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C. Tonin · P. Vandenkoornhuyse · E.J. Joner
J. Straczek · C. Leyval

Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover

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Abstract The ability of arbuscular mycorrhizal (AM) fungi from a metal-tolerant plant (*Viola calaminaria*, violet) to colonise and reduce metal uptake by a non-tolerant plant (*Trifolium subterraneum*, subterranean clover) in comparison to a metal-tolerant AM fungus isolated from a non-tolerant plant was studied. AM spores from the violet rhizosphere and from violet roots were characterised by polymerase chain reaction (PCR) amplification of the SSU rDNA, and sequencing. Subterranean clover was grown in pots containing a soil supplemented with Cd and Zn salts and inoculated either with a mixture of spores extracted from the violet rhizosphere or with spores of a Cd-tolerant *Glomus mosseae* P2 (BEG 69), or non-inoculated. The diversity of fungi, including AM fungi, colonising clover roots was assessed and analysed using terminal-restriction fragment length polymorphism. At least four different *Glomus* species were found in the violet rhizosphere. After 8 weeks in a growth chamber, colonisation of clover roots with spores from the violet rhizosphere increased Cd and Zn concentrations in clover roots without significantly affecting the concentrations of metals in the shoot and plant growth. *G. mosseae* P2 reduced plant growth and slightly increased the Cd concentration. Only one AM fungus (*Glomus* b) from the violet rhizosphere colonised clover roots, but other fungi were present. AM fungi from heavy metal-contaminated soils and associated with metal-tolerant plants may be effective in accumulating heavy metals in roots in a non-toxic form.

Keywords Arbuscular mycorrhizal fungi · *Viola calaminaria* · Terminal-restriction fragment length polymorphism · Heavy metals · SSU rDNA

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C. Tonin · P. Vandenkoornhuyse · E.J. Joner · C. Leyval (✉)
Centre de Pédologie Biologique,
UPR 6831 du CNRS associée à l'Université Henri Poincaré, Nancy I, B.P. 5,
54501 Vandoeuvre-les-Nancy Cedex, France
e-mail: leyval@cpb.cnrs-nancy.fr
Fax: +33-3-83576523

J. Straczek
Laboratoire de Biochimie, CHRU de Nancy,
54035 Nancy Cedex, France

Introduction

Heavy metals in soils can exert an adverse effect on microorganisms and microbial processes (McGrath et al. 1995). Among soil microorganisms, mycorrhizal fungi are the only ones providing a direct link between soil and roots. Mycorrhizal symbioses and fungi occur in almost all habitats and climates (Read 1991; Barea et al. 1997), including disturbed soils (Bundrett et al. 1996). In some heavily polluted mining areas, no inhibition of mycorrhizal root colonisation was observed (Shetty et al. 1994). Arbuscular mycorrhizal (AM) fungi have been shown to improve plant tolerance to heavy metal stress in polluted soils (Leyval et al. 1997). Heavy-metal-tolerant AM fungi have been isolated from polluted soils (Gildon and Tinker 1983; Weissenhorn et al. 1993) and were reported to bind heavy metals (Joner et al. 2000). The alleviation of metal toxicity to plants by AM fungi could contribute to plant metal tolerance on polluted sites. Mechanisms involved in metal tolerance of AM fungi and its stability are still poorly known (Leyval et al. 1997). Most studies have used AM fungi isolated from moderately polluted soils, where heavy metal concentrations permit growth of non-adapted plants, such as maize or clover (Leyval et al. 1997). On highly polluted soils where only adapted plants can grow, mycorrhizal fungi may be associated with metallophyte plants, such as *Viola calaminaria*. This yellow violet is described as an absolute metallophyte plant, which usually colonises Zn- and Pb-rich soils, and accumulates remarkably low levels of metals or none at all,

despite the elevated levels of metals in these soils (Baker 1981). A *Glomus* sp. isolated from the roots of this violet improved maize growth in a polluted soil (Hildebrandt et al. 1999) and reduced root and shoot heavy metal concentrations in comparison to a common *Glomus* isolate or to non-colonised controls (Karldorf et al. 1999).

Only one or a few AM fungi have been considered in most of the studies on heavy metals and mycorrhizas. However, to understand the interactions between heavy metals, AM fungi and plants, it is necessary to study and compare the diversity of AM fungi in heavy metal-polluted and unpolluted soils, when associated with metal-tolerant and non-tolerant plants. Del Val et al. (1999a) showed that AM fungal spore numbers, species richness and diversity depend on the level of contamination of a soil amended with sewage sludge, and that host plant species exert a selective influence on AM fungal population size and diversity. Van der Heijden et al. (1998) showed that AM fungal diversity is a major factor for the maintenance of plant diversity within natural ecosystems.

Molecular techniques, particularly those that utilise the polymerase chain reaction (PCR) to amplify DNA, are more useful than cultures or direct observation for assessing diversity (Alleman et al. 1999; Chelius and Triplett 1999). During the last decade, molecular approaches have been developed for the identification of AM fungi and to analyse their diversity, such as PCR on 18S rDNA (Simon et al. 1993), 25S rDNA (van Tuinen et al. 1998), use of internal transcribed spacer sequences (Lloyd-McGilp et al. 1996) and PCR fingerprinting (Vandenkoornhuyse and Leyval 1998). These methods have permitted the detection of mycorrhizal fungi directly in soil and roots by using specific probes (Claassen et al. 1996; Moutoglis 1997; Chelius and Triplett 1999).

