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Influence of three species of arbuscular mycorrhizal fungi on the persistence of aromatic hydrocarbons in contaminated substrates

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Abstract Aromatic hydrocarbons are pollutants which have mutagenic and carcinogenic properties as well as relatively high hydrosolubility. Their presence in soils makes techniques such as bioremediation an important topic for research. In this work, the effect of arbuscular mycorrhiza (AM) on the persistence of benzene, toluene, ethylbenzene and xylene (BTEX) in artificially contaminated substrates was evaluated. Leek plants were grown with three AM fungal species using a specially designed mesocosm system, in which internal air and substrate samples were analyzed by gas chromatography for BTEX content. Strong reductions in the BTEX concentration in substrates were generally observed in the presence of mycorrhizal plants. Residual BTEX content ranged between nearly total disappearance (<2%) and 40% of the original concentration, whereas there was a high persistence of hydrocarbons in the samples of substrate alone or with non-mycorrhizal plants. These results provide first evidence for an influence of AM activity in reducing pollution of substrates by aromatic hydrocarbons.

Keywords BTEX · Aromatic hydrocarbons · Arbuscular-mycorrhizal fungi · Soil pollution

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Introduction

Arbuscular mycorrhizal (AM) fungi have been widely studied as a potential tool in the reclamation of sites contaminated by various polluting agents (Joner and Leyval 1997, 2001, 2003; Leyval and Binet 1998; Joner et al. 2000; Binet et al. 2001; Leyval et al. 2002; Xiaolin et al. 2002). Whilst data are available concerning the interactions between AM fungi and different heavy metals (Haselwandter et al. 1994; Del Val et al. 1999; Joner et al. 2000; Gonzalez-Chavez et al. 2002; Rivera-Beceril et al. 2002; Liao et al. 2003; Malcovà et al. 2003; Vivas et al. 2003), the only available information about organic pollutants deals with the effect of AM on the fate of polycyclic aromatic hydrocarbons (PAH) in polluted soils (Leyval and Binet 1998; Binet et al. 2000; Joner and Leyval 2001, 2003; Leyval et al. 2002). The positive effect of AM fungi on plant growth, water and nutrient uptake in polluted soils has been postulated by Leyval and Binet (1998) and Joner and Leyval (2001). It has also been proposed that the influence of AM symbiosis on mycorrhizosphere structure may positively relate to the dissipation of pollutants from the soil (Joner et al. 2001). However, there have been no studies on the possible role of AM fungi in soils contaminated by aromatic hydrocarbons.

An important class of aromatic hydrocarbons is represented by benzene, toluene, ethylbenzene, *meta*-, *para*- and *ortho*-xylene (BTEXs). They constitute a significant percentage (up to 18%) of many petroleum derivatives (Christensen and Elton 1996). The spilling of gasoline and diesel fuel from underground leaking oil tanks, in oil distribution and storage stations, is one of the most common sources of BTEX pollution in soils (Chen et al. 1998; Lee et al. 2002; Iturbe et al. 2003, 2004). Due to their relatively high water solubility and vapour pressure (compared to other hydrocarbons), BTEXs can easily enter soils and groundwater. The risk for human health is related to their

acute and long-term toxic effects, including genotoxicity and carcinogenicity (Christensen and Elton 1996; Hayes et al. 1996; Irons and Stillman 1996; Murata et al. 1999). Phytotoxic effects such as inhibition of seed germination and plant growth have also been documented (Henner et al. 1999). The maximum concentrations allowed in soils are 0.1 mg kg^{-1} of dry soil for benzene and 0.5 mg kg^{-1} of dry soil for toluene, ethylbenzene and xylene in land used for habitation, public or private gardens, etc. (decree nos. 471/1999 and 22/1997, Italy, and guideline nos. 91/156/CEE, 91/689/CEE and 94/62/CEE). Values for commercial and industrial soils are 20-fold and 100-fold higher, respectively.

