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SHORT NOTE

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Biodegradation of phenanthrene, spatial distribution of bacterial populations and dioxygenase expression in the mycorrhizosphere of *Lolium perenne* inoculated with *Glomus mosseae*

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Abstract Interactions between the plant and its microbial communities in the rhizosphere control microbial polycyclic aromatic hydrocarbons (PAH) biodegradation processes. Arbuscular mycorrhizal (AM) fungi can influence plant survival and PAH degradation in polluted soil. This work was aimed at studying the contribution of the mycorrhizosphere to PAH biodegradation in the presence of ryegrass (*Lolium perenne* L., cv. Barclay) inoculated with *Glomus mosseae* (BEG 69) by taking into account the structure and activity of bacterial communities, PAH degrading culturable bacteria as a function of the distance from roots. Ryegrass was grown in compartmentalized systems designed to harvest successive sections of rhizosphere in lateral compartments polluted or not with phenanthrene (PHE). Colonization of roots by *G. mosseae* (BEG 69) modified the structure and density of bacterial populations in the mycorrhizosphere, compared to the rhizosphere of non-mycorrhizal plants. *G. mosseae* increased the density of culturable heterotrophic and PAH degrading bacteria beyond the immediate rhizosphere in the presence of PHE, and increased the density of PAH degraders in the absence of the pollutant. Biodegradation was not significantly increased in the mycorrhizosphere, compared to control non-mycorrhizal plants, where PHE biodegradation already reached 92% after 6 weeks. However, dioxygenase transcriptional activity was found to be higher in the immediate mycorrhizosphere in the presence of *G. mosseae* (BEG 69).

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Introduction

Rhizodegradation applied to Polycyclic Aromatic Hydrocarbons (PAH) is a recent technology that uses plants to remedy soils (Anderson and Coats 1994). Rhizodegradation refers to the capacity of plants to increase PAH biodegradation (Günther et al. 1996; Liste and Alexander 2000) through rhizosphere effects on soil microbial communities (Höflich and Günther 2000; Yoshitomi and Shann 2001). Ubiquitous symbiotic partners such as arbuscular mycorrhizal (AM) fungi have been considered in polluted soils for their capacity to physically, chemically, and biologically influence the rhizosphere (Joner and Leyval 2003a). AM fungi are beneficial for the growth and the survival of plants in PAH-polluted soils where they facilitate plant nutrition and water uptake (Binet et al. 2000).

Although no evidence of direct PAH catabolism by AM fungi has been reported yet (Criquet et al. 2000), increased degradation of PAH in the mycorrhizosphere has been observed in pot experiments with *Glomus mosseae* (BEG 69) (Binet et al. 2000; Joner and Leyval 2003a,b). Using phospholipid fatty acid (PLFA) profiles, Joner et al. (2001) showed that this AM fungus can alter microbial community structure and suggested that the mycorrhiza-associated microflora may be responsible for the reduction in PAH concentration in the mycorrhizosphere. Several studies have reported that the development of AM can change bacterial community structure in the rhizosphere (Joner et al. 2001; Marschner et al. 2001; Marschner and Baumann 2003), and as extra-radical hyphal density can range between 1 and 30 m per gram of soil (Smith and Read 1997), AM mycelium may have significant effects, not only on bacterial communities, but also on their biodegradation activities.

By nature, the rhizosphere has a complex ecology with numerous feedback loops that regulate microbial populations (Toal et al. 2000), and interactions between the plant and its microflora control the fate and degradation of organic contaminants. Root exudation, water, and nutrient fluxes create quantitative and qualitative spatial gradients that affect microbial populations (Fang et al. 2001; Butler et al. 2003). Using compartmentalized systems, it was previously shown that PAH biodegradation in the rhizosphere of ryegrass is clearly a function of the distance from roots (Corgié et al. 2003), with increased PHE degradation observed in the immediate proximity of roots and degradation diminishing with increasing distance from them. Furthermore, bacterial densities and community structure changed with distance from roots in polluted and unpolluted rhizosphere (Corgié et al. 2004). Compartmentalized systems permit an accurate description of rhizosphere spatial dimensions of microbial populations, and although this approach simplifies to some extent rhizosphere ecology, compared to real soil environments, they allow easy manipulation of the ecosystem complexity.