Among molecular methods, terminal-restriction fragment length polymorphism (T-RFLP) appears to be a rapid and good method with which to compare and study soil microbial communities (Clement et al. 1998; Marsh 1999). DNA is amplified and fluorescently labelled by PCR. The labelled products are digested with a restriction enzyme and the end-labelled terminal restriction fragments (TRFs) are separated by electrophoresis and detected on an automated DNA sequencer.

The objectives of this work were to study: (1) the diversity of AM fungi in the rhizosphere of a metal-tolerant plant, *V. calaminaria*, (2) the ability of these AM fungi to colonise the roots of a non-metal-tolerant plant, *Trifolium subterraneum* (subterranean clover), and to reduce metal uptake by this plant, (3) to compare these fungi with a Cd-tolerant *Glomus mosseae* (Weissenhorn et al. 1993) with respect to the stimulation of growth of clover. PCR on 18S rDNA and sequencing were used to identify the different AM fungi associated with *V. calaminaria*. Clover was grown in a soil supplemented with heavy metals (Cd, Zn) and was inoculated either with AM fungi from the violet rhizosphere, with the Cd-tolerant *G. mosseae* or non-inoculated. T-RFLP analyses were performed on 18S rDNA extracted from clover roots to estimate fungal diversity and possibly identify AM fungi from the violet rhizosphere.

Materials and methods

Experimental design

Violet plants [*Viola calaminaria* (Gingins) Lej. according to Lambinon and Auquier (1963)] were sampled from Plombières soil (Belgium) (Simon 1978), highly contaminated with heavy metals, particularly Zn and Cd [exchangeable Cd and Zn according to Tessier et al. (1979), 7.1 and 934 mg kg^{-1}; total Cd and Zn concentrations, 41 and 20961 mg kg^{-1}, respectively, J. Colpaert, personal communication], and provided by J. Colpaert (Limburgs Universita Centrum, Belgium). Trap cultures of AM fungi colonising violet roots were grown for 6 months on Plombières soil mixed (1/1, v/v) with sterile Terragreen (oil dry US special type III R; Laporte Absorbents Europe, France). Spores were isolated from the violet rhizosphere and used to inoculate clover seedlings.

A gleyic luvisol was collected from an experimental farm (La Bouzule, Champenoux, France), partially sterilised using gamma irradiation (25 kGy), and mixed with sterile sand (30% fine sand, 30% coarse sand). Soil characteristics are given in Leyval and Binet (1998). Solutions with different levels of $CdSO_4 \cdot 8H_2O$ and $ZnSO_4 \cdot 7H_2O$ (Cd/Zn: 0/0, 2/200, 5/500, 20/2000 ppm, respectively) were added to the soil, placed in 1-l pots.

The design of the experiment was a 3×4 factorial with two different AM inocula and a non-mycorrhizal control, combined with four levels of Cd/Zn addition and three replicates per treatment. A mixture of spores associated with *V. calaminaria* was extracted from the violet rhizosphere using a sucrose gradient, according to Walker et al. (1982), as modified by Vandenkoornhuyse (1998). Roots collected from the same rhizosphere soil sample were dissected and spores collected with fine forceps. Spores of a Cd-tolerant *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe P2 (BEG69), originating from a moderately metal-contaminated soil (Weissenhorn et al. 1993; Weissenhorn and Leyval 1995) and subcultured on leek on the original soil, were used as the second AM inoculum. Before germination, clover seeds (*Trifolium subterraneum* L. var. Mount Baker) were disinfected in 30% H_2O_2 for 20 min, and washed 3 times in distilled water. Two pre-germinated seeds were transplanted to each pot and inoculated with approximately 200 spores (containing all the morphotypes in the same proportion as found in the violet rhizosphere) placed directly on the roots. Clover and violet pots were kept in a growth chamber (24/20 °C day/night, 80% relative humidity, 200–300 $\mu mol\ s^{-1}\ m^{-2}$ photosynthetically active radiation, 18-h day). Plants were watered daily to 60% of water holding capacity with a nutrient solution (Hewitt 1966).

Shoots and roots were harvested separately 56 days after sowing. Sub-samples of fresh roots were taken to assess mycorrhizal colonisation, and to determine the fungi colonising the roots using molecular methods. Fresh weight of total roots and of subsamples was measured. Shoots and remaining roots were dried for 24 h at 80 C and weighed. The percentage water content of remaining roots and total fresh root weight were used to estimate total root dry weight. Root colonisation was estimated after clearing and staining (Koske and Gemma 1989), using the notation system of Trouvelot et al. (1985). Zn and Cd concentrations in dried and ground plant material were determined by inductively-coupled plasma atomic emission spectrometry (Jobin Yvon JY 32) after wet-digestion with 69% HNO_3. NH_4NO_3-extractable and NH_4Ac-EDTA-extractable Zn and Cd concentrations (Symenoides and McRae 1977; Weissenhorn et al. 1995) were assessed in the soil at the end of the experiment.

