Journal of Hazardous Materials 186 (2011) 1206–1217

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria

X.Z. Yu, S.C. Wu, F.Y. Wu, M.H. Wong*

Croucher Institute for Environmental Sciences, Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China

ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form 6 November 2010
Accepted 29 November 2010
Available online 4 December 2010

Keywords:
Bioremediation
Phytoremediation
Dissipation of PAHs
Bioavailability of PAHs
Soil peroxidase activity

ABSTRACT

The major aim of this experiment was to test the effects of a multi-component bioremediation system consisting of ryegrass (*Lolium multiflorum*), polycyclic aromatic hydrocarbons (PAHs)-degrading bacteria (*Acinetobacter* sp.), and arbuscular mycorrhizal fungi (*Glomus mosseae*) for cleaning up PAHs contaminated soil. Higher dissipation rates were observed in combination treatments: i.e., bacteria + ryegrass (BR), mycorrhizae + ryegrass (MR), and bacteria + mycorrhizae + ryegrass (BMR); than bacteria (B) and ryegrass (R) alone. The growth of ryegrass significantly ($p < 0.05$) increased soil peroxidase activities, leading to enhanced dissipation of phenanthrene (PHE) and pyrene (PYR) from soil. Interactions between ryegrass with the two microbes further enhanced the dissipation of PHE and PYR. Mycorrhizal ryegrass (MR) significantly enhanced the dissipation of PYR from soil, PYR accumulation by ryegrass roots and soil peroxidase activities under lower PHE and PYR levels (0 and 50 + 50 mg kg^{-1}). The present results highlighted the contribution of mycorrhiza and PAH-degrading bacteria in phytoremediation of PAH contaminated soil, however more detailed studies are needed.

1. Introduction

In situ bioremediation with bacteria has been recognized as a cost-effective method for the removal of PAHs from soil [1,2]. However, it is difficult to generate sufficient microbial populations with considerable PAHs-degrading capacity in natural soil to achieve an acceptable removal rate of PAHs [2]. The use of high plants in bioremediation processes promises advantages such as: (1) plants improve soil structure and thus aeration and hydrological aspects that may impose limitation to biodegradation; (2) plants may exude oxidative enzymes that contribute to PAH degradation; (3) plants physically translocate organic contaminants into their tissue, and then may transform or mineralize the contaminants; and (4) plant roots provide easily degradable carbon, energy that generally increases microbial activity in soil, which may lead to enhanced degradation of organic pollutants through direct metabolism or co-metabolism [3,4,5].

Arbuscular mycorrhiza fungi (AMF) form ubiquitous symbiosis with most herbaceous plant species, which have drawn considerable attention in the context of phytoremediation of organic compounds [6]. AMF are beneficial for plant establishment on PAH-contaminated site [7]. Joner and Leyval [8] further indicated the potential of AMF to enhance the dissipation of PAHs from soil. It has been shown that PAH dissipation around mycorrhizal roots was faster than that around nonmycorrhizal roots [9]. Although no evidence of direct PAH catabolism by AM fungi has been reported yet [10], increased degradation of PAH in the mycorrhizosphere inoculated with *Glomus mosseae* (BEG69) has been observed in pot experiments [11]; a more recent study revealed that AMF hyphae could facilitate the absorption of PAHs by host plant. In addition, PAHs with a lower molecular weight and higher water solubility seemed to be much easily taken up and translocated into plant root via hyphae [12]. These findings highlight the importance of AM inoculation to enhance plant uptake of organic contaminants [13].

Although fused polycyclic aromatic rings are stable and resistant to biodegradation, a variety of bacteria isolated from PAHs-contaminated soil or sediment have been gradually discovered capable of degrading or transforming PAH compounds under both aerobic and anaerobic conditions [14]. Accordingly, bioremediation (utilizing specific microorganisms to breakdown or to mineralize hazardous pollutants into less harmful or non-toxic compounds) has been proposed to clean up PAHs-contaminated soil [1]. For example, *Mycobacterium* sp. isolated from PAHs-contaminated soil nearby a former gaswork plant metabolized 60% of the pyrene added (0.5 mg/ml) in a mineral salt medium within 8 days [15]. In a soil added with the mixtures of PHE, PYR, and fluoranthene (Flu), inoculation of *Burkholderia* sp. degraded 97% of total PHE after 30 days, and 74% PYR and 88% Flu after 60 days [16]. The consensus is that low molecular weight PAHs (e.g. 2-ring and 3-ring PAHs) are readily degradable using these bacteria, while high molecular

* Corresponding author. Tel.: +852 3411 7746; fax: +852 3411 7743.
E-mail address: mhwong@hkbu.edu.hk (M.H. Wong).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.11.116

weight PAHs with more than three rings are difficult to degrade [16].

The initial reactions of PAH degradation by microorganisms (bacteria and white-rot fungi) are usually ascribed to their extracellular oxidative enzymes, i.e., oxygenase, dehydrogenase and lignolytic enzymes [17], which can catalyze radical formation by oxidation to destabilize bonds in a molecule [18]. In addition, degradation of PAHs by bacteria has been shown stimulated by root exudates and fine root turnover through providing readily available organic matters that would elevate bacterial population [19] and cometabolites (e.g. phenol, salicylate, and benzoate) that induce the cometabolism of PAHs [20]. Ryegrass (*Lolium multiflorum*) is widely used in the phytoremediation of PAH-contaminated sites owing to its fibrous root system and large specific root surface area [21].