In the present study, we have increased the biocomplexity of the system used by Corgié et al. (2004) by introducing the AM fungus, *Glomus mosseae* (BEG 69), as an additional actor in the rhizosphere ecosystem during the biodegradation processes. To evaluate the impact of the resulting mycorrhizosphere, we have compared: (1) the extent of the rhizosphere and the mycorrhizosphere as a function of distance to roots, (2) bacterial communities (density, structure, and activities) in a polluted rhizosphere and mycorrhizosphere, and (3) the resulting effect of *G. mosseae* colonization on PHE degradation and on bacterial catabolism activity measured as the quantity and transcriptional expression of a naphthalene1,2 dioxygenase gene (NDO), known to perform the primary and limiting step for oxidation of PAH in bacterial metabolism (Buchan et al. 2001; Story et al. 2000).

Materials and methods

Experimental system

Compartmentalized systems, as previously described by Corgié et al. (2003), were made with a T-shape PVC tube forming a vertical root compartment (3.5 cm diameter, 250 cm^3). Two horizontal compartments (3.5 cm diameter, 30 cm^3) were inserted at the bottom of the vertical one. The vertical compartment was connected through a mesh to a reservoir of sterile nutrient solution (1 mM NH_4NO_3, 1 mM $Ca(NO_3)_2$, 1 mM Na_2HPO_4 2 H_2O, 1 mM K_2SO_4, 0.75 mM $MgSO_4$ 7 H_2O, 12.5 μM H_3BO_3, 2.5 μM $MnSO_4$ H_2O, 0.3 μM $CuSO_4$ 5 H_2O, 1 μM $ZnSO_4$ 7H_2O, 0.05 μM Na_2MoO_4 2 H_2O, 0.2 μM $CoSO_4$ 7 H_2O, 20 μM Fe-EDTA; pH 6) that was refreshed on a weekly basis. To restrict root entry, the lateral compartments were separated from the central compartment by a 37-μm nylon mesh.

Two hundred spores of *Glomus mosseae* Gerd. & Trappe (BEG 69) (Weissenhorn et al. 1993) were inoculated during the transplantation of a 2-week-old seedling of ryegrass (*Lolium perenne* L. cv. Barclay) in the central compartment. *G. mosseae* BEG 69 was previously shown to improve plant growth in the presence of PAH (Binet et al. 2000) and to increase their disappearance in the rhizosphere (Joner and Leyval 2003b). Devices were preincubated for 2 weeks in a growth chamber (24/20°C day/night, 16-h light period, 60% RH, 300–350 mmol m^{-2} s^{-1} PAR) with lateral compartments containing sterile washed sand and saturated with sterile nutrient solution. Fresh lateral compartments containing polluted (500 mg kg^{-1} phenanthrene) or unpolluted inoculated sand were then inserted in the central compartment.

The microbial inoculum (final concentration of 10^5 bacteria per gram sand) was prepared as described by Corgié et al. (2003) from a PAH contaminated soil from the North of France (Joner et al. 2002). Five treatments (four replicates each) were performed: non-planted treatments with phenanthrene in the lateral compartments (no plant); planted pots without phenanthrene and inoculated (PHE-myc+) or not (PHE-myc−) with *G. mosseae* BEG 69; planted pots containing phenanthrene in the lateral compartments and inoculated (PHE+myc+) or not (PHE +myc−) with the AM fungus. After 6 weeks, lateral compartments were harvested in consecutive sections of 5 mm each. The sections 0–5 mm, 10–15 mm, and 20–25 mm were kept for analysis. One gram was frozen at −80°C before molecular analysis, 2 g were air-dried for PHE analysis, and 1 g was immediately used for bacterial enumeration.