Identification of spores associated with *V. calaminaria* using SSU rDNA analysis

Spores were extracted from the rhizosphere and from the roots of violet, grouped by morphotype, washed and crushed (Vandenkoornhuyse 1998) before molecular analysis. PCR amplification of 18S rDNA was performed directly on crushed spores (about 200 of each morphotype) with MH2 and MH4 primers (Vandenkoornhuyse and Leyval 1998) in a MiniCycler PTC150 (MJ Research). Amplification products (amplicons) were cloned using a plasmid vector (pCRII, Invitrogen). SSU rDNA of 64 positive clones was digested with restriction enzymes: *Hinf*I, *Hae*III (5 U, 3 h at 37 °C; Boehringer-Mannheim), and *Taq*I (5 U, 4 h at 65 C; Boehringer-Mannheim). Different patterns of restriction fragments, referred to as ribotypes, were obtained. One clone of each ribotype was sequenced. To sequence these clones, corresponding PCR products were purified using the High Pure purification PCR product kit (Boehringer-Mannheim) and quantified using a DNA dipstick kit (Invitrogen). Finally, 25 ng was used as a template for the sequencing reaction (ABI PRISM dye terminator cycle sequencing ready reaction kit; Perkin Elmer) using MH2, MH4 or MhPh1 primers (Vandenkoornhuyse and Leyval 1998). The sequences were analysed using an automatic sequencer (ABI-PRISM, model 373A; Perkin Elmer). Sequences are available on the GenBank database (for accession numbers, see Table 1). A multiple alignment was performed on-line using the algorithm Multalin (Corpet 1989) at http://www.toulouse.inra.fr/multalin.htlm. A phylogenetic tree was constructed using parsimony analysis with exhaustive tree search as explained in Vandenkoornhuyse and Leyval (1998). The sequences were compared to the available sequences of the international bank of data by performing a basic local alignment search tool (BLAST) search.

Estimation of fungal diversity in clover roots using 18S rDNAs T-RFLP

Non-inoculated clover roots and clover roots inoculated with the mixture of spores from the violet rhizosphere, from the control and the polluted soil, were carefully collected immediately after harvest, and washed with sterile distilled water. Total root DNA was extracted with DNeasy plant mini kit (Qiagen). PCR amplification of 18S rDNA was performed with MH2 and MhPh1 primers (as above) end-labelled with the fluorescein dyes 6-FAM and HEX, respectively. PCR products were purified with the High Pure PCR product purification kit. Purified amplification products (10 μl) were digested with selected restriction enzymes, *Taq*I (5 U, 4 h at 65 C; Boehringer-Mannheim) and *BseD*I (5 U, 3 h at 37 °C; Fermentas). They both can generate polymorph TRFs (positive and negative strands). The obtained TRFs were compared to the expected TRF lengths (positive and negative strands) corresponding to the AM spores from the violet rhizosphere, deduced from the restriction site of the enzyme and the fungal SSU rDNA sequences. The digested products (10 μl) were precipitated with 2 μl of 2 M acetate buffer (pH 4.6), and 50 μl of absolute ethanol. Each pellet was washed in 70% ethanol and dried under vacuum. The length of the TRFs was determined by electrophoresis with an ABI-PRISM 373A automated sequencer (Applied Biosystems). Pellets were suspended in 4 μl formamide/EDTA, 50 mM, pH 8.0 (5/1) and were added to 0.5 μl loading buffer, and 0.5 μl internal lane-size standard GENESCAN-2500ROX (Perkin Elmer). TRFs were sized using GeneScan software (Applied Biosystems).

Data analyses

Data means were compared by Student's *t*-test. Colonisation data were arcsine transformed, according to St John and Koske (1988). Terminal restriction patterns from all samples were scored as 1 or 0, corresponding to the absence or presence, respectively, of a given TRF in the positive or negative strand. AM fungal diversity in clover roots was evaluated by the TRF richness (where R = no. of TRFs found in the sample), and the Shannon-Wiener index, which combines two components of diversity, richness and evenness of individuals among the species (Krebs 1985). There were three replicates for each treatment and each enzyme digestion. The Shannon-Wiener index, as affected by heavy metals and AM inoculation, was estimated by calculating Ho: $Ho = -\Sigma\pi \log_2 \pi$, where π represents the TRF frequency.

Results

Four different morphological types (morphotypes) of spores were found in the violet rhizosphere based on colour and grouping: yellow spores inside roots, free yellow spores, yellow spores grouped in sporocarps, and black spores joined to common hyphae (Table 1). Microscopic observations showed a non-complex parietal structure for all spore types. Amplification of the four morphotypes, cloning and digestion with restriction enzymes of the 64 positive clones (18 for yellow spores in roots, 19 for free yellow spores, 14 for yellow spores in sporocarps, 13 for black spores) gave five ribotypes (Fig. 1). Digestion with *Taq*I of SSU rDNAs from black spores gave two different patterns (Fig. 1 lanes a, b; Table 1). The free yellow spores gave two patterns with *Hinf*I (Fig. 1, lanes c, e; Table 1). One of them was identical to the pattern of the yellow spores in roots (Fig. 1, lane e; Table 1). Digestion with *Hinf*I and *Taq*I showed different patterns for yellow spores grouped in sporocarps in comparison to free yellow spores and yellow spores in roots (Fig. 1, lanes c, d; Table 1). The five morphotypes gave the same pattern with *Hae*III. One clone of each of the ribotypes was sequenced, except for the ribotype d (Table 1). The BLAST analysis of the resulting sequences revealed that the mixture of spores associated with *V. calaminaria* was composed of at least four different species of AM fungi and that they all belonged to the genus *Glomus*.