Biodegradation of BTEX aromatic hydrocarbons by fungi is well documented for non-symbiotic, saprotrophic species (Fedorak and Westlake 1986; Hemida et al. 1993; Middelhoven 1993; Yadav and Reddy 1993; Weber et al. 1995; Song 1997; Prenafeta-Boldú et al. 2001; Qi et al. 2002; Han et al. 2004; Prenafeta-Boldù et al. 2004). The degrading activity of bacteria, and especially of pseudomonads, is also widely reported, and this has been linked to the expression of genes encoding catabolic enzymes, like the *tou* operon (Bertoni et al. 1996, 1998; Chauhan et al. 1998). Positive interactions between AM and beneficial pseudomonads have been shown (Gamalero et al. 2004), and bacteria phylogenetically close to pseudomonads are known to be obligate endosymbionts of *Gigaspora margarita* (Bianciotto et al. 1996). The aim of this work was to evaluate the effects of leek plants colonized by three different AM fungi on the persistence of BTEX hydrocarbons in artificially polluted soils in a specially designed mesocosm system and to analyse the fungal genome for the presence of *tou* genes involved in BTEX metabolism.

Material and methods

Selection of a substrate suitable for plant growth and able to retain BTEX hydrocarbons

Three different substrates were tested: a natural soil rich in autochtonous mycorrhizal fungi (Massa et al. 2002) collected in the province of Alessandria, Italy, bentonite Saline Seal (Cetco, Merseyside, UK) and vermiculite (BPB Italia, Milano, Italy). Each substrate was mixed with quartz sand (50% v/v), and 500 ml of each mixture was amended with 1% w/w active carbon and supplemented with 200 µl benzene (>99.5), toluene (>99), ethylbenzene (>99) or xylene (>98.5) (Fluka, Buchs, Switzerland). Five grams of each sample was transferred into sealed tubes, and after heating at 60°C for 30 min, a 100-µl sample of the "head space" was collected and injected into a GC Variant 3400 gas chromatograph with a flame ionization detector (FID) and HS850 autosampler (CE Instruments). An SPB5 chromatographic column (Supelco, 30 m length, 0.53 ID, 3 µm film) was used (EPA 5021 method). The analysis was performed as follows: 37°C, 4 min; 37–95°C, 4°C min^{-1}; 95°C, 0.1 min; 95–145°C, 8°C min^{-1}; 145°C, 8 min; and 145–200°C, 25°C min^{-1}. Benzene, ethylbenzene, toluene and xylene (Fluka) were used as standards. The residual concentrations of the four hydrocarbons were evaluated (n=3) at time 0 and after 2, 5, 8, 24, 72 and 144 h.

To verify if the hydrocarbons adsorbed on the active carbon and mixed to the substrate were bioavailable, the vermiculite-based substrate was supplemented with a suspension of BTEX-degrading bacteria (BLF5500, Biofuture Ltd., Dublin, Ireland) under sterile conditions. One millilitre of bacterial suspension (10^6 UFC ml^{-1}) was supplemented to 50 ml of substrates. Samples without bacteria were used as negative controls. Head space gas chromatography was performed as described above to measure the residual BTEX concentration (n=3) after 0, 1, 5 and 11 days, incubation at room temperature. Differences in comparison to the respective control were evaluated by one-way ANOVA (p<0.05).

Plant culture in mesocosms

All materials used in the experiments were first sterilized overnight in commercial bleach (20% v/v in distilled water). *Allium porrum* L. seeds were surface-disinfected in commercial bleach (20% v/v in distilled water) for 3 min, washed 5 and 20 min (repeated six and four times, respectively) with sterile water and germinated on paper moistened with water in Petri dishes. Four-day-old plantlets were transferred into 50-ml tubes containing a 1:1 (v/v) mixture of sterile quartz sand and vermiculite. At this stage, each plantlet was inoculated with a minimum of 200 spores of either *Glomus mosseae* (Nicol. & Gerdemann) Gerd. & Trappe BEG12, *Gigaspora rosea* Gerd. & Trappe BEG9, or *Gi. margarita* Gerd. & Trappe BEG34. All the spores were surface-sterilized as described by Bécard and Piché (1992). Non-inoculated plants were prepared as controls. Plants were grown in a growth chamber (24°C, 16 h photoperiod, 150 µE m^{-2} s^{-1}) and fed three times per week with a Long Ashton nutrient solution (Hewitt 1966) modified according to Trotta et al. (1996) and containing 32 µM P. The nutrient solution was added at 3, 4 and 5 ml $plant^{-1}$ on the 1st, 22nd and 29th day of culture, respectively.

