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Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland

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Abstract Analysis of the community of arbuscular mycorrhizal (AM) fungi in roots of *Fragaria vesca* growing in a heavy metal contaminated site was carried out on a Zn waste site near Chrzanow (southern Poland). The waste substratum was characterized by high contents of Pb, Zn, Cd, Cu and As, and by low levels of N, P and organic matter. Spores of Glomales were isolated by wet sieving and DNA was isolated from individual spores. Nested polymerase chain reaction (PCR) with taxon-specific primers was used to identify the species *Glomus mosseae*, *Glomus intraradices* and *Glomus claroideum*. Spores of other fungi were morphologically characterized and new taxon-discriminating molecular probes were developed for two of them (*Glomus* sp. HM-CL4 and HM-CL5) based on variations in the large ribosomal subunit (25S rDNA). High sequence similarities were found between *Glomus* sp. HM-CL4 and *Glomus gerdemanii*, and between *Glomus* sp. HM-CL5 and *Glomus occultum*. The designed primers were used to characterize the population of AM fungi colonizing the roots of *F. vesca* collected from the Zn waste site. The analysis, carried out on roots stained with trypan blue, showed that the most effective colonizer was closely related to *G. gerdemannii*. *G. claroideum* and the *G. occultum*-like fungus were slightly less common whilst frequencies of *G. intraradices* and *G. mosseae* in roots were much lower. The analysis of mycorrhiza stained with rhodizoniate to localize heavy metal accumulation showed that the stain does not influence the PCR reaction. Seventy percent of the root samples containing positively stained fungal hyphae were found to be colonized by *G. mosseae*. The data obtained demonstrate the usefulness of nested PCR for studies carried out in polluted areas. It will enable selection of AM fungi which are able to colonize plant roots under heavy metal stress conditions, as well as the identification of fungi showing high in situ accumulation of potentially toxic elements.

Keywords Arbuscular mycorrhiza · Heavy metal waste · Nested polymerase chain reaction · Trypan blue stain · Rhodizoniate test

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Introduction

Mycorrhizal fungi are known to be able to accelerate the revegetation of industrially affected areas, such as coal mines or waste sites containing high levels of heavy metals (Marx 1975; Marx and Altman 1979). Ectomycorrhizal trees can be established in such areas and introduced fungi can be fairly easily monitored using morphological or molecular methods (Henrion et al. 1994; Erland 1995). Where intensive wind erosion occurs, however, it is essential to create a stabilizing plant cover composed of herbaceous species which are active in phytoremediation processes and produce high levels of root and shoot biomass (Zak and Parkinson 1982; Leyval et al. 1997). Plants which appear spontaneously in such places are frequently devoid of mycorrhizal symbiosis and are mostly characterized by poorly developed root and shoot biomass when heavy metals are present (Pawlowska et al. 1996). The first arbuscular mycorrhizal (AM) plants to appear on Zn waste sites investigated in southern Poland took 2 or more decades to become established after deposition of industrial

dust, and there are several waste sites which are over 40 years old where no AM plants have so far been observed (unpublished data). The introduction of an AM fungal inoculum into these areas could be a strategy for enhancing the establishment of mycorrhizal herbaceous species. AM fungal isolates differ in their effect on heavy metal uptake by plants (Leyval et al. 1997) and the selection of appropriate isolates could be of importance for a given phytoremediation strategy. Such fungal isolates can be isolated from areas which are either naturally enriched by heavy metals or are old mine/industry waste sites in origin. One isolate has recently been obtained from *Viola calaminaria* L., molecularly characterized [polymerase chain reaction (PCR)-restriction fragment length polymorphism] and shown to confer heavy metal tolerance onto plants including maize, alfalfa, barley and others (Hildebrandt et al. 1999). However, there are usually several AM fungal types which may colonize the same root systems in a given ecosystem (Clapp et al. 1995). The selection of effective isolates should, therefore, include investigations of their colonization abilities in the sites where the inoculum is to be introduced. The identification of AM fungi is mainly based on the morphology of spores which are formed around or within root systems. Evaluation of the success of a fungal isolate as a plant colonizer, however, requires recognition of the fungal partner within root systems. This has recently become possible by the development of PCR primers, based on variability of the 5′ end of the large ribosomal subunit, which subsequently can be used in a nested PCR reaction for the identification of different AM fungi in trypan blue-stained root fragments (van Tuinen et al. 1998a). This method has been successfully used to monitor a mixed population of mycorrhizal fungi in substrates containing sewage sludge enriched or not with heavy metal or organic pollutants (Jacquot et al. 2000).