Table 1 Correspondence between the morphotypes of arbuscular mycorrhizal (AM) spores, the restriction patterns in Fig. 1, and the sequence accession numbers. *ND* Not determined

Morphotype	Black spores joined on common hyphae		Yellow spores in sporocarps	Free yellow spores	Yellow spores in roots
Ribotype	a	b	d	c	e
Genbank no.	AF 229770	AF 229771	N.D.	AF 229772	AF 229773

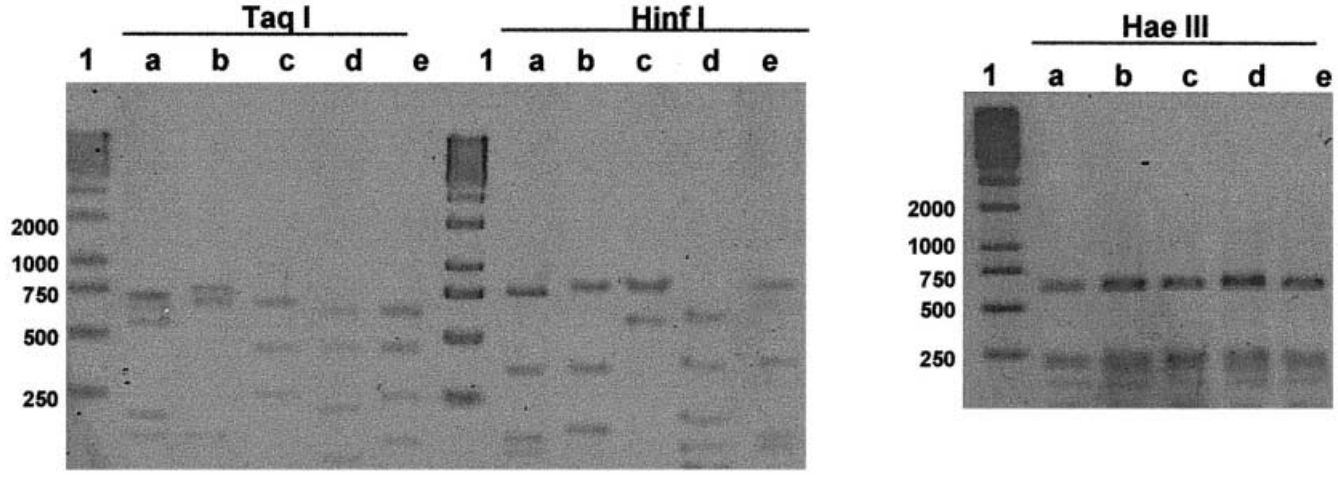

Fig. 1 Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of the 18S rDNA of arbuscular mycorrhizal (AM) fungi isolated from the violet rhizosphere. The SSU rDNA was amplified from purified rDNA of spores from the violet rhizosphere. PCR products were cleaved with *Taq*I, *Hinf*I, and *Hae*III, and separated on a 1.5% agarose gel in 0.5×TAE. *Numbers to the left of panels* indicate base pairs. *Lane 1* Size marker (1-kb ladder; MBI Fermentas), *lanes a*, *b* patterns of black spores joined to common hyphae, *lane c* pattern of free yellow spores, *lane d* pattern of yellow spores in sporocarps, *lane e* pattern of free yellow spores and of yellow spores in roots

Table 2 Mycorrhizal colonisation of *Trifolium subterraneum* roots. Mean values are presented with SD in parentheses and data in the *same column* followed by a *different letter* show a significant difference ($P=0.05$). *F* Frequency of colonised roots, *A* arbuscular richness of roots, *NM* non-mycorrhizal, *P2 Glomus mosseae* P2, *AMv* spores from the violet rhizosphere

	F (%)	A (%)
Control soil		
NM	0	0
P2	5 (5.8) a	1.9 (1.2) a
AMv	16.7 (10) a	3.9 (6) a
Polluted soil (+200 ppm Zn/2 ppm Cd)		
NM	0	0
P2	5 (7.1) a	0.3 (0.4) a
AMv	4.4 (2) a	0.2 (0.3) a

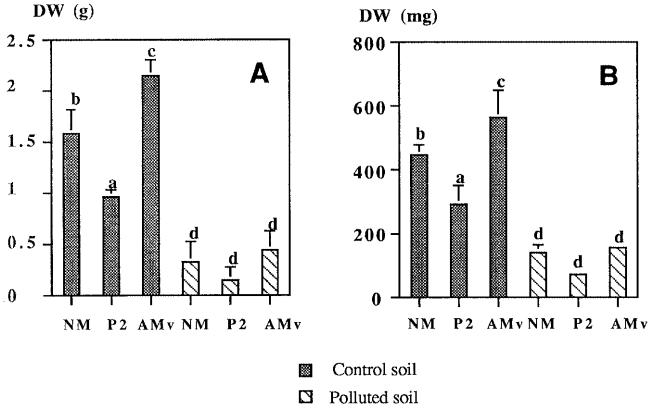

Fig. 2 Shoot (**A**) and root (**B**) dry weight (*DW*) of clover plants in control and polluted soil. *Bars* indicate SDs and *different letters* indicate significant differences ($P=0.05$). *NM* Non-mycorrhizal, *P2 Glomus mosseae* P2, *AMv* spores from the violet rhizosphere ($n=3$)

Clover planted in soil with 500 ppm Zn/5 ppm Cd, and with 2000 ppm Zn/20 ppm Cd, died within 10 days. Only the results of the soil contaminated with 200 ppm Zn and 2 ppm Cd, referred to as "polluted soil", are presented. The NH_4NO_3-extractable fractions in this soil reached 0.2–0.4 μg g^{-1} Cd and 35–50 μg g^{-1} Zn and the NH_4Ac-EDTA-extractable Cd and Zn 1.4–1.7 μg g^{-1} and 160–180 μg g^{-1}, respectively.