PAH quantification

Extraction and quantification of remaining phenanthrene were performed at the IFA (Institute for Agrobiotechnology, Tulnn, Austria) on 2 g of dried samples from the lateral compartment. Phenanthrene was extracted with an automated Soxhlet extractor (Gerherdt Soxtherm extractor, model 2000 automatic, Bonn, Germany) according to Rost et al. (2002) and quantified by HPLC (Hewlett Packard) coupled with a HP 1100 series three-dimensional fluorescence detector (Hewlett Packard).

Microbial analysis

Hyphal length was measured by flotation using the intersection method on a membrane filter (Jakobsen et al. 1992). The results were expressed in meters of mycelium per gram (dry weight) of sand. The density of culturable heterotrophic bacteria (enumerated on Nutrient Broth), including PAH degrading bacteria (enumerated on a mixture of 4 PAH as sole source of C), was quantified with an enumeration procedure in microplates (Corgié et al. 2003), and the results were expressed as the most probable number (MPN) per gram of dry sand. Total DNA and total RNA were isolated from 1 g of sand (Corgié et al. 2004). The partial sequence of the *16S* gene (V6–V8) was

amplified by PCR using the bacterial universal primers 968f and 1401r (Heuer et al. 1999) as described by Corgié et al. (2004). Analysis of bacterial community structure was performed by TTGE (Dcode, Biorad) on polyacrylamide gel [6% (wt/vol) acrylamide, 0.21% (wt/vol) bisacrylamide, 8 M urea, 1.25×TAE and 0.2% (vol/vol) glycerol] at a constant voltage (100 V), with a temperature gradient from 57 to 67°C and an increment of 0.7°C per hour (Corgié et al. 2004).

Quantification of the Rieske [2Fe–2S] gene of the naphthalene1,2 dioxygenase

The Rieske [2Fe–2S] protein (Takizawa et al. 1994) is the active site of naphthalene1,2 dioxygenase (NDO), the sequence of which is highly conserved among the PAH dioxygenase family (Buchan et al. 2001). The protein sequence of the bacterial naphthalene dioxygenase (reference 1.14.12.12, Ligand database, Institute for Chemical Research, Kyoto University, Japan, http://www.genome.ad.jp/) was used to determine the sequence of the active site of the enzyme [protein Rieske (2Fe–2S)]. Alignments of the target sequence (EMBL-EBI, Cambridge College, U. K., http://www.ebi.ac.uk/embl/) were performed with Blastn software on the Genbank database to determine the most conserved region (*Pseudomonas putida*, position 104-369): 5′-GGTTTCGTTTGCAGTTATCACGGCTGG GGCTTCGGCTCCAACGGCGAACTGCAGAGCGTTC CATTCGAAAAAGAGCTGTACGGCGAGTCGCTCAAC AAAAAATGTCTGGGGTTGAAAGAAGTCGCTCGCGT AGAGAGCTTCCATGGGTTCATCTATGCCTGCATCGA TCAGGAGGCCCCTTCTCTTATGGACTATCTCGGTGA CGCTGCTTGGTACCTGGAACCCATCTTCAAACATTCA GGCGGTTTAGAACTGGTAGGCCCTCC-3′.

Two primers (NDO265scf: GGTTTCGTTTGCAGTTAT CA and NDO265scr: ATCTTGACCATCCGGGAGG) were designed to amplify the sequence from a reference PAH degrading bacterial strain (*Pseudomonas putida*, strain 8063 DSMZ). The PCR mix consisted in 50 mM buffer, 3 mM MgCl2, 0.2 mM dNTP, 1.2 mM of each primer, and 2U taq polymerase (Fastart, RocheDiagnostic). The probe (265 bp expected) was amplified from *P. putida* (strain 8063 DSMZ) with an iCycler (Biorad) using the following amplification program: 94°C for 5 min (1 cycle), 94°C for 30 s, 56°C for 30 s, 72°C for 30 s (35 cycles), and 72°C for 5 min (1 cycle). The purified probe was coupled with an alkaline phosphatase using the Gene Image Alkphos Direct labeling (Amersham Biosciences), according to the manufacturer's instructions, and stored at −20°C in 50% glycerol.