The aim of this study was to use nested PCR to identify AM fungi colonizing roots of plants occurring spontaneously on a 20-year-old Zn waste site in Poland, so that effective AM fungi could be targeted for isolation from the field site. Spores of AM fungi were isolated and specific primers designed from large ribosomal subunit sequences for nested PCR on DNA from root fragments stained with trypan blue/lactophenol and rhodizoniate. Rhodizoniate is an indicator of heavy metal accumulation within biological material. It has been applied to localize lead at acid pH in algae (Silverberg 1975; Theiss 1987), lichens (Garty and Theiss 1990) and in arbuscular mycorrhiza (Turnau 1998). However, with increasing pH, rhodizoniate forms a complex also with Zn, Fe, Cd and Cu salts. The combination of nested PCR with rhodizoniate staining could therefore provide a useful approach for ecological studies of interactions between heavy metals and mycorrhizal development.

Materials and methods

Biological material

The investigations were carried out on a 20-year-old industrial waste site located in Chrzanow (southern Poland, 30 km west of Cracow) colonized by *Fragaria vesca* L., *Euphorbia cyparissias* L., *Hippophae rhamnoides* L., *Arrhenatherum elatius* (L.) J. and C. Presl., *Trifolium repens* L. and *Viola tricolor* L. The solid fraction of the waste, on which observations were carried out, has a sandy soil texture and contains up to 15,000 mg kg^{-1} Zn, 7,000 mg kg^{-1} Pb, 22,000 mg kg^{-1} Fe, 24,000 mg kg^{-1} Al_2O_3, 80 mg kg^{-1} Cd, 500 mg kg^{-1} As, and 200 mg kg^{-1} Cu (Lekan and Piotrowska 1988). Plants were collected from slopes of the waste site where the soil characteristics were pH (H_2O) 7.4, organic matter content 1.12%, N content 0.044%, P_2O_5 content 74 mg kg^{-1}, K_2O content 69 mg kg^{-1}, and CaO content 2,509 mg kg^{-1}. Plants of *F. vesca, T. repens* and *V. tricolor* were collected at the beginning of May 1999. Roots and soil were soaked in water, mixed carefully for 20 min and spores collected by wet sieving while the roots were collected for staining. Spore morphotypes were grouped together using a stereomicroscope on the basis of morphological characters. Roots were stained in 0.05% trypan blue in lactophenol for 20 min (Phillipps and Hayman 1970). Stained roots were immediately placed in distilled water for storage. For each plant at least 30 stained root fragments were randomly taken and scored for percentage presence/absence of mycorrhizal fungi, mycorrhizal colonization (M%) and arbuscule abundance (A%) as an average of all root fragments, using light microscopy (Trouvelot et al. 1986).

Root samples of *F. vesca* were also stained with rhodizoniate salt, a cytochemical indicator of lead in light microscopy. Rhodizoniate (50 mg) was dissolved in 25 ml buffer (pH 2.8) containing 1.5 g tartaric acid and 1.9 g sodium bitartrate in 100 ml distilled water. Washed, fresh roots were soaked in this solution for 5 h, transferred to the buffer without rhodizoniate and observed under a stereomicroscope. Roots containing distinctly red stained hyphae were selected and washed in water.