Trypan blue root staining confirmed AM fungi colonisation of clover roots which were inoculated with *G. mosseae* P2, and with the mixture of spores from the violet rhizosphere. The presence of arbuscules suggested that both symbioses were active, although the colonisation percentage was rather low (5–17%, Table 2). No mycorrhizal colonisation was detected in non-inoculated treatments. A significant reduction in shoot weight was observed in the polluted soil (Fig. 2A). Shoot dry weight (DW) of clover inoculated with spores from the violet rhizosphere was higher than for non-mycorrhizal clover and clover colonised with *G. mosseae* P2. However, the shoot DW difference was significant in the control soil but not in the polluted soil. Clover root dry weight also significantly decreased in the polluted soil (Fig. 2B). As for shoots, the dry weight of roots with the violet inoculum was significantly higher, and with *G. mosseae* P2 inoculation lower, than in the non-inoculated plants, but these differences were only significant in the control soil.

Clover inoculated with spores from the violet rhizosphere had higher Cd root concentrations than the other treatments (Fig. 3). Similar results were obtained for Zn concentrations in plants (Fig. 3). Inoculation with *G. mosseae* P2 significantly increased plant Cd concentrations, but had no effect on Zn concentrations.

Fungal diversity within roots of non-inoculated clover and clover inoculated with spores from the violet rhi-

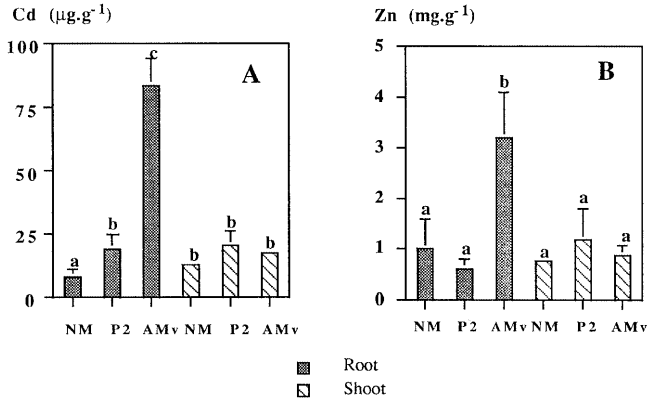

Fig. 3 Cd (**A**) and Zn (**B**) concentrations in shoots and roots of clover. Abbreviations as in Fig. 2

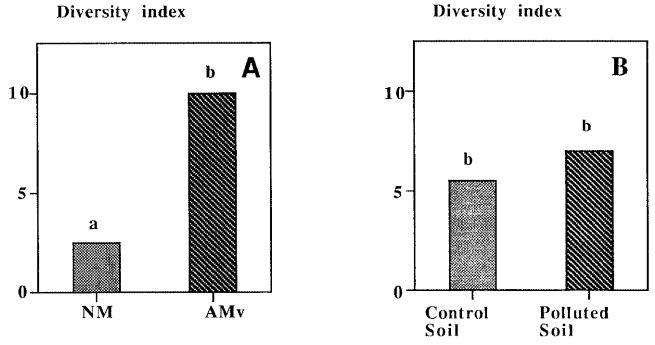

Fig. 5 Effects of mycorrhizal colonisation (**A**) and heavy metal addition (**B**) on the diversity, measured by the Shannon-Wiener index, of fungi colonising clover roots. Abbreviations as in Fig. 2

zosphere in the control and in the polluted soil was estimated by TRF numbers. Only results with *Taq*I were analysed because of the high background noise in the patterns obtained with *BseD*I. As the primers used are specific for fungi, but not for AM fungi (Vandenkoornhuyse 1998), and only a partial soil sterilisation was performed before the experiment, one or more fungal communities were found in all clover roots, including non-inoculated roots. The number of fungal TRFs was, however, significantly higher in the roots inoculated with the violet inoculum than in the control soil (Fig. 4). The number of TRFs for mycorrhizal roots in polluted and in control soil was not significantly different. For non-mycorrhizal clover, this number was significantly higher in polluted soil compared to control soil. The Shannon-Wiener index also increased when clover was inoculated with AM fungi from the violet rhizosphere (Fig. 5). Heavy metal pollution tended to increase the diversity index, but differences were not significant.

The TRFs obtained with clover roots inoculated with spores from the violet rhizosphere were compared to the expected ones from the sequences found in the violet rhizosphere (Table 3). It appeared that only one of the four AM fungi (*Glomus* b) identified in the violet rhizosphere had colonised clover roots in the polluted and in the control soil. The sequence of this *Glomus* b corresponded to one of the sequences of black spores, which accounted for 10% of the total number of spores extracted from the violet rhizosphere.