Mesocosms were set up using materials sterilized as described above. After 50 days of growth, plants were transferred with the substrate into 750-ml pots containing the same sand/vermiculite substrate mixed with active carbon (1% w/w) onto which the different BTEX had been adsorbed before at the same concentrations as in the substrate tests. Pots containing only the substrates were prepared as negative controls. All pots were transferred immediately into a mesocosm system designed and constructed to prevent the loss of pollutants by evaporation and to protect the operator from exposure to BTEX (Fig. 1).

Five mesocosms were set up, one for each of the hydrocarbons plus one without any pollutant for reference. Each mesocosm contained five pots per treatment (substrate only, non-mycorrhizal plants, plants inoculated with *G. mosseae*, *Gi. rosea* or *Gi. margarita*) that were placed on separate trays according to the fungal treatment to avoid

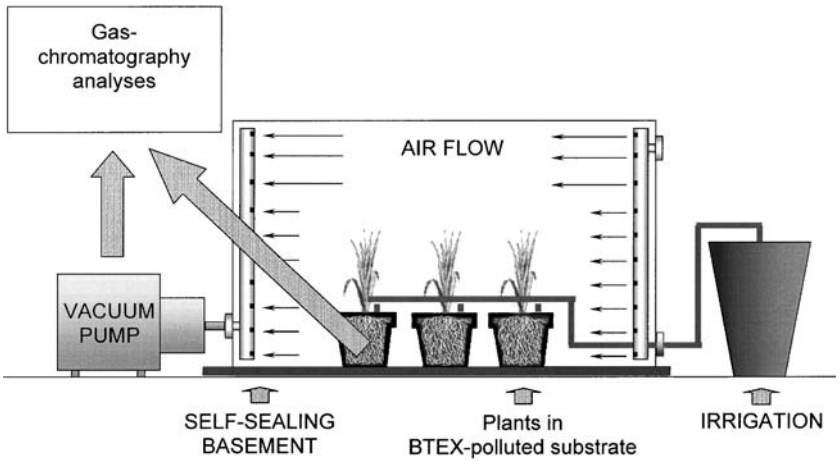

Fig. 1 Scheme of the mesocosm culture system. It consisted of a glass cabinet containing separate trays for the different fungal treatments. A set of tubes allowed the watering of plants without opening the cabinet. A second set of tubes was used to collect the air at the end of the experiment

cross-contamination (125 pots, 25 different treatments). To minimize hydrocarbon loss, watering was performed by means of plastic tubes (one for each pot) installed at the start of the mesocosm experiments. The mesocosms were placed in a glass-walled room, and plants were grown at 21°C, under natural daylight, for 17 days. Pots were supplemented with a Long Ashton solution modified as described above (100 ml pot^{-1}, three times per week).

A system of internal metal tubes was set up within the mesocosm to collect the internal air at the end of the experiment and to sample for BTEX which possibly evaporated from the substrates.

Air, substrate and plant analyses

All the air present in each cabinet was collected at the end of the experiment by means of a vacuum pump and run through a carbon cartridge to adsorb the BTEX (NIOSCH 1501 method). For substrate analyses, 5–10 g of substrate was sampled from each pot and transferred into sealed glass tubes. Gas chromatography analyses of residual BTEX were performed on air and substrate samples as described above.

Root and leaf fresh weight were measured. Roots were then fixed in a mixture of 25% acetic acid and 75% ethanol (v/v), for 1 h and stored in 70% ethanol. Mycorrhizal colonization and arbuscule frequency within the root systems were evaluated according to Trouvelot et al. (1986) after staining with 1% methyl blue in lactic acid. Significant differences between samples within the same treatment were evaluated by one-way ANOVA ($p<0.05$).

Analyses for *tou* genes in *Gi. margarita*

Polymerase chain reaction analyses were performed to check *Gi. margarita* (including endosymbiotic bacteria) for sequences homologous to the *Pseudomonas stutzeri* OX1 *tou*A, B C, D, E and F genes coding a toluene/*o*-xylene monooxygenase (Bertoni et al. 1996, 1998). Ten spores of *Gi. margarita* were rinsed in sterile H_2O, crushed in 40 µl Tris–ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and heated at 95°C for 10 min in the presence of 10 µl 20% Chelex-100 (Sigma). The crude DNA suspension was separated from cellular fragments by centrifugation at 12,000 *g* for 5 min, and 1/10 of volume was used for PCR amplification.