Identification of AM fungi

One to three spores of individual morphotypes were crushed in 40 µl TE buffer (10 mM TRIS-HCl, pH 8, 1 mM EDTA) and heated at 100 °C for 3 min in the presence of 10 µl of 20% Chelex 100 (BioRad). The crude DNA suspension was separated from cellular fragments and Chelex by centrifugation at 3,000 *g* for 1 min, followed by cooling on ice, and 5 µl of the supernatant was used as target DNA in the first set of PCR amplifications. This was performed in a final volume of 25 µl containing 10 mM TRIS-HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM dNTP, 500 nM of each primer, 0.25 U Taq DNA polymerase (Appligen Oncor, Illkirch) and 5 µl of extract. The reaction was overlaid or not with mineral oil depending on the themocycler used. The amplification program was set at: initial denaturation cycle 95 C (3 min), annealing at 60 C (1 min), extension at 72 °C (1 min), 30 cycles of denaturation at 93 C (1 min), annealing at 60 C (1 min) and extension at 72 C (1 min); the last cycle was followed by a final extension of 72 °C for 5 min. The first PCR was performed with the primer pair ITS3 (White et al. 1990) and with the eukaryote-specific primer NDL22 (van Tuinen et al. 1998b). Five microlitres of the first amplifications product, diluted 1/500, was used as a template for a second reaction with the eukaryotic LR1 (van Tuinen et al. 1998b) and with the fungal specific primer FLR2 (Trouvelot et al. 1999). The second amplification product was diluted 1/500 and amplified using taxon-specific primers in combination with LR1 or FLR2 as described above. Available taxon-specific primers were used for *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (5.25-FLR2, van Tuinen et al. 1998b) and *Glomus intraradices* Schenck and Smith (8.22-LR1, van Tuinen et al. 1998a). Other primers were obtained as described below. The am-

plification products were separated by gel electrophoresis on 1.4% agarose gel in TAE buffer (40 mM TRIS, pH 7.8, 20 mM acetic acid, 2 mM EDTA) and DNA was visualized after staining with ethidium bromide (Sambrook et al. 1989).

Cloning of PCR products and primer design

Specific primers were developed, as previously described (van Tuinen et al. 1998a), for those morphotypes for which taxon-specific primers were not already available. Nested PCR was carried out to enhance the efficiency of the amplification in order to increase the amount of DNA available for cloning using the primer pair ITS3 (White et al. 1990) and NDL22 (van Tuinen et al. 1998b) for the first amplification step and LR1 and NDL22 for the second. PCR products were cloned into the pGEM-T vector, according to the manufacturer's recommendations (Promega, Madison, Wis.). The presence of cloned inserts was checked by PCR directly on bacterial colonies diluted 20 times in water, using the LR1 and NDL22 primers. The recombinant plasmids were purified and both strands were sequenced (Genome Express, Grenoble, France). Sequences were first aligned with the ClustalW program, and the alignment was further improved manually. For *Glomus claroideum* Schenck and Smith, spores of the isolates BEG14 and BEG31 from the Banque Européenne des Glomales were treated as described above. Taxon-specific primers were deduced for each fungus. Predicted specificity was tested by simulation with the program Amplify (Bill Engels, University of Wisconsin, Madison, Wis.) and confirmed using DNA from spores.

Detection of fungal species within plant roots

Roots stained with trypan blue in lactophenol or with rhodizoniate salts were used for nested PCR analysis. One-centimetre-long pieces of roots with visible AM fungal structures were selected under a dissecting microscope. Over 60 trypan blue- and ten rhodizoniate-stained mycorrhizal root fragments were taken from five *F. vesca* specimens sampled randomly across the Zn waste site. The roots were rinsed in 100 mM TRIS buffer, pH 8, crushed in 90 μl of the same buffer and heated at 95 C for 10 min in the presence of 20% Chelex-100. Chelex was precipitated by cooling on ice for 5 min and centrifuged for 1 min at 1,200 *g*. Crude DNA (1/10) was used as the template for a first amplification reaction with ITS3/NDL22 primers, a second with LR1/FLR2 and a third with fungal specific/(FLR2 or LR1) primers. Amplification products obtained after the first and second PCR steps were used at a 500 times dilution in water and the PCR reactions were carried out as described above for all three steps. Frequency of the presence of the different AM fungi within the root systems was calculated according to van Tuinen et al. (1998a).