However, the interactions among AMF, PAH-degrading bacteria and plant as well as their effects on PAH dissipation are still poorly understood. The objectives of this experiment were therefore to investigate: (1) the interaction of AMF, PAH-degrading bacteria and ryegrass, and their interactions on PAH dissipation and (2) the accumulation of PAH by ryegrass under the influence of AMF and PAH-degrading bacteria. Some possible mechanisms related to enhanced bioremediation including oxidative enzymes activity as well as PAH availability and its relationships with dissolved organic matter, plant accumulation and PAH dissipation were also studied.

2. Materials and methods

2.1. Preparation of PHE and PYR contaminated soil

The sandy loam (with clay 4.4; silt 19.7; sand 75.9%) soil used for this experiment was collected from Loi Tung Village, the New Territories of Hong Kong, and characterized by 1.26% organic matter, a pH of 6.20 and undetectable levels of PAHs [22]. After being air-dried, sieved through a 2 mm mesh and steam-sterilized at 121 °C for 2 h, appropriate concentrations of the mixtures of PHE and PYR (0, 50 + 50, 100 + 100, and 200 + 200 mg kg^{-1}) were spiked to soils to achieve the desired PAH concentrations. The soils were then kept in the dark at room temperature (about 20 °C) for 2 weeks prior to the experiment. Before starting the experiment, initial concentrations of PHE and PYR in spiked soil were analyzed, which were 39.01 ± 1.03, 69.39 ± 0.94, and 158.42 ± 4.20 mg kg^{-1} for PHE, 44.05 ± 1.10, 83.69 ± 2.40, and 189.73 ± 2.58 mg kg^{-1} for PYR in 50 + 50, 100 + 100, and 200 + 200 mg kg^{-1} spiked soils, respectively.

2.2. Isolation and inoculation of PAH-degrading bacteria

The PAH-degrading bacterium used in this experiment was isolated from PAH-contaminated soil obtained from Guiyu (an intensive electronic-waste recycling site in South China, Yu et al. [23]). The dissipation rates of PHE and PYR in the medium by *Acinetobacter* sp. over a period of two weeks were tested, and results showed that 32.2% PHE, and 15.8% PYR could be degraded separately as sole carbon sources, and when PHE and PYR were simultaneously added into the medium, their dissipation rates were 29.5 and 18.3%, respectively.

The isolated PAH-degrading bacterium was then cultured in a liquid medium (0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 0.6 g Na_2HPO_4, 0.02 g $MgSO_4 \cdot H_2O$, 1.0 g NH_4NO_3 and 0.1 g PHE per liter, pH 7.0, 28 ± 1 °C and 200 rpm) in the presence of 50 mg L^{-1} PHE and 50 mg L^{-1} PYR. The bacteria cells were harvested in log phase using a centrifuge (Beckman, USA) at 6000 rpm and washed twice with decreasing concentrations of the medium solution, and re-suspended in 5 ml of sterilized deionized water. Finally, the inoculum was transferred into the pots designed for bacterial inoculation treatment with an initial concentration of approximately 3.3×10^6 CFU g^{-1} dry soil.

2.3. Mycorrhiza inoculum and host plants

The AM fungi inoculum, *G. mosseae* obtained from Biorize Sarl, Dijon (France) was sand-based and rich in mycorrhizal root fragments and spores. The inoculum was thoroughly mixed into the soil (50 g kg^{-1} soil). Seeds of ryegrass (*L. multiflorum*), obtained from Nanjing Agriculture University, Jiangsu Province (China), were surface sterilized (10%, v/v, solution of hydrogen peroxide) for 10 min. After germination on a moist filter paper placed in Petri-dishes, they were transplanted to the pots (10 cm × 8 cm × 8 cm, L × W × H), each containing 15 one-week-old seedlings.

2.4. Experimental design

The experiment consisted of four levels of PAH-contamination (PHE + PYR): 0, 50 + 50, 100 + 100, and 200 + 200 mg kg^{-1} and six treatments: control, bacterial inoculation (B), ryegrass cultivation (R), ryegrass–bacteria cultivation (BR), ryegrass–mycorrhiza cultivation (RM) and ryegrass–mycorrhiza–bacteria (BMR), resulting in 24 treatments with 4 replicates each. After bacterial inoculation, AMF inoculation and transplant of ryegrass (15 seedlings each pot), all the pots were arranged randomly in a greenhouse, with temperature control (25–30 °C), and supplemented with additional illumination (with a light intensity of 250 μmol m^{-2} s^{-1}, under a 14/10 h – light/dark cycle). Mineral nutrients were added once at the rate of 162 mg N (urea), 126 mg K and 50 mg P (K_2HPO_4) kg^{-1} soil. The moisture of all pots was adjusted regularly to 70% of field water capacity with deionized water. Soil samples were collected at different intervals of 1, 3, 5, 10, 20, 40, and 60 days using a sterile cork borer for enumerating PAH-degrading bacteria population. After a growth period of 60 days, shoots and roots of ryegrass were harvested separately, and washed with deionized water. Parts of fresh root samples were randomly collected from each pot to determine the mycorrhiza infection rate of roots. Other root and shoot samples were then freeze-dried and ground, in preparation for PHE and PYR analyses. Soil samples were air-dried and analyzed for residual PHE and PYR after Soxhlet extraction. Soil samples were extracted with mild solvents (methanol or n-butanol) to estimate available fractions of PHE and PYR in soil. In addition, polyphenol oxidase activities, peroxidase activities and dissolved organic carbon (DOC) content in soil samples were determined.

