizosphere was removed, whereas the decomposing organic material at HS-OH and RS was included. Soil pH was determined after stirring 10 mg of the air-dried samples in 25 ml, 0.01 M $CaCl_2$ for 1 h. The total metal content of soil samples dried at 105°C was determined by X-ray fluorescence analysis with a SPECTRO X-LAB 2000 spectrometer (Spectro, Kleve, Germany) as described by Schmid and Wiegand (1998).

Sampling, EM morphotyping and quantitative analysis of EM community structure

Ectomycorrhizas were collected as described by Kaldorf et al. (2002), excluding roots from other plants by tracing birch roots from the trunks. Four samples, each consisting of one root segment (50 cm long, diameter 5 mm) together with the connected rootlets and EM, were collected at each site in May, July and September 2001, respectively. To differentiate tree vs site-specific effects, the 12 samples from each site originated from six trees sampled in duplicate, with both of the duplicate samples taken at the same date. The distance between double samples was at least 1 m to obtain spatially independent samples.

Morphotyping of EM was done as described by Agerer (1991). EM color, surface structure and shape were observed under a dissecting microscope, whereas hyphal mantle structures, cystidia and emanating hyphae were described based on hand sections, using a Zeiss Axioplan light microscope at 400–1,000× magnification. For quantitative EM community analyses, ~300 EM from each sample were classified. The mycorrhization rate was defined as the percentage of EM presence among all root tips. Root tips were classified as ectomycorrhizal only when a well-developed hyphal mantle was present, excluding uncolonized, weakly colonized and apparently degenerated roots. The Shannon–Wiener index of diversity (Shannon and Weaver 1949) was calculated to estimate EM diversity, whereas the Sørensen (1948) index was used to compare the similarity of EM communities.

DNA analysis

Genomic DNA was isolated after homogenization of single EM (fresh weight 0.1–1 mg) in 100 µl of CTAB extraction buffer, following the protocol of Doyle and Doyle (1990). Purified DNA was dissolved in 100 µl H_2O and stored at 4°C. Amplification of fungal is nuclear ribosomal internal transcribed spacer (ITS) regions by polymerase chain reaction (PCR) using the primers ITS1 and ITS4 (White et al. 1990) was performed as described previously (Kaldorf et al. 2004). Restriction fragment length polymorphism (RFLP) patterns were generated by digestion of 4 µl PCR product each with *Alu*I, *Eco*RI, *Bsu*RI, *Hinf*I or *Msp*I, respectively (all from MBI Fermentas, St. Leon-Rot, Germany). Restriction fragments were separated by electrophoresis on 2% agarose gels at 10 V/cm. Cloning of PCR products was performed with the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany), following the manufacturer's protocol. Sequencing was done on a LI-COR DNA Sequencer Long Reader 4200 using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia, Little Chalfont, England). The BLASTN program (Altschul et al. 1997) was used to compare sequence data with the GenBank database.

Results

Heavy metal contents of the experimental sites

Soil analyses (Table 1) revealed elevated concentrations of several heavy metals at the reference site (RS). Beside

Table 1 Element content and pH in soils of three sites located in the uranium mining area close to Ronneburg (Thuringia, Germany) in mg/kg (mean±SD), as measured by X-ray fluorescence analysis

Elements	Bare heap site	Heap site with organic horizon	Reference site	Limits for non-elevated element content in soil
Antimony	25.2±13.6	6.1±4.8	8.1±1.3	2
Arsenic	55.0±19.8	24.0±14.3	19.5±1.3	20
Bismuth	<0.8	<0.8	<0.8	1
Cadmium	0.5±0.6	0.4±0.3	0.2±0.1	0.7
Cesium	51.9±25.4	15.7±4.7	22.5±2.6	–
Chromium	134.6±28.4	75.9±8.9	132.0±2.4	100
Cobalt	38.3±6.2	47.2±16.6	35.0±9.3	15
Copper	104.3±67.3	63.5±15.0	164.0±0.85	35
Lead	31.1±6.7	26.6±6.7	61.7±18.7	40
Mercury	<2	<2	<2	0.1
Molybdenum	21.8±12.3	3.2±4.7	0.5±0.1	5
Nickel	81.1±40.2	48.1±9.4	56.7±4.5	50
Selenium	2.8±1.9	0.3±0.2	0.7±0.2	1
Silver	<0.4	<0.4	<0.4	1
Tellurium	5.6±2.7	0.5±0.6	2.0±0.4	–
Uranium	27.1±16.6	13.0±5.6	3.8±0.8	1
Vanadium	393.9±92.7	158.6±81.3	216.5±11.2	35
Zinc	103.1±42.5	90.2±10.8	87.1±1.4	100
Soil pH	3.5±0.7	4.3±0.6	3.7±0.1	