Results

Identification of AM fungal spores

After wet sieving of the Zn substratum collected with root samples, the most common spores were grouped into six morphotypes. The amplification products obtained for each by nested-PCR with the primers LR1 and FLR2 were cloned and sequenced, and each sequence compared to those available in the EMBL database. On the basis of sequencing, two morphotypes matched [>98% over 760 nucleotides (nt)] 25S rDNA sequences that have already been identified for *G. mosseae* (accession no. Y07656) and *G. claroideum* (accession nos. AJ271928 and AJ271929). A taxon-specific primer was designed for *G. claroideum* and named 38.21 (5′-TGG-GCT-CGC-GGC-CGG-TAG-3′) and the specificity was confirmed by PCR against other Glomales (Table 1). Only the PCR reaction performed on the spores of the aforementioned morphotype or of *G. claroideum* BEG14 and BEG30 gave the expected specific amplification product of 632 bp with FLR2 (Table 1). No amplification was obtained with any of the other Glomales tested. Taxon-specific primers were developed for each of the remaining fungal morphotypes. According to EMBL sequencing data, one of the morphotypes had a sequence (accession no. AJ271925) close (71% over 634 nt) to *Glomus gerdemannii* Rose, Daniels and Trappe (Accession number AJ2711712) and was named *Glomus* sp. HM-CL4. The primers for this fungus were: cad 4.1 (5′-TCG-AGT-ATT-GCT-GCG-ACG-A-3′) and cad 4.2 (5′CTC-AAG-TGT-CCA-CAA-CTG-C-3′), and they were used in combination with FLR2 and LR1, respectively, for PCR. The two remaining morphotypes gave identical sequences and were grouped together as *Glomus* sp. HM-CL5. This fungal sequence (accession no. AJ271926) showed high similarity (>84.6% over 716 nt) to *Glomus occultum* Walker (accession no. AJ271713) and two primers were designed: cad 5.1 (5′-GAA-GTC-TGT-CGC-AGT-CTG-3′) and cad 5.3 (5′-TCG-CGA-AAG-CTT-GTG).

Table 1 Specificity test of the *Glomus claroideum* primer (38.21) in combination with FLR2 on spores of different Glomales

Isolate no.	Species	PCR product size (bp) (38.21-FLR2)
BEG30	*Glomus claroideum*	632
This study	*G. claroideum*	632
BEG1	*Scutellospora castanea*	0
BEG8	*Acaulospora longula*	0
BEG9	*Gigaspora rosea*	0
BEG11	*Glomus geosporum*	0
BEG12	*Glomus mosseae*	0
BEG25	*G. mosseae*	0
BEG29	*G. mosseae*	0
BEG83	*G. mosseae*	0
BEG84	*G. mosseae*	0
BEG13	*Acaulospora laevis*	0
BEG17	*Gigaspora candida*	0
BEG20	*Glomus caledonium*	0
BEG22	*Glomus coronatum*	0
LPA8	*Glomus intraradices*	0
BEG34	*Gigaspora margarita*	0
BEG35	*Scutellospora heterogama*	0
BEG47	*Glomus versiforme*	0
BEG78	*Acaulospora lacunosa*	0
LPA 20	*Scutellospora pellucida*	0

The primer combinations used and the size of the nested PCR amplification products for each fungal isolate analysed are given in Table 2. On the basis of root observations, the presence of *G. intraradices* was suspected and thus the corresponding taxon-specific primer 8.22 was used (van Tuinen et al. 1998a). No cross amplification between isolates was obtained and there-

Table 2 Size of amplification products obtained by nested polymerase chain reaction using discriminating primers for five morphotypes of arbuscular mycorrhizal fungi selected from a Zn waste site

Source of DNA	LR1		FLR2				
	8.22	Cad 4.2	5.25	38.21	Cad 4.1	Cad 5.1	Cad 5.3
G. mosseae			372				
G. intraradices	455						
G. claroideum				632			
Glomus sp. HM-CL4		660			321		
Glomus sp. HM-CL5						597	296

fore the primers were considered taxon-specific for the AM fungi investigated.