Table 3 Predicted terminal restriction fragments (TRFs) and observed TRFs after *Taq*I digestion of DNA extracted from clover roots. Only the observed TRFs with a size close to the predicted TRFs are presented. For *Glomus* a, b, c and e, see Fig. 1. For abbreviations, see Table 2

	Fragment size (bp)	
	Positive strands	Negative strands
Predicted TRFs		
Glomus a	56	106
Glomus b	56	258
Glomus c	468	188
Glomus e	56	188
Observed TRFs		
Control soil		
NM	–	258
AMv	56	258
Polluted soil		
NM	58	257
AMv	56, 469	258

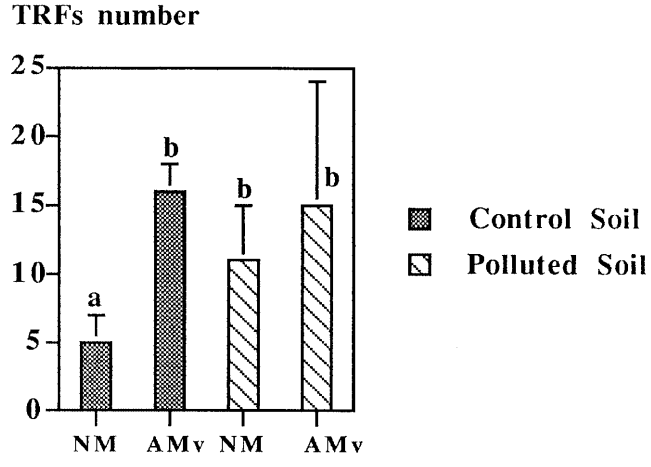

Fig. 4 Species richness estimated by the number of terminal restriction fragments (*TRFs*) in clover. Abbreviations as in Fig. 2

Discussion

The literature gives little information about AM fungal ecotypes adapted to high heavy metal concentrations in soil (Gildon and Tinker 1983; Weissenhorn et al. 1994; Diaz et al. 1996). Morphological observations of spores extracted from the violet rhizosphere, and sequencing of their SSU rDNA, showed that there were at least four *Glomus* spp. in the violet rhizosphere. This num-

ber was probably an underestimate, since minor, non-sporulating or late-sporulating species may have been neglected during the trap culture and spore extraction (Stutz and Morton 1996). In contrast, Hildebrandt et al. (1999) found only one *Glomus* species in the roots and adjacent soil of *V. calaminaria*. In our study, the AM spores were isolated from the rhizosphere, and only one type was extracted from the violet roots. 18S rDNAs of the free yellow spores and of the yellow spores inside roots were digested with *Hinf*I, *Hae*III and *Taq*I. Their patterns were similar with the three enzymes. These two morphotypes were the most numerous in the violet rhizosphere. T-RFLP analysis showed that these fungi did not colonise clover roots, in contrast to a black-spored fungus which was less numerous (*Glomus* b).

The two highest metal concentrations in soil (500 ppm Zn/5 ppm Cd and 2000 ppm Zn/20 ppm Cd) completely inhibited clover growth. The addition of 200 ppm Zn/2 ppm Cd to the soil, which is very close to the EC, United-Kingdom and German limits for Zn and Cd for agricultural soils (Official Journal of the European Communities no. L364/23), produced a significant decrease in shoot and root biomass (Fig. 2). Soil pH did not differ significantly between treatments (5.8–6.2) (data not shown). However, these metals were added as salts, which are highly available. The NH_4Ac-EDTA- and NH_4NO_3-extractable fractions corresponded to 75% and 25% of the total metal concentrations (200 ppm Zn/2 ppm Cd) in soil. The high trace element concentrations in the soil solution and the readily exchangeable fraction, as reflected by the NH_4NO_3 extraction (Sauerbeck and Styperek 1985; Leyval et al. 1995), confirmed the high availability of the metals in the soil.

Although Zn and Cd availability was high, and plant growth was reduced in the polluted soil, functional mycorrhizal structures, arbuscules, were found in clover roots with both mycorrhizal inocula. AM colonisation and arbuscule formation were low, but were not significantly different in the control and the polluted soil. In laboratory experiments, Del Val et al. (1999b) found that high levels of metals could affect mycorrhizal colonisation of leek and sorghum. Weissenhorn and Leyval (1995) reported no correlation between AM abundance in maize and the degree of metal pollution found in a field soil. In contrast, with the adapted plant *V. calaminaria*, Hildebrandt et al. (1999) reported a higher mycorrhizal colonisation in strongly contaminated sites.

Previous studies have reported that AM fungi decrease metal accumulation in plants, thus protecting them against heavy metal toxicity and helping them grow (Gildon and Tinker 1981; Diaz et al. 1996). Bradley et al. (1981) showed that Cu and Zn were sequestered in roots of *Calluna vulgaris* which were colonised by ericoid mycorrhizas. Reduced metal translocation from roots to shoots in the presence of AM fungi has also been shown (Schüepp et al. 1987; Joner and Leyval 1997). The accumulation of heavy metals in the fungal structures as suggested by their high heavy metal-binding capacity (Joner et al. 2000) could represent a biological barrier. In our experiment the AM inoculum from the violet rhizosphere was very efficient in sequestering metals in the roots. Clover roots inoculated with AM fungi from the rhizosphere of the metallophyte plant contained eightfold higher Cd and threefold higher Zn concentrations compared to non-inoculated plants, without any significant difference in plant biomass and concentrations of metals in shoots. Hildebrandt et al. (1999) reported that *Glomus* Br1 isolated from the same metallophyte plant allowed plants like maize, alfalfa, barley and others to grow in heavy metal-rich soils. These results suggest that AM fungi associated with metal-tolerant plants, e.g. metallophytes, may contribute to the accumulation of heavy metals in plant roots in a non-toxic form.