Limits for non-elevated element contents after Fiedler and Rösler (1993)

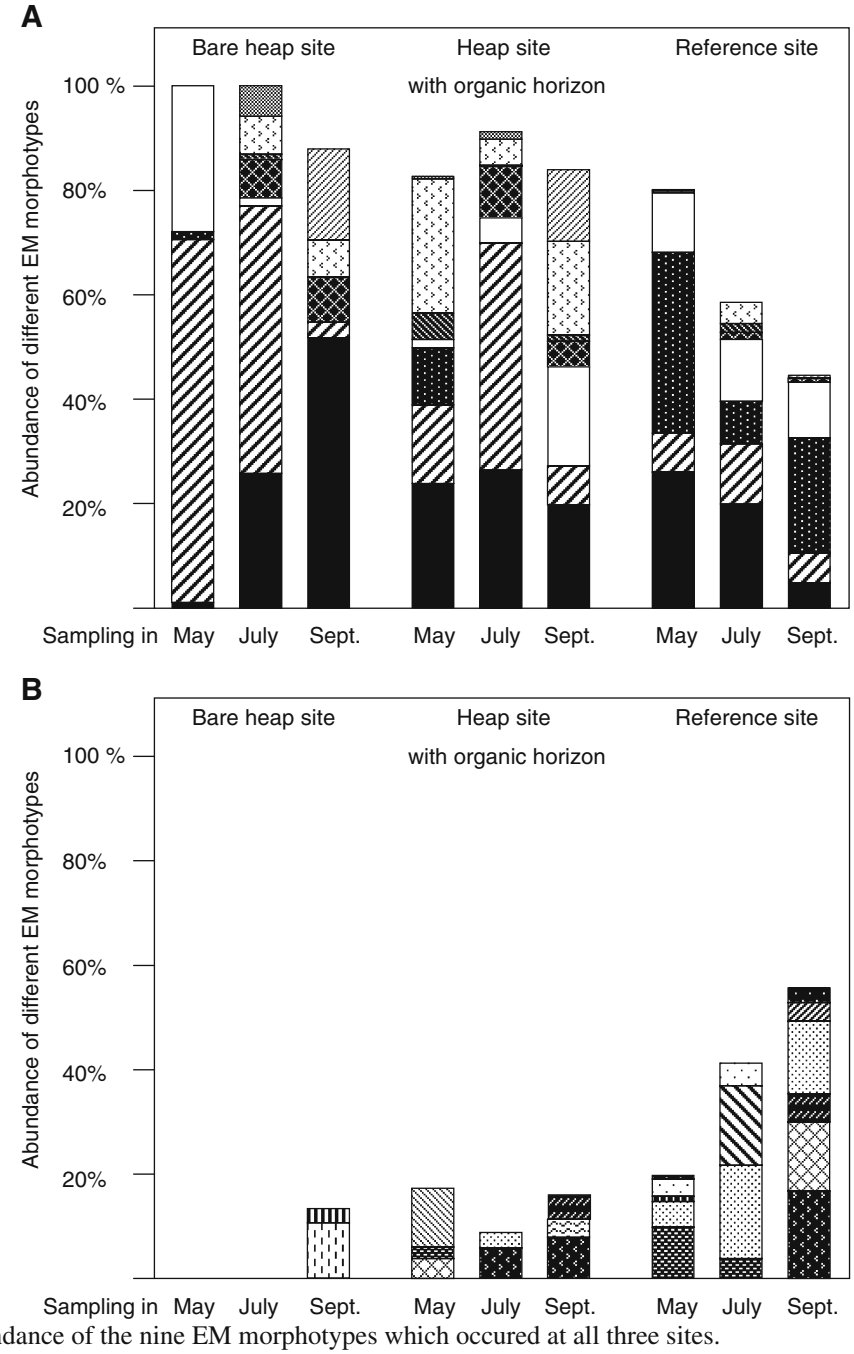
antimony, chromium, cobalt, copper, nickel, vanadium and zinc, even the uranium content at RS was above the limits for soils with normal element contents (Fiedler and Rösler 1993). Highest concentrations of uranium and most other heavy metals with the exception of cobalt and lead occurred at the bare heap site (BHS). The heavy metal concentrations in the substrates from the heap site with organic horizon (HS-OH) and RS were in the same range and lower compared to BHS.