Reverse transcriptions were performed on crude extracts of nucleic acids. For each reaction, 8 µl of extracts were incubated for 30 min at 37°C with DNAse (RQ1 RNAse-free DNAse, Promega) in buffer plus inhibitors of RNAse (Recombinant Rnasin Ribonuclease Inhibitor, Promega). One microliter of STOP DNAse was added into each tube and the samples were incubated for 15 min at 70°C, then chilled on ice for 10 min. Reverse transcription was immediately performed with the Reverse Transcription System (Promega) with 11 µl of nucleic acid crude extract DNA-free to a final volume of 21 µl (1× buffer, 2.5 mM $MgCl_2$, 1 mM of each dNTP, 1 U/µl of Recombinant Rnasin Ribonuclease Inhibitor, 1.5 U/µl AMV Reverse Transcriptase, 25 ng/µl random primer).

Reverse transcription was performed with an iCycler (Biorad) using the following program: 10 min at 24°C, 60 min at 42°C, 5 min at 95°C, and, finally, 5 min at 4°C. DNA sample (20 µl) or cDNA sample (10 µl) were mixed with 10 µl of 20× SSC buffer and completed with water to a final volume of 100 µl. Samples were incubated for 5 min in boiling water, chilled on ice, and transferred to the blotting membrane (Hybond-N+, Amersham Biosciences) with a transfer cassette (48 wells, PR 648, Amersham Biosciences). Membranes were incubated at 80°C for 2 h.

The hybridization procedure was performed according to manufacturer's instructions with 10 ng of labeled probe per milliliter of hybridization buffer at 55°C overnight. Detection of hybridized NDO265 probe was performed with the Chemiluminescent CDP-Star Kit (Amersham Biosciences), and luminescence was revealed on a photographic film (HyperfilmTM, ECLTM, Kodak) exposed for 2 h to DNA blots and 12 h for cDNA blots. Films were developed, scanned and intensity of the hybridized probe signal was quantified using the Kodak 1D 3.5.2. software. The quantity of NDO265 DNA or cDNA was calculated comparatively to standards and expressed in femtogram per gram of sand (dry weight).

Statistical analysis

Analysis of variance (StatView) was performed on microbial numbers and PHE concentration. Principal component analyses were performed on relative band intensity from TTGE profiles (Corgié et al. 2004), and analysis of variance was made on coordination plots. Non-parametric tests were performed on quantities of NDO hybridized with crude DNA or cDNA from reverse transcript mRNA.

Results and discussion

The use of compartmentalized systems allows studies of the spatial distribution of rhizosphere communities and activities. When ryegrass plants were inoculated with *G. mosseae* (BEG 69), hyphal length in lateral compartments was similar (approximately 3.7 (±1.1) m g^{-1} sand) in the presence or absence of PHE, but was significantly higher (P=0.05) in the section between 0 and 5 mm, where hyphal density reached 4.9 (±1.8) m g^{-1} sand. No hyphae were detected in non-inoculated treatments. Jakobsen et al. (1992) showed that hyphal density could extend up to 11 cm in compartmentalized systems and decreased with distance to roots.

Bacterial community structure, compared using PCA analysis based on relative species abundances from TTGE gels (Fig. 1), showed variations arising from the presence of *G. mosseae*, the presence of PAH, and the distance from

roots. The first component (51% of total variance) separated the treatments as a function of distance from roots and the presence/absence of *G. mosseae*. The second component (32% of total variance) corresponded to the presence/absence of PHE. Community structures in the sections farthest from roots, at 10–15 mm and 20–25 mm, were always close, and they were similar to the one of the non-planted treatment in the PHE+myc− treatment. However, both were always significantly different (P=0.05) from those observed closest to the roots (0–5 mm) and which varied between treatments (Fig. 1). These results concur with those from previous experiments with similar compartmentalized systems of non-mycorrhizal plants, where bacterial community structures were shown to vary up to 9 mm from roots (Corgié et al. 2004). Treatment with PHE appeared to consistently affect bacterial communities in sections farthest from roots.