2.5. Chemical analyses

Soil subsamples were extracted with butanol or methanol according to the methods described by Johnson et al. [24] and Liste and Alexander [25], with minor modifications. Briefly, 2.5 g soil sample and 1 g anhydrous sodium sulfate were mixed with 10 ml of n-butanol or methanol. The mixture was suspended on a vortex mixer for 2 min, and centrifuged at 3000 rpm. The obtained suspension was passed through a 0.20 μm polyether ester elastomer (TPFE) membrane filter, and then analyzed using HPLC (Agilent 1100). A 20 μl injection of the standard or extract was separated on the C-18 column (Agilent, 5 μm, 4.6 mm × 150 mm), heated constantly at 30 °C. The mobile phase was a mixture of methanol and water at a ratio of 85–15, with a flow rate of 1.0 ml/min. PHE and PYR were detected by a fluorescence detector (FLD) at excitation wavelength of 273 and 330 nm, emission wavelength of 350 and 390 nm, respectively. For the detection of total PHE and PYR in soil, root and shoot, samples were extracted according to Standard Method 3540C [26]. Subsamples were extracted according to the standard method. Florisil column was used for purifying the concentrated extract (Standard Method 3620B) [27]. The eluant was evaporated

Table 1
Mycorrhizal infection rates of ryegrass roots (% of total root length infected).

Treatments	PAHs addition (PHE + PYR) mg kg^{-1}			
	0	50 + 50	100 + 100	200 + 200
R	0	0	0	0
BR	0	0	0	0
MR	18%b	14%a	20%a	8%a
BMR	26%a	16%a	15%a	14%a

R, ryegrass cultivation; BR, bacteria–ryegrass cultivation; MR, AMF–ryegrass cultivation; BMR, bacteria–AMF–ryegrass cultivation.
Values within the same column followed by the same letter are not significantly ($p < 0.05$) different ($n = 4$).

to less than 2 ml prior to analysis. PHE and PYR concentrations were analyzed using GC–MS (Agilent GC 6890N with 5390 Mass Selective detector), based on Standard Method 8270C [28]. Mycorrhiza infection rate of roots (infected root length as percentage of total root length) was evaluated with a line-intersect method after staining with trypan blue [29]. The DOC of fresh soil samples was extracted with deionized water (soil:water = 1:5), and analyzed with a total organic carbon (TOC) analyzer (Shimadzu TOC-Vcph, Japan).

2.6. Enumeration of PAH-degrading bacteria

The population of PAH-degrading bacteria in soil during the experiment was estimated using the medium described for bacterial culture, which contained 100 mg L^{-1} PHE. There were three replicates for each dilution, and all plates were incubated at 28 °C. The colonies formed in all plates were counted after 5 days. The number was expressed as colony-forming units (CFU) g^{-1} dry soil.

2.7. Enzyme activity analysis

Soil polyphenol oxidase (E.C. 1.10.3.1) activity was measured according to the colorimetric method described by Chen et al. [30] with minor modifications. Pyrogallic acid was employed as a substrate. The mixture of 1 g soil and 10 ml 1% pyrogallic acid was incubated at 30 °C for 2 h, and 4 ml citric–phosphoric acid buffer (pH 4.5) was added to the mixture. The purpurigallin produced was extracted with ether, and then measured by a spectrophotometer (SHIMADZU, UV-1601) at 430 nm. Peroxidase (EC 1.11.1.7) activity was analyzed following the same procedure as polyphenol oxidase activity, except that 2 ml 0.5% H_2O_2 together with 10 ml 1% pyrogallic acid was used as a substrate.

2.8. Statistical analyses

Means of data were compared using Student–Newman–Keul's multiple comparison test at the 5% probability level. To investigate the effects of PAH addition, PAH-degrading bacteria, AMF and their interactions on accumulation of PAH by ryegrass shoots and roots, three-way analyses of variance (ANOVA) were used. The statistical analyses were carried out using SPSS 11.0.0. The correlation analyses of mild solvent extractable PAH with PAH dissipation rate, PAH content in ryegrass and soil DOC content were performed using Sigmaplot 9.0.1. The PAH dissipation rate was calculated as: (initial PAH concentration − residual concentration) × 100/initial PAH concentration.

3. Results

3.1. Mycorrhiza infection rates of ryegrass roots and biomass of the plants

Mycorrhizal colonization was only found in the treatments with AMF inoculation (MR treatment and BMR treatment). Colonized roots comprised 8–26% of the total root length (Table 1). In the MR treatments (50 + 50 and 100 + 100 mg kg^{-1}), no significant ($p > 0.05$) adverse effects of PAHs on the mycorrhiza infection rates were observed. At the highest level of PAH (200 + 200 mg kg^{-1}), the mycorrhiza infection rates were significantly lower ($p < 0.05$) than that of unspiked control (at PAH level of 0 mg kg^{-1}). Inoculation with PAH-degrading bacteria *Acinetobacter* sp. significantly ($p < 0.05$) increased the mycorrhiza infection rates of ryegrass roots in unspiked soil, but did not significantly ($p > 0.05$) increase the mycorrhiza infection rates in soil spiked with PAHs. The inoculation of AMF significantly stimulated the growth of ryegrass while the biomass of plant was significantly decreased due to the presence of PAHs (Table 2). In general, the shoot and root biomass of ryegrass in all treatments decreased with increasing of PAHs concentration

3.2. Bacterial population

The populations of PAHs-degrading bacteria (*Acinetobacter* sp.) were monitored in all treatments throughout the 60-day experiment. No target bacteria as well as other PAHs-degrading

Table 2
Effects of PAH-degrading bacteria (*Acinetobacter* sp.) and *Glomus mosseae* on shoot and root biomass of ryegrass growing on PAHs-spiked soils (mean, $n = 4$).