Detection of fungal species in mycorrhizal root systems

Mycorrhizal root fragments of a few specimens of each plant of the three species collected from the Zn waste site were stained with trypan blue. DNA extracted from mycorrhizal root fragments was used as a template for nested PCR reactions. Satisfactory amplification was only obtained with roots of *F. vesca,* which was therefore selected for further investigations. Analyses were carried out on five plants taken randomly across the polluted field site. The mean M% was 67% and A% was 53%. Usually no band corresponding to fungal rDNA was detected after the first PCR, but the specific fungal amplification products were often detected after the second and/or the third amplification. An example of amplification products obtained from nested PCR with the taxon-specific internal primers is given in Fig. 1.

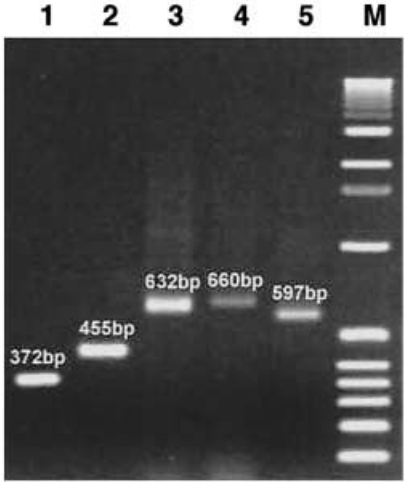

Fig. 1 Amplification products obtained after nested polymerase chain reaction (PCR) of DNA from mycorrhizal root fragments of *Fragaria vesca* using for the second PCR amplifications the primer-specific pair for: *Glomus mosseae* (*1*); *G. intraradices* (*2*); *G. claroideum* (*3*); *Glomus* sp. HM-CL4 (*4*); *Glomus* sp. HM-CL5 (*5*); molecular size marker (*M*). The respective amplification sizes (bp) are indicated on the figure

The results obtained using taxon-specific primers suggest that among the five different AM fungi analysed, the most effective colonizer of roots of *F. vesca* from industrial waste sites was *Glomus* sp. HM-CL4 (Fig. 2). This fungus was detected with an average frequency of 52% of the whole root system whilst *G. claroideum* and *Glomus* sp. HM-CL5 were slightly less common (32–34% of the root system). The frequencies of *G. intraradices* and *G. mosseae* were much lower and they were detected 3–5 times less often than the other three AM fungi. Fifty percent of the analysed root fragments were colonized by only one of the five fungi, and this was mostly *Glomus* sp. HM-CL4. A total of two fungi were found in 19% of the analysed root pieces and of three in 14.5% of root pieces. No taxon-specific PCR product was obtained in 12% of the analysed root segments, despite the presence of arbuscules within