The presence of *G. mosseae* (BEG 69) modified the structure of bacterial communities in both PAH-polluted and non-polluted sand. Changes in bacterial community structure in rhizospheres colonized by AM fungi have already been reported (Joner et al. 2001; Marschner et al. 2001; Marschner and Baumann 2003). Without PHE, the communities of non-mycorrhizal and mycorrhizal plants were clearly different, coordinated in the left and the right of the PCA plot, respectively (Fig. 1), suggesting that microbial communities were selected by the presence of *G. mosseae*.

Colonization of the lateral compartment by *G. mosseae* (BEG 69) increased the number of culturable heterotroph and PAH degrading bacteria up to 25 mm from the root mat in the presence of PHE (Table 1). In the PHE+myc+ treatment, heterotroph and PAH degrader densities were also higher at 10–15 and 20–25 mm from the root compartment, compared to non-mycorrhizal plants (PHE +myc−). Without phenanthrene, *G. mosseae* increased the number of PAH degraders at all distances from roots, compared to the non-mycorrhizal treatment. The quantity of NDO265 probe hybridizing to the crude DNA was always higher for the treatments with PHE (Table 1), especially for the sections 0–5 and 10–15 mm of the PHE +myc− treatment. In the PHE+myc+ treatment, quantities were similar to the ones of the non-planted treatment. The quantity of NDO transcripts (cDNA) was higher in the first section of the PHE+myc+ treatment and represented approximately 47% of the quantity of genomic DNA, indicating that after 6 weeks, the NDO gene was highly expressed in that particular zone of the mycorrhizosphere.

Naphthalene1,2 dioxygenase is a α3β3 hexamer protein in which each subunit contains a (2Fe–2S) cluster (Colbert et al. 2000). The probe hybridizes with the *pahAc* gene (position 5578-5843, *Pseudomonas putida*) coding for the iron sulphur protein large subunit, part of the active site of the enzyme. This sequence is highly conserved in the PAH dioxygenase family, and the enzyme is involved in bacterial degradation pathway of PAH, including PHE (Buchan et al. 2001; Story et al. 2000). The quantity of NDO gene increased in the presence of PHE, especially in the sections 0–5 and 10–15 mm of the PHE+myc+ treatment. However, here the quantity of NDO transcripts reached only 3% of the NDO gene.

A significant gradient of PHE biodegradation was observed for the planted treatments (PHE+myc−, PHE+myc+) (Table 1). In the presence of *G. mosseae*, PHE