Treatments	Shoot biomass (g pot^{-1}, dry weight) PAHs addition (PHE + PYR) mg kg^{-1}				Root biomass (g pot^{-1}, dry weight) PAHs addition (PHE + PYR) mg kg^{-1}			
	0	50 + 50	100 + 100	200 + 200	0	50 + 50	100 + 100	200 + 200
R	6.16	4.88	5.25	4.55	2.11	1.37	1.58	0.87
BR	6.42	5.21	5.23	4.06	1.72	1.13	1.40	0.66
MR	7.35	5.83	5.89	5.00	3.76	1.61	1.31	1.41
BMR	6.88	5.73	5.65	5.41	2.86	1.63	1.36	2.59

Analyses of variance	*F*-values	Sig.	*F*-values	Sig.
PAH	75.3	0.000***	36.0	0.000***
Mycorrhiza	64.0	0.000***	50.2	0.000***
Bacteria	0.191	0.664	0.673	0.416
PAH × mycorrhiza	0.723	0.543	13.4	0.000***
PAH × bacteria	0.344	0.794	5.32	0.003**
Mycorrhiza × bacteria	0.440	0.510	2.97	0.091
PAH × mycorrhiza × bacteria	3.626	0.091	3.85	0.015*

R, ryegrass cultivation; BR, bacteria–ryegrass cultivation; MR, AMF–ryegrass cultivation; BMR, bacteria–AMF–ryegrass cultivation.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.000$.

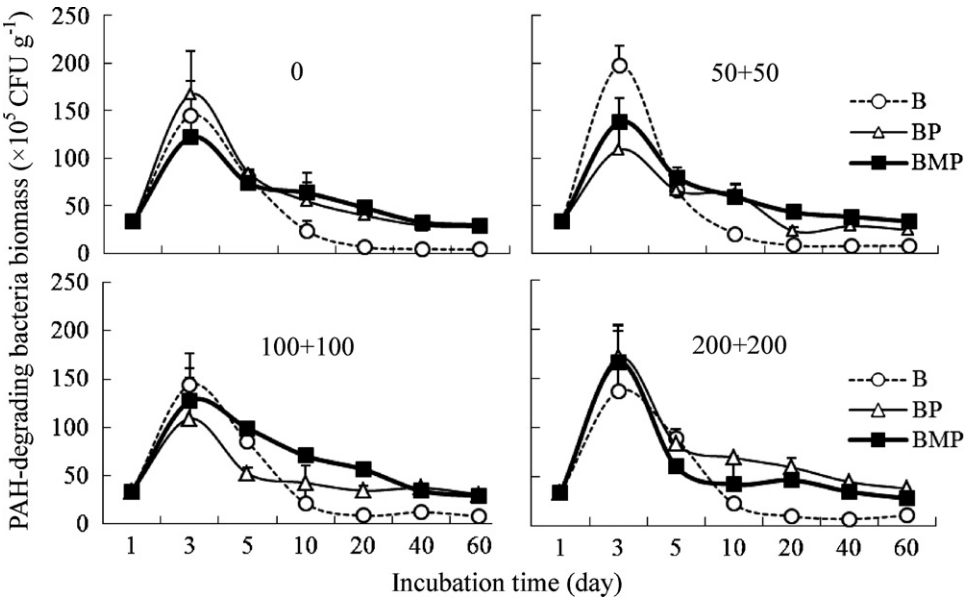

Fig. 1. PAH-degrading bacteria population. Vertical bars represent standard deviations. B, bacterial inoculation; BR, bacteria–ryegrass cultivation; BMR, bacteria–AMF–ryegrass cultivation.

bacteria were detected in the soil from treatments without bacterial inoculation (treatments of control, ryegrass cultivation and AMF–ryegrass cultivation). The population size of *Acinetobacter* sp. from treatments with bacterial inoculation (treatment of bacterial inoculation, bacteria–ryegrass cultivation and bacteria–AMF–ryegrass cultivation) ranged from 10^5 to 10^7 CFU g^{-1} dry soil (Fig. 1). In the treatment with only inoculation of *Acinetobacter* sp., the population increased to the highest density of 10^7 CFU g^{-1} dry soil on the third day, and then decreased sharply and eventually remained at 10^5 CFU g^{-1} dry soil regardless of the concentrations of PAHs. However, when ryegrass was planted (treatments of bacteria–ryegrass cultivation and bacteria–AMF–ryegrass cultivation), the population of *Acinetobacter* sp. was maintained at 10^6 CFU g^{-1} dry soil until harvest. According to the statistical results, ryegrass cultivation significantly ($p < 0.01$) improved the bacterial population, while spiked PAHs or inoculated AMF had no marked effect ($p > 0.05$) on the growth of *Acinetobacter* sp.