Specific oligonucleotides were designed for each *P. stutzeri* OX1 *tou* gene: *tou*A [forward (F) 5′-GGATGCATATGCTAAAACGTGAAG-3′; reverse (R) 5′-GACTAGTGTTAACAAGCCGCGTTGGTTTTTTTCTGG-3′], 1,494-bp amplification product; *tou*B (F 5′-GGATGCATATGGCGACGTTCCCGATTATG-3′; R 5′-GACTAGTGTTAACAGTTATCCATAAAAATAATATCGAGT-3′), 258-bp amplification product; *tou*C (F 5′-GGATGCATATGGCATTCGAAAAAATTTGCAC-3′; R 5′-GACTAGTGTTAACAGCTGTGGGACTTGAACGGTT-3′), 336-bp amplification product; *tou*D (F 5′-GGATGCATATGACAACCAACACAGTTCAAA-3′; R 5′-GACTAGTGTTAACAAAGGTGCTTATTGAAGTAGAAA-3′), 330-bp amplification product; *tou*E (F 5′-GGATGCATATGTCAGAACAACAACCTGAAG-3′; R 5′-GACTAGTGTTAACACAGCCCCATCATTTGGC-3′), 990-bp amplification product; and *tou*F (F 5′-GACTAGTGTTAACACAGCCCCATCATTTGGC-3′; R 5′-GGATGCATATGAGCAATAAGATAAAAATTGCC-3′), 1,023-bp amplification product.

Reactions were performed in a final volume of 50 µl containing 50 mM Tris–HCl, pH 9.0, 15 mM $(NH_4)SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 nM deoxynucleoside 5c-triphosphates (dNTPs), 200 nM of each primer, 1.5 U of *Taq* polymerase (Finnzymes) and 5 µl of DNA. Each reaction was performed in a thermal cycler (PCR Express, HYBAID) programmed as follows: initial denaturation cycle at 95°C (5 min) followed by 35 cycles of denaturation at 95°C (30 s), annealing at 55°C (30 s) and extension at 72°C (30 s); the last cycle was followed by a final extension at 72°C for 10 min.

A nested PCR reaction was performed for the *tou*A, E and F genes to enhance the efficiency of the amplification. Five µl of the first PCR amplification, diluted 1/1,000, served as template for the second reaction using specific internal sequence-coding oligonucleotides. The following primers were used: *tou*A (touAi F 5′-GGACCGTCGCT

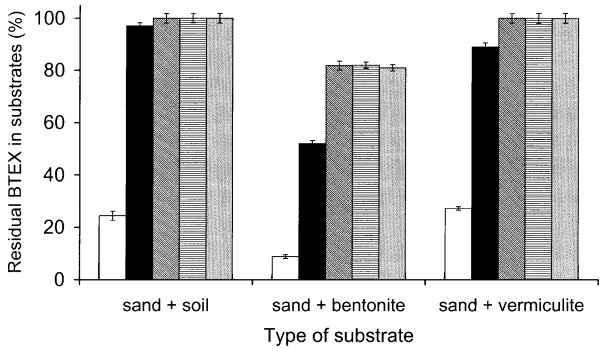

Fig. 2 Residual concentration of the aromatic hydrocarbons on three different substrates measured by gas chromatography after 144 h. Soil- and vermiculite-based substrates showed the highest persistence of hydrocarbons (□ indicates benzene; ■, toluene; ▨, ethylbenzene; ▤, *meta*- and *para*- xylene; and ▥, *ortho*-xylene)

CAAGATCCTG-3′;touA R as above), 782-bp amplification product; *tou*E (touEi F 5′-TGCGCTGGCTAACTCA CAC-3′; touE R as above), 503-bp amplification product; and *tou*F (F 5′-CTGACTGTCCCGGGTATTGAAGG-3′; touF R as above), 612-bp amplification product. The amplification conditions were as above except for 30 amplification cycles and an annealing temperature of 54°C.

Amplification products were separated by gel electrophoresis on 1.2% agarose gel Tris–acetate–EDTA (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). Controls consisted of PCR using the same primers on the *tou* genes cloned as cDNA in a pBlueScript expression vector (positive control) or water (negative control).