In the present experiment, *G. mosseae* P2 colonisation was not beneficial to, and even reduced, clover growth. The higher Cd concentration in plants inoculated with P2 could be a consequence of the reduction in biomass rather than a higher Cd uptake. This is not in agreement with our previous results, where plants inoculated with *G. mosseae* P2 showed decreased shoot and root Cd concentrations (in maize) and decreased Cd translocation from roots to shoots (in clover) (Weissenhorn et al. 1995; Joner and Leyval 1997). The fungus did not significantly influence plant growth and metal uptake, suggesting that under these experimental conditions, including a rather low light intensity, this fungus was inefficient.

T-RFLP analysis of DNA extracted from non-inoculated clover roots showed the presence of fungi. Since the soil treatment to eliminate indigenous AM fungi (25 kGy gamma irradiation) was not a total sterilisation, these fungi were probably of soil origin. The presence of fungi has also been shown in seeds of subterranean clover (data not shown). The role of non-mycorrhizal fungi in the interactions between AM fungi, roots and heavy metals in soil, is unknown. Fungal diversity within uninoculated clover was lower than in clover inoculated with AM fungi from the violet rhizosphere (Fig. 4), suggesting competition between fungi.

Among the four *Glomus* spp. detected in the violet rhizosphere, only *Glomus* b, which represented 10% of the spores extracted from the violet rhizosphere, colonised clover roots. The colonisation of roots by AM fungi may depend on the host plant (Bever et al. 1996), but also on fungal colonisation dynamics, interactions between plant and fungal species, and experimental conditions. In this experiment heavy metal addition did not affect AM fungal diversity in clover roots.

Our results confirm that T-RFLP analysis is a useful tool for assessing the diversity of complex microbial communities. This method facilitates comparisons of structure and diversity of different ecosystems (Liu et al. 1997) and permits assessment of subtle genetic differences between strains (Marsh 1999). In this experi-

ment, universal fungal primers were used for T-RFLP, so the diversity was estimated within fungi as a taxon, not within the *Glomales*, and only previously sequenced AM fungi could be identified. The diversity within the *Glomales* can be assessed by using T-RFLP with AM fungi-specific primers (Simon et al. 1993; van Tuinen et al. 1998). Our results show that non-mycorrhizal fungi can be present at a relatively high level in roots and should be taken into account in studies on plant-soil-microorganism interactions. The role of AM fungal diversity within roots and its relation to plant functions also needs to be highlighted.

AM fungi isolated from a metal-tolerant plant, *Viola calaminaria*, exerted a significant effect on heavy metal accumulation in plant roots, apparently restricting metal transfer from roots to shoots. Such AM fungi could contribute to phytoremediation processes, by preventing elevated concentrations of heavy metals in aerial plant parts.

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References

Allemann C, Hoegger P, Heiniger U, Rigling D (1999) Genetic variation of *Cryphonectria* hypoviruses (CHV1) in Europe assessed using restriction fragment length polymorphism (RFLP) markers. Mol Ecol 8:843–854

Baker AJM (1981) Accumulators and excluders – strategies in the response of plants to heavy metals. J Plant Nutr 3:643–654

Barea JM, Azcòn-Aguilar C, Azcòn R (1997) Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil-plant systems. In: Gange AC, Brown VK (eds) Multitrophic interactions in terrestrial systems. Cambridge University Press, Cambridge, pp 65–77

Bever JD, Morton JB, Antonovics J, Schultz PA (1996) Host specificity and diversity of arbuscular fungi in *Glomales*: an experimental approach using an old field soil. J Ecol 84:71–82

Bradley R, Burt AJ, Read DJ (1981) Mycorrhizal infection and resistance to heavy metal toxicity in *Calluna vulgaris*. Nature 292:335–337

Bundrett MC, Ashwath N, Jasper DA (1996) Mycorrhizas in the Kadadu region of tropical Australia. II. Propagules of mycorrhizal fungi in disturbed habitats. Plant Soil 184:173–184

Chelius M, Triplett E (1999) Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. Mycorrhiza 9:61–64

Claassen V, Zasoski R, Tyler B (1996) A method for direct soil extraction and PCR amplification of endomycorrhizal fungal DNA. Mycorrhiza 6:447–450

Clement BG, Lucia EK, Debord KL, Kitts CL (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J Microbiol Methods 31:135–142

Corpet F (1989) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–10890

Del Val C, Barea JM, Azcòn-Aguilar C (1999a) Diversity of arbuscular mycorrhizal fungus populations in heavy metal contaminated soils. Appl Environ Microbiol 99:718–723

Del Val C, Barea JM, Azcòn-Aguilar C (1999b) Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage sludge-contaminated soils. Appl Soil Ecol 11:261–269

Diaz G, Azcón-Aguilar C, Honrubia M (1996) Influence of arbuscular mycorrhiza on heavy metal (Zn and Pb) uptake and growth of *Lygedum spartum* and *Anthyllis cytisoides*. Plant Soil 180:241–249