3.3. PAH dissipation from soil

Fig. 2 shows the dissipation rate of PHE and PYR in each treatment. The presence of *Acinetobacter* sp. (B), ryegrass (R), or AMF (M) as well as their combined cultures significantly ($p < 0.05$) enhanced the dissipation of PHE and PYR from soil when compared with control. The dissipation of PHE from soil was dramatic with the loss of 58–71% in control soil, and 91–97% PHE in B and R treatments at the end of 60 days. The dissipation rates were even much

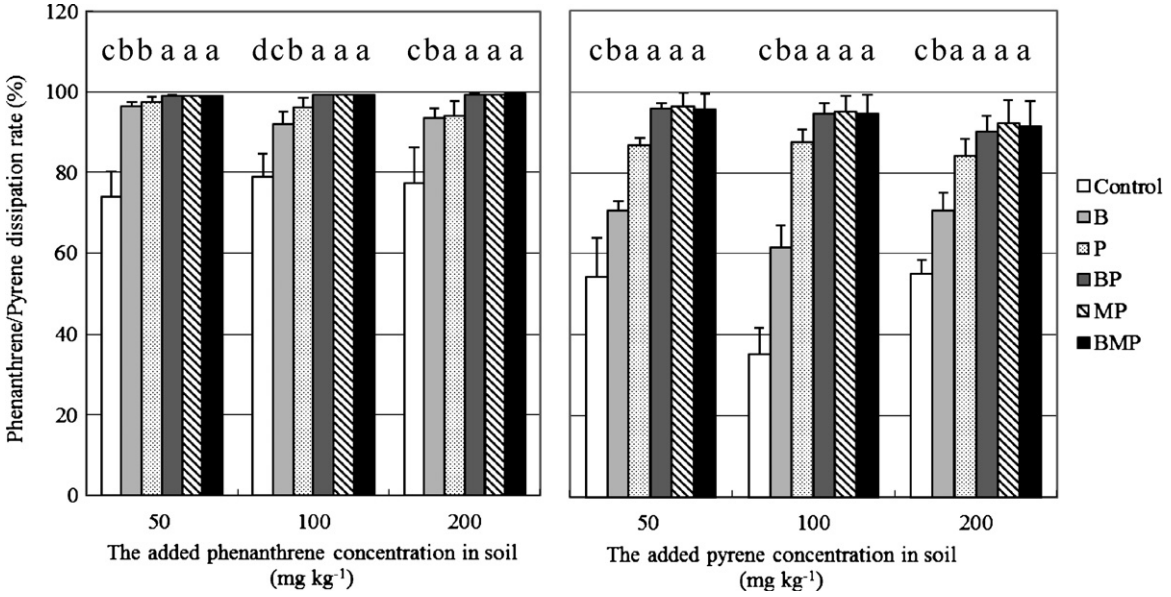

Fig. 2. Dissipation rates of PHE and PYR from soil. Vertical bars represent standard deviations. Bars with the same letter at the same PAHs level are not significantly ($p < 0.05$) different (4 replicates) according to the SNK test. Control: without bacteria, AMF and ryegrass; B, bacterial only; R, ryegrass only; BR, bacteria–ryegrass; MR, AMF–ryegrass; BMR, bacteria–AMF–ryegrass.

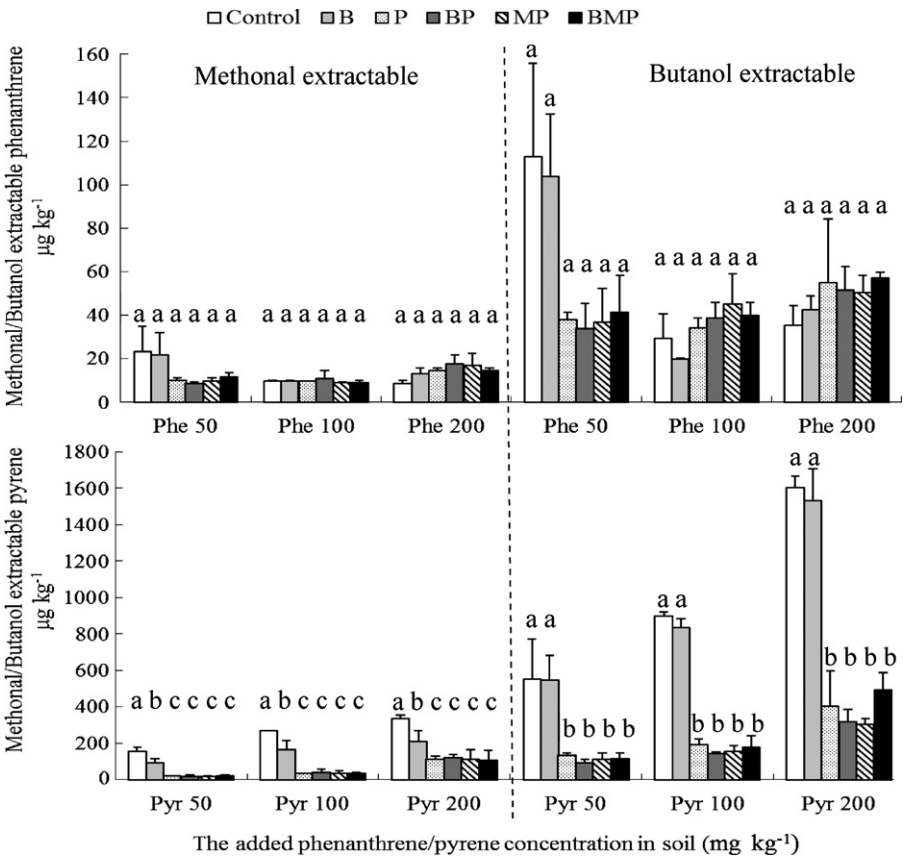

Fig. 3. Methanol and butanol extractable PHE and PYR in soil. Vertical bars represent standard deviations. Bars with the same letter at the same PAHs level are not significantly ($p < 0.05$) different (4 replicates) according to the SNK test. Control: without bacteria, AMF and ryegrass; B, bacterial only; R, ryegrass only; BR, bacteria–ryegrass; MR, AMF–ryegrass; BMR, bacteria–AMF–ryegrass.