Morphotyping and identification of birch EM

Twenty-three different EM morphotypes of birch growing at the experimental sites were described (Electronic supplementary material, Table S1) that could be divided into four separate groups. Group A contained white to brownish morphotypes with hydrophilic hyphal mantles and few or missing emanating hyphae. Within this group, EM A24 was easy to differentiate based on its color and mantle surface, whereas EM A4, EM A8 and EM A17 could only

Table 2 Identification of the mycobionts of birch (*Betula pendula* Roth) ectomycorrhiza morphotypes from a uranium mining heap and from the corresponding reference site, located in the Ronneburg uranium mining area

Morphotype	Accession number	Best BLAST hit (accession number, % identity)	Classified as
EM A4	AJ549961	*Lactarius fulvissimus* AF204679, 95%	*L. decipiens*
EM A8	AJ549962	*Russula velenovskyi* AY061721, 96%	*Russula* sp.
EM A24	AJ549963	*Russula ochroleuca* AF418617, 98%	*Russula* sp.
EM B6	AJ549964	*Amanita muscaria* AB080983, 97%	*A. muscaria*
EM B11 (I)	AJ549965	*Cortinarius alboviolaceus* AF325597, 97%	*Cortinarius* sp.
EM B11 (II)	AJ549966	*Tricholoma muricatum* AF458438, 97%	*Tricholoma* sp.
EM B19 (I)	AJ549967	*Hebeloma incarnatulum* AF430291, 98%	*Hebeloma* sp.
EM B19 (II)	AJ549968	*Cortinarius atrocoeruleus* AY083178, 96%	*Cortinarius* sp.
EM B22	AJ549969	Thelephoraceae sp. AF184742, 97%	Thelephoraceae
EM B23	AJ549970	*Tuber* sp. AJ534705, 96%	*Tuber* sp.
EM C9	AJ549971	Thelephoraceae sp. AF184743, 98%	Thelephoraceae
EM C16	AJ549972	*Thelephora terrestris* U83486, 97%	*Thelephora* sp.
EM D1	AJ549973	Ectomycorrhizal isolate AJ410863, 95%	–
EM D2	AJ549974	*Phialophora finlandia* AJ534704, 98%	*Phialophora* sp.
EM D5	AJ549975	*Cenococcum geophilum* AY112935, 97%	*C. geophilum*
EM E7	AJ549976	*Hebeloma edurum* AF124698, 98%	–



A: Abundance of the nine EM morphotypes which occured at all three sites.

Phialophora sp. EM D2, *Cenococcum geophilum* EM D5, EM E7,
Lactarius decipiens EM A4, *Russula* sp. EM A8, *Thelephora* sp. EM C16,
EM B11, Thelephoraceae EM B22, *Amanita muscaria*, EM B6

B: Abundance of the 14 EM morphotypes restricted to one or two of the three sites.

Tuber sp. EM B23, *Russula* sp. EM A24, EM E12, EM C14,
Thelephoraceae EM C9, EM B19, EM D1, EM C25,
EM C21, EM D18, EM C20, EM C26, EM D3, EM A17

Fig. 1 Abundance of different EM morphotypes in percent of the total EM at two uranium mining heap sites near Ronneburg (Germany) compared to a reference site

be differentiated by microscopic features of their hyphal mantle and emanating hyphae. Group B summarized silvery morphotypes with hydrophobic hyphal mantles and included all morphotypes with rhizomorphs. In group B, only EM B22 and EM B23 were easy to define, whereas EM B6, EM B11 and EM B19 were difficult to differentiate due to transitional expression of some characters. The common character of group C was the presence of cystidia. EM C9, EM C25 and EM C26 were defined by the shape of their cystidia, whereas the other four morphotypes had similar, needlelike cystidia. EM C14 and EM C20 were recognized based on their hyphal mantle structure. Two morphotypes of group C, EM C16 and EM C21, were not defined by a single character, but only by the combination of branching patterns and the frequency of emanating hyphae and cystidia, making their classification difficult. All five morphotypes of group D were black or dark grey and could unequivocally be recognized, based on the presence or absence of clamps and on the hyphal mantle structure. Two morphotypes did not fit into the four groups. EM E12 was clearly defined, whereas EM E7 summarized brown, branched EM without cystidia and a poorly developed hyphal mantle, which showed no further distinctive characters.