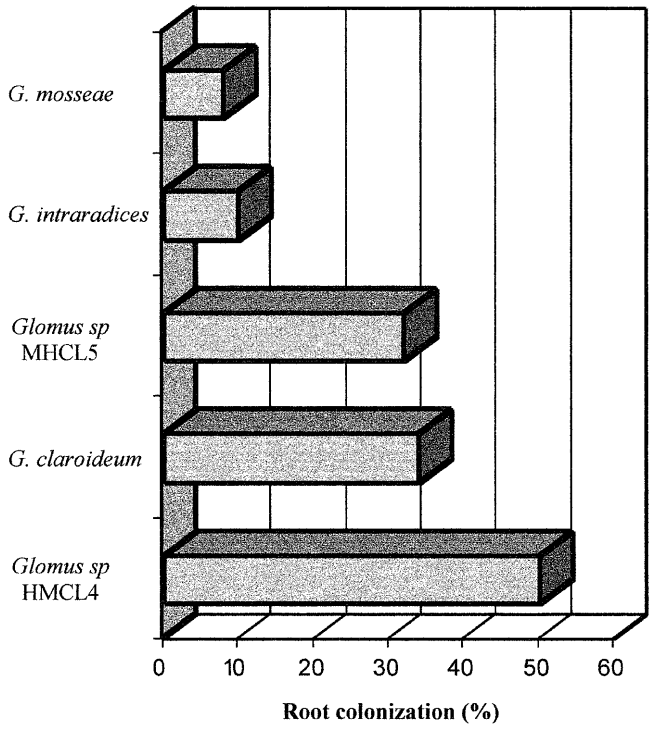

Fig. 2 Mycorrhizal colonization of *Fragaria vesca* roots by different mycorrhizal fungi in a Zn waste site, detected by nested PCR using taxon-specific primers for each fungus

them, indicating that AM fungi other than the five identified morphotypes were probably also present in the root systems of *F. vesca*.

The analysis of rhodizoniate-stained mycorrhiza showed that staining did not influence the PCR reaction (data not shown). The staining was observed in only about 5% of the root system. Seventy-eight percent of the rhodizoniate-stained root samples were found to be colonized by *G. mosseae* and only 35% by the other AM fungi investigated. In the stained root fragments, *G. mosseae* was always accompanied by other AM fungi, mainly *Glomus* sp. HM-CL4 and HM-CL5. The latter fungus was detected alone only in one sample. Three fungi were detected within a single root fragment in 50% of the samples.

Discussion

Nested PCR using taxon-specific primers for AM fungal species (morphotypes) is a highly sensitive method which allows detection of fungal hyphae present in roots as well as from soil (van Tuinen et al. 1998a; Jacquot et al. 2000). This opens up new possibilities for investigating mycorrhizal community structure, and competition between different fungal species within roots and within soil. The present study clearly shows the usefulness of the method to evaluate the behaviour of field communities of AM fungi under stress conditions such as in heavy metal-polluted wastes. It overcomes the limitations linked to simply evaluating the presence of spores in the vicinity of roots, which does not give any information about the level of root colonization of each individual species. Methods developed for staining intraradicular mycelium can, at best, only broadly discriminate between AM families, based on the differences in arbuscule morphology, and presence/absence of vesicles (Brundrett et al. 1996). Furthermore, as shown in this study for *G. intraradices*, the absence of detectable spores does not necessarily indicate the absence of the fungus from roots.

The simultaneous use of nested PCR with rhodizoniate staining could provide a basis for discriminating between AM fungi with respect to their ability to accumulate heavy metals within roots under natural conditions. In the present study staining was observed in only about 5% of the root system despite the fact that roots were strongly colonized by mycorrhizal fungi. *G. mosseae* was the only fungus which was always present in stained root fragments, suggesting that AM fungi may differ in their capacity for heavy metal accumulation. At the same time, this species was not so frequently detected in roots stained with trypan blue as compared to the other investigated fungi. However, *G. mosseae* was always accompanied by at least one of the other AM fungi, and whether *G. mosseae* is really the heavy metal-accumulating species needs further research.

Nested PCR with taxon-specific primers carried out on trypan blue-stained roots has already been successfully used in pot cultures where the fungal species were known (van Tuinen et al. 1998a; Jacquot et al. 2000). In one case, the influence of sewage sludge enriched with heavy metals and organic pollutants on the community structure of arbuscular mycorrhizal fungi was investigated (Jacquot et al. 2000). The present study is the first to use this analytical approach on material originating from a field site where the AM fungi present were initially unknown and where the plant root system was probably less amenable for molecular studies than that from controlled culture conditions. This could partly explain why three sequential PCR reactions were necessary in order to obtain characteristic PCR products. In a previous study performed in a reconstituted microcosm devoid of toxic elements (van Tuinen et al. 1998a), an amplification product could be readily distinguished after one amplification with eukaryotic-specific primers. Various factors such as plant growth conditions, plant species or age could affect the amplification efficiency (Zhao et al. 2000), as well as inhibitory compounds present in the soil. This difference emphasizes the necessity to adapt the procedure to the experimental material used.