higher than expected. In BR, MR and BMR treatments, the dissipation rates of PHE were higher than 99%. A lower dissipation rate of PYR from control soil was found, with only 35–55%. Ryegrass (R) alone or inoculation with *Acinetobacter* sp. (B) significantly ($p < 0.05$) enhanced the dissipation rate of PYR, while the highest dissipation rates (90–96%) were observed in the treatments of BR, MR and BMR.

3.4. Mild solvent extractable PAHs

Fig. 3 shows that the mild solvent extractable PHE concentrations ranged from 9 to 23 $\mu g\,kg^{-1}$ (methanol extraction) and from 20 to 113 $\mu g\,kg^{-1}$ (butanol extraction), respectively, which were much lower than mild solvent extractable PYR concentrations. There were no significant differences of mild solvent extractable PHE concentrations between each treatment (control, B, R, BR, MR and BMR treatments). Significantly higher ($p < 0.05$) mild solvent extractable PYR (92–334 $\mu g\,kg^{-1}$) was detected in control and bacteria inoculated soil, when compared with other treatments. Treatments of R, BR, MR and BMR had similar concentrations of mild solvent extractable PYR (methanol extraction: 16–120 $\mu g\,kg^{-1}$ and butanol extraction: 93–496 $\mu g\,kg^{-1}$).

3.5. PAH accumulation by ryegrass

PHE and PYR concentrations in ryegrass shoots and roots are shown in Tables 3 and 4, respectively. Low concentrations of PHE and PYR were detected in ryegrass shoots (but not in roots), even in those grown in un-spiked soil. In soil spiked with PAHs, PHE and PYR concentrations of shoots were higher than those in unspiked soil. PYR concentrations of shoots in spiked soil co-cultivated with *Acinetobacter* sp. and AMF (BMR) were significantly ($p < 0.05$) higher than those of R, BR and MR. PYR concentrations in ryegrass roots increased with the increase in spiked PAH, which were much higher than PHE in corresponding treatments. Inoculation with AMF alone or together with *Acinetobacter* sp. significantly ($p < 0.05$) enhanced PYR accumulation by ryegrass roots.

3.6. Soil enzyme activities

Peroxidase activities in un-spiked control soil did not differ significantly from those in PAH-spiked control soil (Fig. 4). The growth of ryegrass significantly ($p < 0.05$) increased peroxidase activities in soils of R, BR, MR and BMR treatments, when compared with control soil. Inoculation of AMF in soil with ryegrass significantly ($p < 0.05$) increased peroxidase activities under lower PAH levels (0 and 50 + 50 $mg\,kg^{-1}$), but no significant differences among R, BR, MR and BMR treatments were found under higher levels (100 + 100 and 200 + 200 $mg\,kg^{-1}$). Inoculation with *Acinetobacter* sp. alone had no significant impacts on peroxidase activities when compared with control at 0, 100 + 100, and 200 + 200 $mg\,kg^{-1}$ PAHs.

Polyphenol oxidase activities in PAH-spiked control soil were much lower ($p < 0.05$) than those in control soil (Fig. 4). Inoculation with *Acinetobacter* sp. significantly increased polyphenol oxidase activities when compared with control soil. However, polyphe-

Table 3
Effects of bacteria and mycorrhiza on PAH accumulation in ryegrass shoot (mg kg^{-1} dry matter basis).

Treatments	PHE				PYR			
	0	50 + 50	100 + 100	200 + 200	0	50 + 50	100 + 100	200 + 200
R	0.021a	0.086 a	0.059a	0.054a	0.049a	0.144b	0.089b	0.173b
BR	0.025a	0.089a	0.052ab	0.057a	0.040a	0.159b	0.097b	0.177b
MR	0.028a	0.089a	0.049b	0.063a	0.052a	0.158b	0.156a	0.177b
BMR	0.029a	0.091a	0.056ab	0.058a	0.044a	0.212a	0.144a	0.246a

Analyses of variance	*F*-values	Sig.	*F*-values	Sig.
PAH	210	0.000***	73.3	0.000***
B	0.316	0.578	4.01	0.054
M	1.98	0.169	18.7	0.000***
PAH × B	0.250	0.860	2.46	0.080
PAH × M	1.20	0.326	2.13	0.115
B × M	0.020	0.889	1.98	0.169
PAH × B × M	1.77	0.172	1.59	0.212

R, ryegrass cultivation; BR, bacteria–ryegrass cultivation; MR, AMF–ryegrass cultivation; BMR, bacteria–AMF–ryegrass cultivation.
Values within the same column followed by different letters were significantly ($p < 0.05$) different among different treatments at the same PAHs concentration according to the SNK test.
*** $p < 0.000$.

nol oxidase activities in treatments with ryegrass (R, BR, MR and BMR) were all significantly ($p < 0.05$) lower than those in control soil. There were no significant differences of polyphenol oxidase activities among R, BR, MR and BMR treatments.