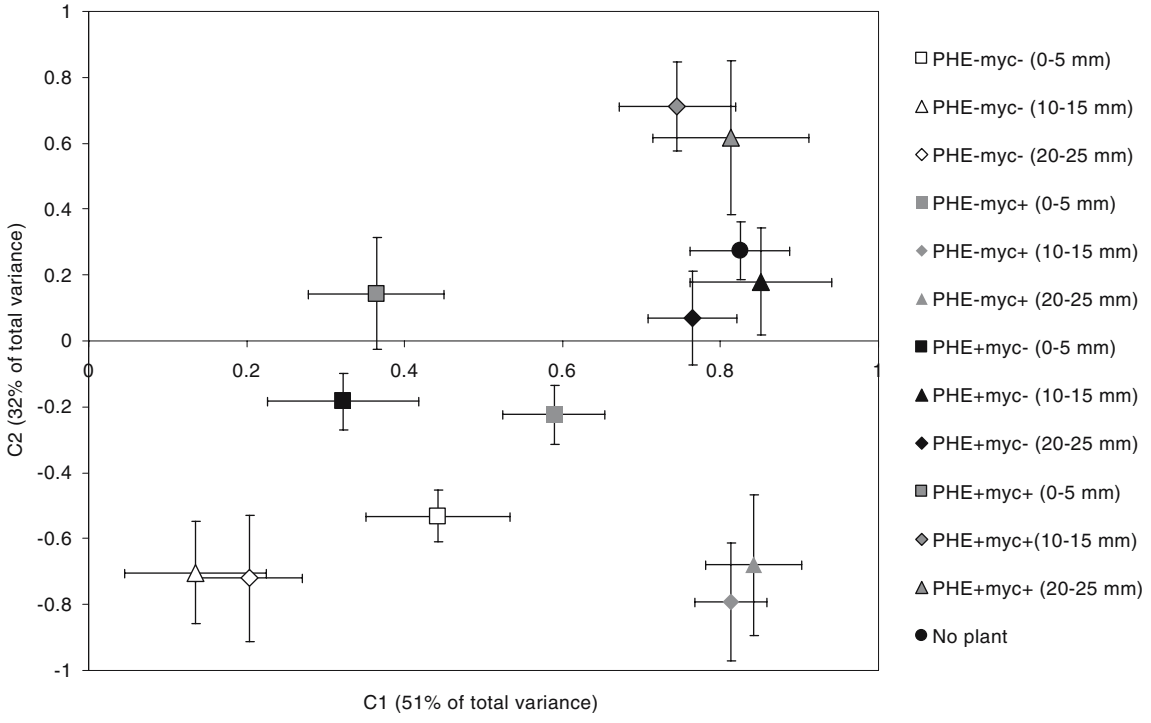

Fig. 1 PCA analysis of relative specie abundance of TTGE analysis. Data points represent the mean coordinate values (n=4) of corresponding sections within each treatment; *bars* are SEM

Table 1 Number of culturable heterotrophic bacteria, PAH degrading bacteria per gram of sand, quantity (fg g^{-1} sand) of probe NDO265 hybridized to DNA and cDNA, and PHE biodegradation (% of initial value and standard deviation)

	Sections	Heterotroph bacteria (per g sand)	PAH degrading bacteria (per g sand)	NDO (DNA) fg g^{-1} sand	NDO (cDNA) fg g^{-1} sand	PHE biodegradation	
						Percentage	(±) SD
No plant		$1.25\ 10^{7}$(c)	$7.07\ 10^{5}$(c)	$9.35\ 10^{3}$ (b)		76.92 (c)	5.32
PHE-myc−	0–5 mm	$6.31\ 10^{7}$(b)	$6.76\ 10^{3}$(e)	$3.41\ 10^{3}$(c)	$2.65\ 10^{3}$ (b)		
	10–15 mm	$1.90\ 10^{6}$(e)	$4.07\ 10^{2}$(f)	$5.38\ 10^{3}$ (c)	$1.85\ 10^{3}$ (b)		
	20–25 mm	$2.05\ 10^{6}$(e)	$4.57\ 10^{2}$(f)	$3.20\ 10^{3}$ (c)	$1.02\ 10^{3}$ (b)		
PHE-myc+	0–5 mm	$2.09\ 10^{7}$(b)	$6.45\ 10^{5}$(c)	$6.58\ 10^{3}$ (c)	$2.00\ 10^{3}$ (b)		
	10–15 mm	$3.74\ 10^{6}$(e)	$1.69\ 10^{4}$(d)	$4.69\ 10^{3}$ (c)	$2.77\ 10^{2}$ (c)		
	20–25 mm	$5.75\ 10^{6}$(d)	5.88 104(d)	$3.25\ 10^{3}$ (c)	$1.82\ 10^{3}$ (b)		
PHE+myc−	0–5 mm	$8.12\ 10^{7}$(a)	$8.71\ 10^{6}$(a)	$7.83\ 10^{4}$ (a)	$2.41\ 10^{3}$ (b)	92.95 (a)	0.93
	10–15 mm	$1.03\ 10^{7}$(c)	$7.41\ 10^{5}$(c)	$6.68\ 10^{4}$ (a)	$3.08\ 10^{3}$ (b)	82.12 (b)	4.31
	20–25 mm	$8.75\ 10^{6}$(c)	$5.75\ 10^{5}$(c)	$1.37\ 10^{4}$ (b)	$2.33\ 10^{3}$ (b)	77.36 (c)	5.34
PHE+myc+	0–5 mm	$1.69\ 10^{8}$(a)	$3.09\ 10^{7}$(a)	$1.11\ 10^{4}$ (b)	$5.23\ 10^{3}$ (a)	92.69 (a)	4.04
	10–15 mm	$3.02\ 10^{7}$(b)	$3.54\ 10^{6}$(b)	$1.06\ 10^{4}$ (b)	$1.95\ 10^{3}$ (b)	75.84 (c)	5.09
	20–25 mm	$5.20\ 10^{7}$(b)	$3.09\ 10^{6}$(b)	$1.29\ 10^{4}$ (b)	$1.36\ 10^{3}$ (b)	73.43 (c)	5.94