The ITS regions were successfully amplified by PCR from single EM for 14 of the 23 morphotypes. The RFLP patterns of morphotypes EM A4, EM B6, EM B22, EM B23, EM C9 and EM D2 were reproducible, whereas only one PCR product was obtained for EM A8, EM A24, EM C16, EM D1, EM D5 and EM E7, respectively. Two PCR products with different RFLP patterns were found for EM B11 and for EM B19. All PCR products were cloned and sequenced to identify the corresponding mycobionts (Table 2). The original RFLP patterns matched those deduced from the sequences in all cases except EM E7. The sequence-based identification of three morphotypes was confirmed at the species level, either by finding fruit bodies and EM with identical RFLP patterns (*Amanita muscaria*=EM B6 and *Lactarius decipiens*=EM A4) or by morphological and anatomical characters of the mycorrhizas (*Cenococcum geophilum*=EM D5).

Comparison of the EM communities at the different sites

Analysis of the EM community compositions within each of the three sites revealed no significant differences between individual trees. The similarity between double samples from one tree was 66±20% (mean of Sørensen similarity index values±SD, n=17), being only marginally above the Sørensen similarity of 62±15% (n=34) recorded for samples from different trees at the same site. Thus, the duplicate samples taken from the same tree could be considered as spatially independent.

Irrespective of the presence of an organic soil horizon, the four EM morphotypes *Phialophora* sp. EM D2,