Gildon A, Tinker PB (1981) A heavy metal-tolerant strain of a mycorrhizal fungus. Trans Br Mycol Soc 77:648–649

Gildon A, Tinker PB (1983) Interactions of vesicular-arbuscular mycorrhiza infections and heavy metals in plants. II. The effects of infection on uptake of copper. New Phytol 95:263–268

Heijden MGA van der, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Roller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Technical communications no. 22, 2nd edn. Commonwealth Agricultural Bureau, London

Hildebrandt U, Karldorf M, Bothe H (1999) The zinc violet and its colonization by arbuscular mycorrhizal fungi. J Plant Physiol 154:709–717

Joner EJ, Leyval C (1997) Uptake of ^{109}Cd by roots and hyphae of a *Glomus mosseae*/*Trifolium subterraneum* mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytol 135:253–360

Joner EJ, Leyval C, Briones R (2000) Metal binding capacity of arbuscular mycorrhizal mycelium. Plant Soil (in press)

Karldorf M, Kuhn AJ, Schröder WH, Hildebrandt U, Bothe H (1999) Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. J Plant Physiol 154:718–728

Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 92:486–505

Krebs CJ (1985) Ecology: the experimental analysis of distribution and abundance, 3rd edn. Harper and Row, New York

Lambinon J, Auquier P (1963) La flore et la végétation des terrains calaminaires de la Wallonie Septentrionale et de la Rhénanie Aixoise. Nat Mosans 16:113–131

Leyval C, Binet P (1998) Polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants. J Environ Qual 27:402–407

Leyval C, Singh BR, Joner EJ (1995) Occurrence and infectivity of arbuscular mycorrhizal fungi in some Norwegian soils influenced by heavy metals and soil properties. Water Air Soil Pollut 84:203–216

Leyval C, Haselwandter K, Turnau K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 7:139–153

Liu W, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lloyd-McGilp SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of ribosomal internal transcribed spacers within and among isolates of *G. mosseae* and related mycorrhizal fungi. New Phytol 133:103–112

Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplifications products. Curr Opin Microbiol 2:323–327

McGrath SP, Chaudri AM, Giller KE (1995) Long-term effects of metals in sewage sludge on soils, micro-organisms and plants. J Ind Microbiol 14:94–104

Moutoglis P (1997) Genetical aspects of vesicular-arbuscular mycorrhizal fungi. PhD thesis. University of Montréal, Canada

Read DJ (1991) Mycorrhizas in ecosystems. Experentia 47:376–391

Sauerbeck DR, Styperek P (1985) Evaluation of chemical methods for assessing the Cd and Zn availability from different soils and sources. In: Leschber R, Davies RD, L'Hermite P (eds) Chemical methods for assessing bioavailable metals in sludges and soils (EUR9538). Elsevier, London, pp 49–66

Schüepp H, Dehn B, Sticher H (1987) Interaktionen zwischen VA-Mykorrhizen und Schwermetallbelastungen. Angew Bot 61:85–96

Shetty KG, Hetrick BAD, Figge DAH, Schwab AP (1994) Effects on mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environ Pollut 86:181–188

Simon E (1978) Heavy metals in soil, vegetation development and heavy metal tolerance in plant population from metalloferous areas. New Phytol 81:175–188

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and consequence with vascular land plants. Nature 363:67–69

St John TV, Koske RE (1988) Statistical treatment of endogonaceous spore counts. Trans Br Mycol Soc 91:117–121

Stutz JC, Morton JB (1996) Successive pot cultures reveal high species richness of arbuscular endomycorrhizae fungi in arid ecosystems. Can J Bot 74:1883–1889

Symenoides C, Mc Rae SG (1977) The assessment of plant-available cadmium in soils. J Environ Qual 6:120–123

Tessier A, Campbell PGC, Bisson M (1979) Sequential extraction procedure for the speciation of particulate trace metals. Anal Chem 51:844–851

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1985) Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Aspects physiologiques et génétiques des mycorhizes. Proceedings of the 1st European Symposium on Mycorrhizas, Dijon, 1–5 July 1985. INRA, Paris, pp 217–221

Tuinen D van, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Vandenkoornhuyse P (1998) Approche de la diversité inter- et intraspécifique des champignons mycorhiziens à arbuscules. PhD thesis. University of Nancy, France

Vandenkoornhuyse P, Leyval C (1998) SSU rDNA sequencing and PCR-fingerprinting reveal genetic variation within *Glomus mosseae*. Mycologia 90:791–797

Walker C, Mize CW, McNabb HS (1982) Populations of endogonaceous fungi at two locations in central Iowa. Can J Bot 60:2518–2529

Weissenhorn I, Leyval C (1995) Root colonization of maize by a Cd-sensitive and a Cd-tolerant *Glomus mosseae* and cadmium uptake in sand culture. Plant Soil 175:233–238

Weissenhorn I, Leyval C, Berthelin J (1993) Cd-tolerant arbuscular mycorrhizal (AM) fungi from heavy metal polluted soils. Plant Soil 157:247–256

Weissenhorn I, Glashoff A, Leyval C, Berthelin J (1994) Differential tolerance to Cd and Zn of arbuscular mycorrhizal (AM) fungal spores isolated from heavy metal-polluted and unpolluted soils. Plant Soil 167:189–196

Weissenhorn I, Leyval C, Belgy G, Berthelin J (1995) Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil. Mycorrhiza 5:245–251