Previous studies on the influence of heavy metals on AM fungi have tended to select species which sporulate easily in trap cultures. However, lack of sporulation does not necessarily mean absence from a site. For example, mycelium of *G. intraradices* was detected in roots of *F. vesca*, in the present study, but spores were not found in any of the soil samples. The molecular evaluation of AM fungal populations within roots is therefore a promising approach for identifying naturally occurring fungi which efficiently colonize plant roots in field sites like heavy metal-polluted wastes or soils, so that effective AM fungi can be isolated and selected as inocula for the revegetation of such stressed environments with appropriate plant species.

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References

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhiza in forestry and agriculture. ACIAR monograph no. 32. Australian Centre for International Agricultural Research, Canberra, Australia

Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of the fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265

Erland S (1995) Abundance of *Tylospora fibrillosa* ectomycorrhizas in a south Swedish spruce forest measured by RFLP analysis of the PCR-amplified rDNA ITS region. Mycol Res 99:1425–1428

Garty J, Theiss HB (1990) The localization of lead in the lichen *Ramalina duriarei* (De Not.) Bagl. Bot Acta 103:311–314

Henrion B, Di Battista C, Bouchard D, Vairelles D, Thompson BD, Le Tacon F, Martin F (1994) Monitoring the persistence of *Laccaria bicolor* as an ectomycorrhizal symbiont of nursery-grown Douglas fir by PCR of the rDNA intergenic spacer. Mol Ecol 3:571–580

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

Jacquot E, Tuinen D van, Gianinazzi S, Gianinazzi-Pearson V (2000) Monitoring species of arbuscular mycorrhizal fungi in planta and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil (in press)

Lekan S, Piotrowska M (1988) Evaluation of the agricultural environment in the vicinity of Szyb Wlodzimierz mining wastes (in Polish). Szyb Wlodzimierz report no. 413-34/87. Panstwowy Instytut Ochrony Srodowiska (PIOS), Pulawy, Poland

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

Marx DH (1975) Mycorrhizae and the establishment of trees on strip-mined land. Ohio J Sci 75:288–297

Marx DH, Altman JD (1979) *Pisolithus tinctorius* ectomycorrhiza improve survival and growth of pine seedlings on acid coal spoil in Kentucky and Virginia. Reclam Rev 2:23–37

Pawlowska TB, Blaszkowski J, Rühling A (1996) The mycorrhizal status of plants colonizing a calamine spoil mound in southern Poland. Mycorrhiza 6:499–505

Phillips JM, Hayman DS (1970) Improved procedures for clearing and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, New York

Silverberg BA (1975) Ultrastructural localization of lead in *Stigeoclonium tenue* (*Chlorophyceae, Ulotrichales*) as demonstrated by cytochemical and X-ray microanalysis. Phycologia 14:265–274

Theiss HB (1987) Histochemischer Nachweis von Blei in Cladophora. Mikrokosmos 76:108–109

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1986) Mesure du taux de mycorhisation VA d'un systeme radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Mycorrhizae: physiology and genetics. INRA Press, Paris, pp 217–221

Trouvelot S, Tuinen D van, Hijri M, Gianinazzi-Pearson V (1999) Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization. Mycorrhiza 8:203–206

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

Tuinen D van, Zhao B, Gianinazzi-Pearson V (1998b) PCR in studies of AM fungi: from primers to application. In: Varma AK (ed) Mycorrhizal manual. Springer, Berlin Heidelberg New York, pp 387–399

Turnau K (1998) Heavy metal uptake and arbuscular mycorrhiza development of *Euphorbia cyparissias* on Zn wastes in south Poland. Acta Soc Bot Pol 67:105–113

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sminski JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic Press, San Diego, Calif., pp 315–322

Zak JC, Parkinson D (1982) Initial vesicular-arbuscular mycorrhizal development of the slender wheatgrass on two amended mine soils. Can J Bot 60:2241–2248

Zhao B, Tuinen D van, Gianinazzi-Pearson V (2000) DNA extraction and PCR detection of the AM fungus *Glomus intraradices* in mycorrhizal root systems. J Huazhong Agric Univ (in press)