Values followed by the same letter within a column are not significantly different ($p>0.05$, $n=4$ for bacterial counts and PHE biodegradation, $n=3$ for NDO quantifications)

biodegradation was the highest in the first (0–5 mm) and last (20–25 mm) sections of the hyphal compartment. However, biodegradation was not significantly different in the presence or absence of *G. mosseae* for these sections, and was even lower in the 10–15-mm section, compared to the non-mycorrhizal plants (Table 1). Although *Glomus mosseae* BEG 69 has been previously reported to increase PHE degradation in the mycorrhizosphere (Binet et al. 2000; Joner and Leyval 2003b), degradation was not improved in the hyphal compartment in our experimental conditions. In pot experiments using industrial contaminated soils, Joner and Leyval (2003b) observed a region of the rhizosphere in which PAH dissipation was lower, or even nil, compared to bulk soils; however, this inhibition did not occur in the presence of an AM fungus in their study. In the present study, high degradation already by control non-mycorrhizal plants (92% in 6 weeks) may have prevented mycorrhiza from giving any further significant effect on biodegradation.

No correlation was observed between the numbers of heterotrophs and PAH degraders and the quantity of NDO from DNA or RNA analysis. As dioxygenase enzymes are involved in the early steps of PAH catabolism, NDO transcription should have been higher at the beginning of the experiment (Wilson et al. 1999) than at the end when 70 to 90% of PHE was degraded. Also, NDO expression was increased closest to roots in the presence of *G. mosseae* BEG 69. NDO transcription may not be directly related to PHE biodegradation as other enzymes, often linked to phthalate and catechol degradation (Ringelberg et al. 2001; Sei et al. 1999), are involved in the complete mineralization of PAH.

An extensive study of genes coding PAH degrading enzymes should be undertaken to identify bacterial catabolic activities in the rhizosphere (Buchan et al. 2001) and mycorrhizosphere.

Interactions between AM fungi and rhizosphere bacteria in compartment systems with restricted carbon sources (root exudates and PHE) may also contribute to the lower degradation of PAH in the rhizosphere of mycorrhizal plants. Competition for water and mineral nutrients between roots and soil microorganisms (George et al. 1992; Kaye and Hart 1997), or modification of root exudation by mycorrhizal roots (Laheurte et al. 1990; Azaizeh et al. 1995), may also be limiting factors to biodegradation. Vazquez et al. (2000) showed that bacterial activities could be affected by AM fungi and Olsson et al. (1996) attributed the reduction of bacterial activity to inhibitory compounds produced by ectomycorrhiza. AM fungi are not known to have direct biodegrading activities compared to saprophytes such as the white rot fungus (Bezalel et al. 1996; Zheng and Obbard 2000).

In conclusion, *G. mosseae* BEG 69 PAH increased degrading bacterial populations and the expression of the naphthalene dioxygenase gene in the mycorrhizosphere of ryegrass. However, extra-radical hyphae had no significant effect on PHE biodegradation compared to non-mycorrhizal root exudates in the present experimental conditions. Further studies should focus on effects of other environmental parameters controlling PAH biodegradation such as nutrients levels, soil carbon content, or the initial composition of bacterial communities. The compartmentalized system used here could be useful for screening different AM fungi in pure culture or mixed communities, and understanding of the biodegradation processes and effectiveness of bacterial communities in the mycorrhizosphere.

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