3.7. Correlations of mild solvent extractable PAHs with root PAHs, PAHs dissipation rate and soil DOC

Fig. 5 shows that PHE or PYR contents of ryegrass roots (R, BR, MR and BMR) were significantly (r^2: 0.584–0.753, $p < 0.01$) correlated with methanol or butanol extractable soil PHE or PYR. A highly significant (r^2: 0.663, 0.582; $p < 0.001$) hyperbola relationship ($y = y0 + ax/(b + x)$) was found between dissipation rates of PHE and available proportions of PHE (mild solvent – PHE/Soxhlet – PHE) (Fig. 6). Dissipation rates of PYR and available proportions of PYR (mild solvent – PYR/Soxhlet – PYR) were linearly correlated, but not statistically significant ($r^2 = 0.207$, $p > 0.05$; $r^2 = 0.202$, $p > 0.05$) (Fig. 6). DOC content of soil was significantly ($p < 0.05$) correlated with methanol or butanol extractable soil PYR, while no significant correlations were observed between DOC content and mild solvent extractable PHE (Fig. 7).

4. Discussion

4.1. Effects of PAHs and PAH-degrading bacteria on mycorrhiza infection rates of ryegrass roots

In this study, no significant adverse effects of PAHs on the mycorrhiza infection rates of ryegrass roots were detected at 50 + 50 and 100 + 100 mg kg^{-1} PAH. At the highest PAH level of 200 + 200 mg kg^{-1}, the mycorrhiza infection rate was significantly ($p < 0.05$) lower than that of unspiked control (Table 1). This is in agreement with results of Gaspar et al. [31], who found that there was a restriction of fungal hyphal growth in the presence of PHE. Cabello [32] also noted soil hydrocarbon contamination had negative effects on AMF-symbiosis, in terms of arbuscles percentage, vesicle number and entry point number.

In addition, inoculation with PAH-degrading bacteria significantly increased the mycorrhizal infection rates in control but not in the treatments spiked with PAHs. Fitter and Garbaye [33] has already reported that some soil microorganisms can act as 'mycorrhization helpers', which improve the ability of mycorrhizal fungi to colonize plant roots. The mechanisms by which these soil microorganisms stimulate AM colonization are still not fully understood.

Table 4
Effect of bacteria and mycorrhiza on ryegrass root PAH concentration (mg kg^{-1} dry matter basis).

Treatments	PHE				PYR			
	0	50 + 50	100 + 100	200 + 200	0	50 + 50	100 + 100	200 + 200
R	ND	0.369a	0.338a	0.759b	ND	3.59b	4.67b	26.7c
BR	ND	0.393a	0.358a	0.696b	ND	3.87b	4.53b	44.5b
MR	ND	0.546a	0.388a	0.855b	ND	5.27a	5.90 a	109.9a
BMR	ND	0.470a	0.419a	1.19a	ND	4.87a	6.56a	127.3a

Analyses of variance	*F*-values	Sig.	*F*-values	Sig.
PAH	254	0.000***	595	0.000***
B	2.32	0.137	8.58	0.006**
M	28.3	0.000***	201	0.000***
PAH × B	2.55	0.073	8.32	0.000***
PAH × M	8.17	0.000***	183	0.000***
B × M	3.01	0.092	0.001	0.979
PAH × B × M	5.95	0.002**	0.012	0.998

R, ryegrass cultivation; BR, bacteria–ryegrass cultivation; MR, AMF–ryegrass cultivation; BMR, bacteria–AMF–ryegrass cultivation.
Values within the same column followed by different letters were significantly ($p < 0.05$) different among different treatments at the same PAHs concentration according to the SNK test.
** $p < 0.01$.
*** $p < 0.000$.

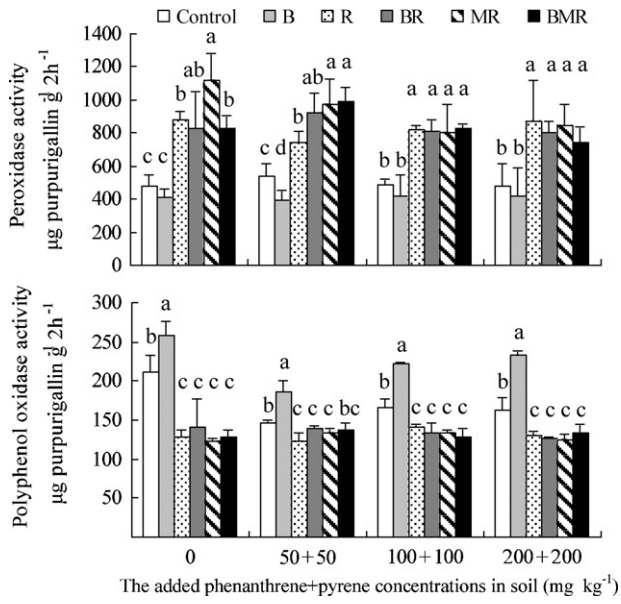

Fig. 4. Peroxidase and polyphenol oxidase activities in soil. Vertical bars represent standard deviations. Bars with the same letter at the same PAHs level are not significantly ($p < 0.05$) different (4 replicates) according to the SNK test. Control: without bacteria, AMF and ryegrass; B, bacterial only; R, ryegrass only; BR, bacteria–ryegrass; MR, AMF–ryegrass; BMR, bacteria–AMF–ryegrass.