Results

Choice of culture substrate

Figure 2 shows the residual percentage of BTEXs in each substrate. A high persistence of hydrocarbons was observed in the soil-based mixture [residual percentages ranging between 24.36% (benzene) and 100% (ethylbenzene, xylenes)] and vermiculite-based mixture [residual percentages ranging between 27.24% (benzene) and 100% (ethylbenzene, xylenes)]. The bentonite-based mixture showed the lowest persistence. Inoculation of the polluted vermiculite-based substrate with BTEX-degrading bacteria resulted in a greater reduction ($p<0.05$) of the BTEX concentrations in comparison with non-treated controls, except for benzene which was the most volatile compound.

After 11 days of incubation, the residual BTEX concentrations of the samples inoculated with bacteria were: benzene, 58%; toluene, 34%; ethylbenzene, 53%; *m*-, *p*-xylene, 56%; and *o*-xylene, 60% of the corresponding controls (without bacteria). The observed reductions were statistically significant ($p<0.05$).

Influence of BTEX on plant growth and mycorrhiza development

Mycorrhizal leek plants showed a typical growth effect compared to non-mycorrhizal plants (30% size increase) when transferred to the BTEX-contaminated substrates. Plants colonized by *G. mosseae* or by *Gi. rosea* grew less than controls in the presence of toluene and ethylbenzene, respectively (Table 1). The ratio between root and shoot fresh weight was the same in all treatments except for an increase induced by *Gi. rosea* colonization in the presence of ethylbenzene (Table 1).

Glomus mosseae and *Gi. rosea* produced the highest levels of root colonization, although variable according to the different BTEX treatments. In contrast, colonization achieved with *Gi. margarita* was always very low. The presence of benzene in the substrate resulted in a significant reduction in colonization by *G. mosseae* in comparison to untreated samples, whilst ethylbenzene, xylene and toluene did not affect colonization (Table 2). The frequency of arbuscule formation showed the same trends as mycorrhizal colonization. No significant effect on root colonization by *Gi. rosea* was observed with any BTEX (Table 2). However, significantly lower arbuscule formation was observed in the presence of ethylbenzene. In all the other cases,

Table 1 Leaf weight and ratio between root and leaf weight in leek plants treated with BTEX, and colonized by *G. mosseae* (BEG12), *Gi margarita* (BEG34) or *Gi. rosea* (BEG9)

	AMF	BEG12	BEG34	BEG9
Leaf weight (g)				
BTEX	0.100±0.015a	0.108±0.022a	0.165±0.003a	0.117±0.035a
Benzene	0.110±0.016a	0.083±0.021a	0.138±0.031a	0.086±0.023a
Toluene	0.123±0.008a	0.085±0.011b	0.114±0.017ab	0.116±0.015ab
Ethylbenzene	0.131±0.013a	0.095±0.015ab	0.126±0.018ab	0.071±0.013b
Xylene	0.081±0.024a	0.091±0.021a	0.146±0.035a	0.083±0.018a
Root/leaf weight ratio				
BTEX	0.898±0.100a	0.960±0.066a	0.634±0.050a	0.650±0.167a
Benzene	1.140±0.229a	1.240±0.386a	1.094±0.205a	1.113±0.229a
Toluene	1.159±0.152a	1.513±0.286a	1.380±0.188a	0.984±0.118a
Ethylbenzene	0.866±0.124a	1.077±0.196a	0.960±0.136a	1.664±0.124b
Xylene	0.798±0.164a	1.156±0.280a	0.954±0.258a	1.093±0.160a

Controls are indicated as AMF (non-mycorrhizal) and BTEX (no hydrocarbons). Different letters indicate significant differences within the same BTEX treatment ($p<0.05$)

Table 2 Degree of mycorrhizal colonization and arbuscule formation in leek plants treated with BTEX and colonized by *G. mosseae* (BEG12), *Gi margarita* (BEG34) or *Gi. rosea* (BEG9)

Controls are indicated as AMF (non mycorrhizal) and BTEX (no hydrocarbons). Different letters indicate significant differences within the same fungus treatment ($p<0.05$)