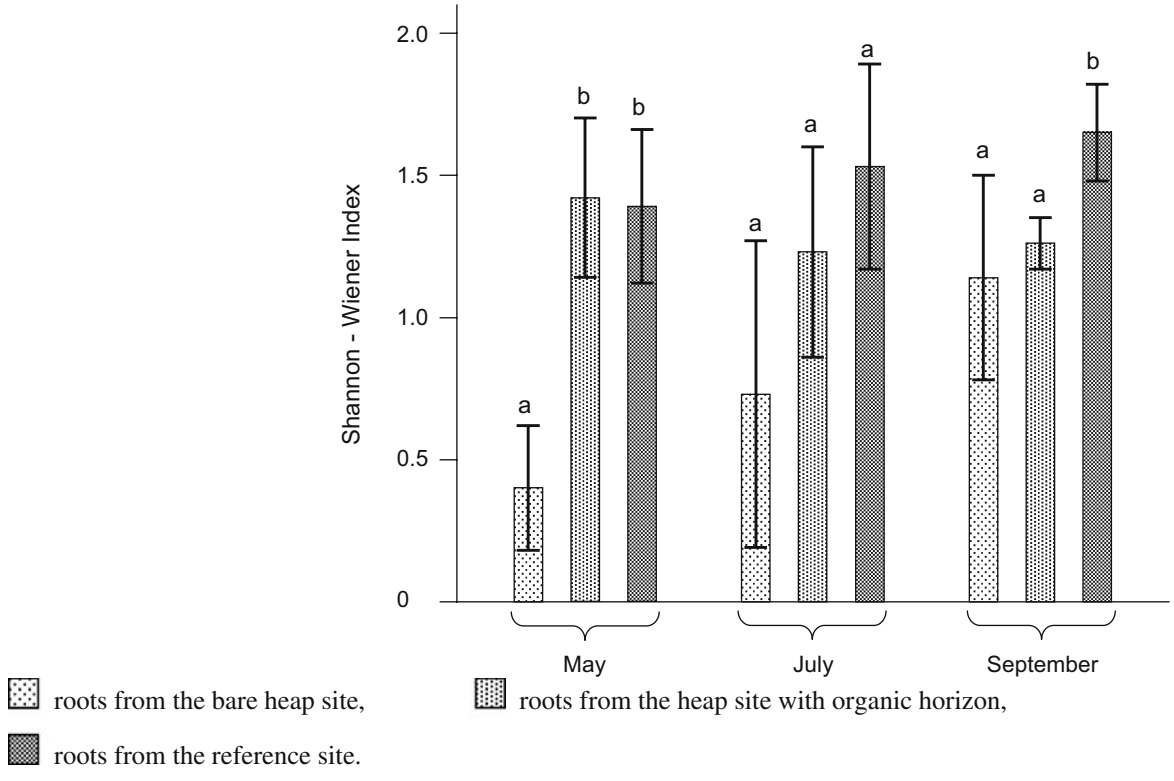


Fig. 2 Ectomycorrhiza (*EM*) diversity at two uranium mining heap sites near Ronneburg (Germany) compared to a reference plot. For each root sample, the Shannon–Wiener index of diversity was calculated, based on the EM morphotyping. Each *column* represents the mean±SD of the Shannon–Wiener index values of the four root samples collected at one site at the same date. Within each sampling date, different *letters* above the *columns* indicate significant differences at P<0.05 (one-way ANOVA, Tukey's test)

L. decipiens EM A4, *A. muscaria* EM B6 and EM B11 dominated at the uranium mining heap. Together, these morphotypes added up to 62–99% of all EM at both heap sites for each of the three samplings. Each of these four morphotypes was also found at RS, but with a lower abundance of only 21–48% (Fig. 1a). Although both heap sites were dominated by the same four morphotypes, the EM community at BHS differed considerably from the ones at HS-OH and RS for all quantitative and qualitative parameters investigated. Mycorrhization rate (see Electronic supplementary material, Table S2) was only 27.7±18.6% at BHS, whereas HS-OH (61.5±24.8%) and RS (65.5±12.5%) displayed significantly higher rates (n=12 root samples for all sites, P<0.01%, Student's t-test). The lower mycorrhization rate at BHS was coupled to a reduced EM diversity. From the 23 morphotypes, only 11 were observed at BHS. Nine of these morphotypes were found at all three sites (Fig. 1a), whereas EM A24 and EM B23 were restricted to BHS. In contrast, 16 and 19 morphotypes were detected at HS-OH and RS, respectively. Two morphotypes were only found at HS-OH, and five morphotypes occurred exclusively at RS (Fig. 1b). The quantification of EM diversity with the Shannon–Wiener index confirmed that the EM diversity was lowest at BHS, intermediate at HS-OH and highest at RS (Fig. 2). Significant reductions of EM diversity were found in May (BHS compared to HS-OH and RS) and in September 2001 (BHS and HS-OH compared to RS).

Although both the total number of different morphotypes and the EM diversity within root samples were reduced at BHS, the similarity between the 12 samples from BHS (quantified by the Sørensen index, Electronic supplementary material, Fig. S1) was the lowest. It was intermediate at HS-OH and the highest at RS. Comparing samples from one site collected at the same date, the mean of the Sørensen indices was in the same range for the three sites (Table 3). Thus, the spatial heterogeneity in the EM communities was about the same at all sites. Even when samples from different dates were compared, Sørensen indices for RS differed not significantly, whereas the similarity between samples was reduced at both heap sites. The lowest similarity was found between the samples taken at BHS in May vs September, being significantly below the other Sørensen index values from BHS (Table 3) and indicating a high temporal variation within the EM community at BHS.

Table 3 Similarity of birch EM community compositions in root samples from two uranium mining heap sites and one reference site within the former uranium mining area close to Ronneburg (Thuringia, Germany)

Samples from	Bare heap site	Heap site with organic horizon	Reference site
The same date	63.6±18.3 a	61.5±18.4 a	64.6±13.9 a
May vs July	45.1±16.4 b	41.5±13.3 b	63.3±16.2 a
July vs September	42.5±11.6 b	51.5±17.0 ab	53.7±14.4 a
May vs September	10.1±15.5 c	42.4±14.9 b	61.8±12.3 a

Means±SD of the Sørensen index values, obtained by pairwise comparison of the EM morphotype composition in root samples collected in May, July and September 2001 (see Fig. S1), are given. Within columns, values with different letters are significantly different at P<0.05 (one-way ANOVA, Tukey's test)