	BTEX	Benzene	Toluene	Ethylbenzene	Xylene
Degree of mycorrhizal colonization (%)					
AMF	–	–	–	–	–
BEG12	51.141±2.636bc	31.292±6.786a	54.312±3.647b	51.060±5.603bc	39.000±2.651ac
BEG34	7.733±2.065a	5.701±1.998a	6.335±2.014a	11.461±3.333a	10.830±5.700a
BEG9	38.318±4.338a	32.409±7.514a	34.883±5.687a	24.702±5.240a	41.067±2.163a
Degree of arbuscule formation (%)					
AMF	–	–	–	–	–
BEG12	35.912±5.156bc	18.010±6.430a	40.374±9.123b	41.515±6.081bc	23.135±5.097ac
BEG34	4.046±0.944a	1.330±0.643a	2.031±1.005a	4.766±1.880a	5.173±3.093a
BEG9	28.369±3.792a	21.984±8.894ab	18.514±3.739ab	10.439±5.668b	31.888±3.369a

colonization and arbuscule formation were comparable to the control. Colonization and arbuscule formation by *Gi. margarita* were low in all cases, and no significant differences were induced by BTEX treatments.

BTEX contents in substrates and air

The residual percentage of benzene and toluene, compared with the original concentration, was 73 and 75%, respectively, in substrate samples in the absence of plants or fungi after 17 days (samples "f-p−", Fig. 3). Percent ethylbenzene and xylene were both about 93% (Fig. 3). This reflected the chemical properties of the hydrocarbons which have different vapour pressures. The substrate of non-mycorrhizal plants (samples "f-p+", Fig. 3) had BTEX concentrations that were never different from those of pots without plants and fungi.

A strong decrease of BTEX concentrations was observed in the substrates in the presence of plants colonized by AM fungi (Fig. 3). The effect was particularly significant for benzene in the case of leeks colonized by *Gi. margarita* on benzene (reduction to the 1.7% of the initial concentration). In the case of toluene and ethylbenzene, the highest reduction was achieved when plants were colonized by *Gi. rosea*, and the least with *G. mosseae*. Xylene showed the highest residual concentrations; values were significantly lower in the case of leeks colonized by *Gi. margarita* and comparable in the other cases. Gas chromatography analyses of air samples revealed high concentrations of hydrocarbons after 17 days except in the case of benzene (Fig. 4). Analysis of samples collected from the non-polluted cabinet showed a low background of BTEX.

Detection of *tou* genes in *Gi. margarita*

No PCR amplification product was observed from DNA extracted from spores of *Gi. margarita* using the primers designed for the amplification of the *tou* (*ABCDEF*) genes coding for a toluene/*o*-xylene monooxygenase in *P. stutzeri* OX1 (Bertoni et al. 1996, 1998; Cafaro et al. 2002) (Fig. 5). As expected, a positive signal was obtained when primers were used with the positive control (*tou* genes cloned into pBlueScript expression vector); no signal was observed with the negative control (water).

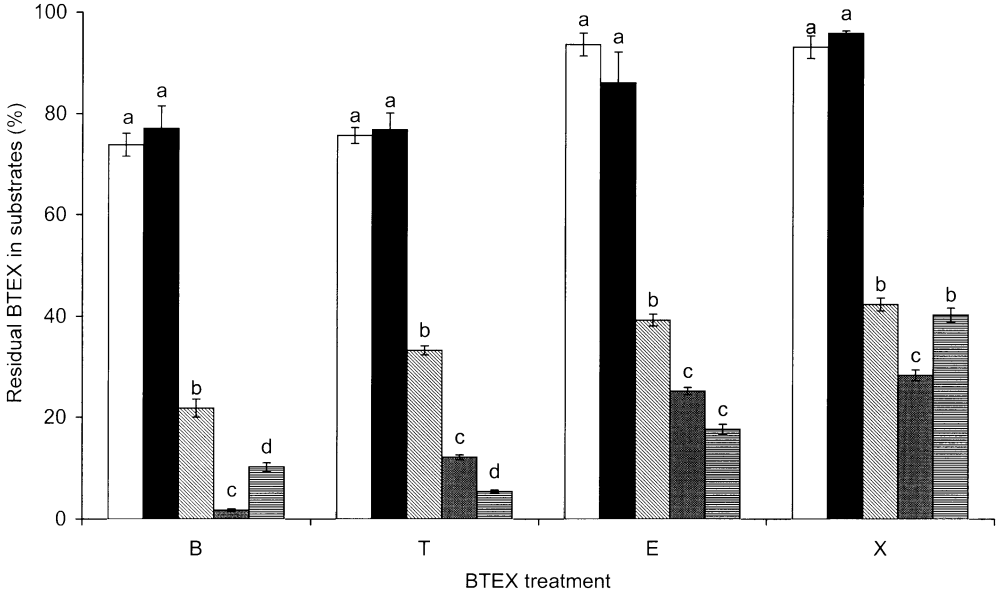

Fig. 3 Chemical analyses on substrates after 17 days of mesocosm cultures. Substrates where mycorrhizal plants had been grown showed a significant reduction of BTEX persistence. □ indicates f-p−; ■, f-p+; ▧, *G mosseae* (BEG12); ▩, *Gi margarita* (BEG34); and ▤, *Gi. rosea* (BEG9). Different *letters* indicate significant differences within the same BTEX treatment ($p<0.05$)

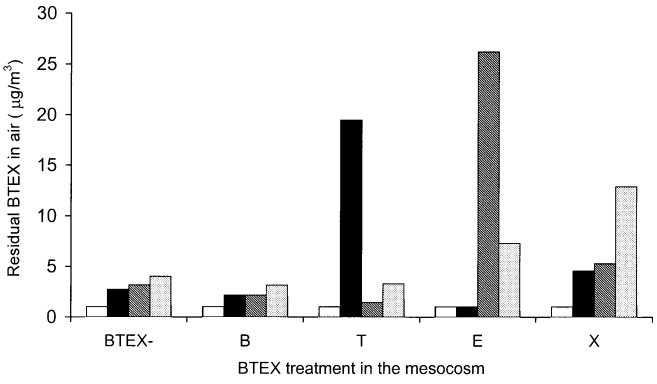

Fig. 4 Benzene, toluene, ethylbenzene and xylene concentration in the air of the mesocosm at the end of the experiment (17 days of culture) measured by gas chromatography. In each cabinet, the most abundant compound corresponded to the BTEX treatment applied, with the exception of benzene that was comparable to the levels observed in the control cabinet (*BTEX−*). □ indicates benzene; ■, toluene; ▨, ethylbenzene; and ⊡, xylene

Discussion

The present results show for the first time that plants colonized by AM fungi are able to strongly reduce the persistence of BTEX hydrocarbons in artificially polluted soils. Following preliminary analyses on candidate substrates using powdered activated carbon to apply hydrocarbon concentrations comparable to those previously documented for polluted soils (Margesin et al. 2003), a vermiculite-based substrate containing 1% w/w active carbon was retained as suitable for subsequent experiments. Good persistence of the BTEX was measured up to 144 h, and only small losses due to evaporation were observed. Evidence for the bioavailability of the hydrocarbons within this material was indicated by the significant decrease in the BTEX concentration in the vermiculite-based substrate after inoculation with bacteria commercially used for hydrocarbon degradation. The low overall persistence of the BTEX in this experiment was due to the smaller amounts of substrate used for the assays and inevitable evaporation of the hydrocarbons from the systems used.

Leek plants were inoculated with AM fungi prior to BTEX exposure to avoid stressful conditions during the establishment of symbiosis. A significant reduction of the BTEX was observed exclusively in the sand/vermiculite substrate containing mycorrhizal plants. BTEX levels remained very high in pots containing only the substrate or non-mycorrhizal plants, and residual concentrations were generally comparable to those observed in the preliminary tests on substrates. Since substrates and materials were sterilized before use, and plants were maintained in a confined environment, it is very unlikely that the consistent reduction in BTEX in pots containing mycorrhizal plants was due to differential bacterial contamination of these, and we therefore conclude that the major driver of BTEX reduction was the mycorrhizal symbiosis. A strong decrease in benzene and xylene was observed in substrates in the presence of *Gi. margarita*, whilst toluene and ethylbenzene concentrations decreased most in the presence of *Gi. rosea*. *G. mosseae* exerted the least effect. There was no obvious relation between root colonization levels and BTEX disappearance. Further experiments are needed to clarify whether these fungal effects are direct or indirect, acting for example through the host plant or linked to variations in physico-chemical conditions in the presence of the AM fungi, a hypothesis already discussed for organic contaminants (Joner et al. 2002).