Discussion

The combination of morphotyping and PCR-based rDNA analysis is well established for the characterization of EM communities (Horton and Bruns 2001). In several studies, molecular methods revealed that apparently uniform EM morphotypes were in fact heterogeneous (e.g. Kårén and Nylund 1997; Pritsch et al. 1997). Three factors contribute to such observations: (1) it is often difficult to differentiate similar EM by morphotyping; (2) one root tip may be colonized by two EM fungi; and (3) the presence of DNA from soil fungi in EM samples can lead to additional contaminating PCR products. In the present work, the molecular data indicated that three morphotypes (EM E7, EM B11 and EM B19) in fact summarize EM formed by different fungi, whereas EM C16 and EM C21 represent different developmental stages of the same EM. The remaining 18 morphotypes represented distinct fungal species that could be recognized unambiguously in the field samples. Thus, the characterization of EM by morphotyping and molecular methods provided a good basis for the comparison of EM communities in the Ronneburg uranium mining area.

Several studies exist on the role of EM and AM in contaminated soils with toxic heavy metal concentrations (e.g. Colpaert et al. 2000; Kaldorf et al. 1999). Compared to these studies, BHS was characterized by moderately enhanced concentrations of up to 46 mg U/kg soil in one BHS sample, which is by far below the limit for plant growth inhibition of 300 mg U/kg soil (Ebbs et al. 1998). However, ecotoxicology of uranium could have been enhanced by additional stress factors, e.g. other heavy metals, low pH, poor nutritional status and high fluctuations in temperature and moisture. Under these unfavorable conditions, a reduced mycorrhization rate and EM diversity were observed at BHS. As the majority of the root tips without fully developed EM were apparently degenerated or dead, the low mycorrhization rate may reflect a rapid turnover of EM rather than a reduced formation of EM at BHS. This interpretation is supported by the high temporal variation at BHS shown by the Sørensen indices. Faster replacement of fungal mycelium and a decreased life span of EM under heavy metal stress have been described previously (Colpaert and van Assche 1993; Turnau et al. 2002) and may be typical for EM in heavy-metal-polluted soils.

An important question for the use of EM fungi in restoration or phytoremediation projects is whether EM communities at contaminated sites are formed by site-adapted EM species. Among the 23 birch EM morphotypes described in this study, 12 and seven were not found at BHS and HS-OH, respectively, while only four were missing at RS. Each of these four morphotypes was only found in a few samples taken at one single date, whereas all mor-

photypes dominating at the heap sites were present also at RS. This distribution pattern of EM morphotypes is in general correspondence with hypothesis (2) mentioned in Introduction that EM fungi able to tolerate uranium contamination, heavy metal contamination, acidic soil conditions and high microclimate fluctuations were selected among EM fungi present in the area. The contrasting hypothesis (1), according to which the EM community at the uranium mining heap might be dominated by fungal species comparable to the metallophytes among plants, which means highly adapted species with a distribution restricted to contaminated soils, is not supported by the data of this study. Considerable differences in heavy metal tolerance of EM fungi, as the most plausible explanation for the reduced EM diversity at the uranium mining heap, have been reported in many studies at the species, strain or ecotype levels (Hartley et al. 1997 and references therein). For example, *A. muscaria* and *Pisolithus tinctorius* were able to tolerate high concentrations of different heavy metals under laboratory conditions (Hartley et al. 1997). Both fungi can form EM on birch, and fruit bodies of these species were found during the fieldwork (data not shown), either at BHS (*Pisolithus*) or at RS (*Amanita*). However, no *Pisolithus* EM were detected, whereas EM formed by *Amanita* were present at all three sites with similar abundance.

In the present study, the analysis of EM community structures was performed at the species level. Laboratory studies on fungal isolates might reveal uranium and heavy metal tolerant strains at the heap site, as demonstrated for fungi isolated from heavily polluted sites (Colpaert et al. 2000, 2004). Further insight into functional aspects of EM symbioses in soil might be gained by using modern molecular biological techniques (e.g. gene expression profiling with DNA microarrays, Gibson 2002) on EM field samples.

Acknowledgements This work was kindly supported by a grant from the Deutsche Forschungsgemeinschaft (Geschäftszeichen Nr. Bu 941/5-1). The authors would like to thank Dr. J. Wiegand for the soil analyses and Dr. G. Walther for the identification of fruit bodies.

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