Root colonization by *Gi. rosea* and *G. mosseae* was generally maintained high in the presence of BTEX, whilst much lower colonization was observed for *Gi. margarita* even in the absence of pollutants. The effect of BTEX exposure on root colonization varied with the type of pollutant and the AM fungus. Decreases in root colonization and arbuscule formation by *G. mosseae* were observed in the presence of xylene and, particularly, benzene, whilst *Gi. rosea* was more sensitive to toluene and ethylbenzene. Such detrimental influence of organics on AM colonization has previously been documented (Cabello 1997). Equal numbers of spores were used for each fungus, and results from preliminary experiments suggest that it is not spore germination that is greatly influenced by the presence of BTEX (data not shown). The decrease in BTEX concentrations observed in the presence of mycorrhizal plants can be due to a (partial or complete) degradation of pollutants

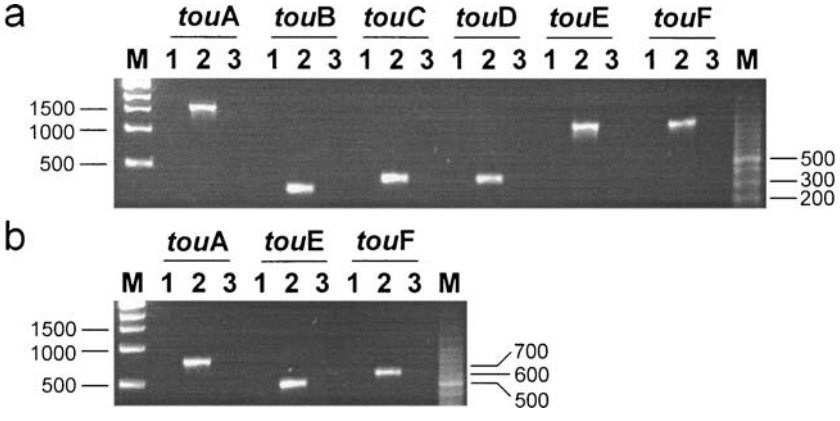

Fig. 5 Amplification products from first- (**a**) and second (**b**) nested PCR reactions on (*1*) water (negative control), (*2*) *tou* genes cloned as cDNA in a pBlueScript expression vector (positive control), (*3*) DNA from *Gi margarita* spores using specific primers for each *P. stutzeri* OX1 *tou*A-F gene (**a**) and specific internal primers for *tou*A, E and F genes (**b**). Amplification products were detected only for the positive control (*lane 2* of each sample)

as observed in several bacterial strains. Other possibilities could be an increased dispersion in the environment (but the low concentration of BTEX inside the cabinets makes this unlikely) or uptake and accumulation in plant tissues or in fungal structures. Plant accumulation of organics has been previously documented, the extent of the phenomenon depending on water solubility of the compound (Gao and Zhu 2004). The analytical techniques applied here do not allow identification of chemical substances derived from the catabolism or degradation of BTEX by the AM fungi or host plants, and therefore, no hypotheses can be proposed as to the fate of the pollutants. Further studies based on GC-MS analyses or using ^{14}C-labelled hydrocarbons can provide such information, as already documented for polycyclic compounds (Harms 1996; Binet et al. 2001). The PCR analyses on DNA from *Gi. margarita* spores (including the genome of the bacterial endosymbiont) clearly show that the genomes do not contain sequences homologous to bacterial genes coding for enzyme toluene/*o*-xylene monooxygenase, and therefore, this BTEX catabolizing activity cannot explain the AM-induced decreases in BTEX levels.

In conclusion, the experimental system developed here has provided first evidence for an effect of AM on the fate of BTEX hydrocarbons in a substrate. It is interesting to note that these effects varied with the AM fungal species and the nature of the BTEX. A number of studies have shown that a same plant species can respond differently to the presence of different AM fungal species, and similarly, a same fungal species can promote different responses in different plant species (Klironomos et al. 1998; Parke and Kaeppler 2000; Helgason et al. 2002) In this context, it would be interesting to evaluate further combinations of AM fungi and host plants for their influence on BTEX persistence in polluted soils.

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