Specialized bacterial activities such as the production of vitamins, amino acids, and hormones may be involved in these interactions [34]. The presence of rhizobacterial inoculation might have assisted in the germination of a large number of spores thus leading to a higher AMF infection percentage [35]. Some endophytic species of plant-growth-promoting-rhizobacteria (PGPR) were known to excrete cellulase and pectinase [36,37] and these enzymatic activities would no doubt aid in mycorrhizal infection. However, such interactions between soil microorganisms and AMF may be interrupted by the presence of PAHs. Gaspar et al. [31] demonstrated that the inoculation of *Rhodotorula glutinis*, a yeast which can degrade PAH, together with AMF did not enhance AMF colonization in the presence of PHE.

4.2. Effects of PAHs and ryegrass on bacterial population

In this study, the introduced PAH-degrading bacteria *Acinetobacter* sp. successfully survived in soil spiked with PHE and PYR. The growth of *Acinetobacter* sp. was not markedly affected by the high concentrations of PHE and PYR (Fig. 1). When isolated from soil containing a high level of PAHs (3200 μg kg^{-1}) [23], the bacteria developed gradually to tolerate PAHs and even utilize PAHs as a source of carbon. In the soil with only inoculation of *Acinetobacter* sp., the bacterial population decreased from 10^7 to 10^5 CFU g^{-1} dry soil after 20 days (Fig. 1), which may have been caused by nutrient shortage due to the depletion of available nutrients in the soil by the sudden blooming of introduced bacteria after inoculation [38]. After approximately one week, ryegrass enhanced the growth of *Acinetobacter* sp. (10^6 CFU g^{-1} dry soil), which may be attributed to specific circumstance in rhizosphere of ryegrass roots compared with control (no plants). By nature, the rhizosphere has a complex ecology with numerous feedback loops that regulate microbial populations [39]. Root exudation, water, and nutrient fluxes create quantitative and qualitative spatial gradients that affect microbial populations [40,41]. This is in agreement with the results obtained by Kirk et al. [42] who indicated that perennial ryegrass increased the number of petroleum degrading microbial population in petroleum contaminated soil.

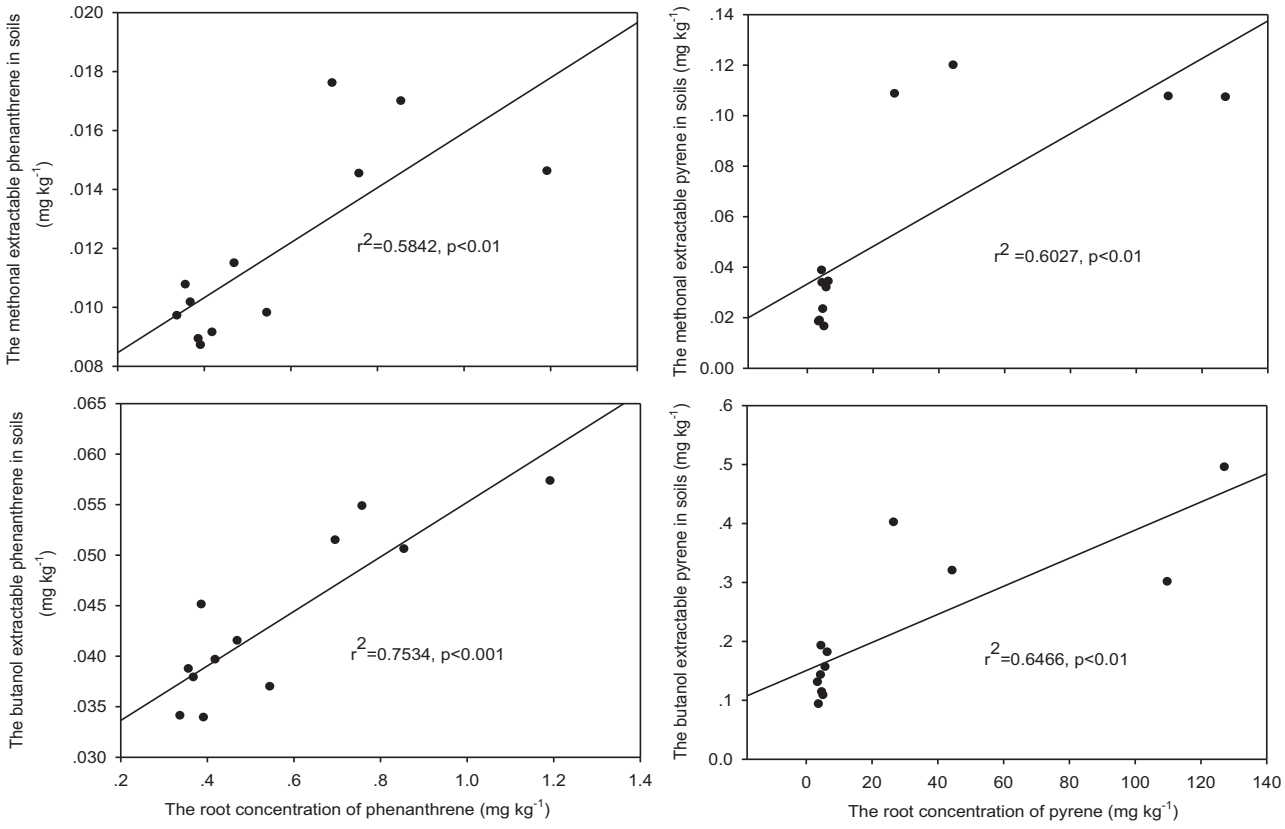

Fig. 5. Correlations between PAHs content in ryegrass roots and mild solvent extractable PAHs in soil.

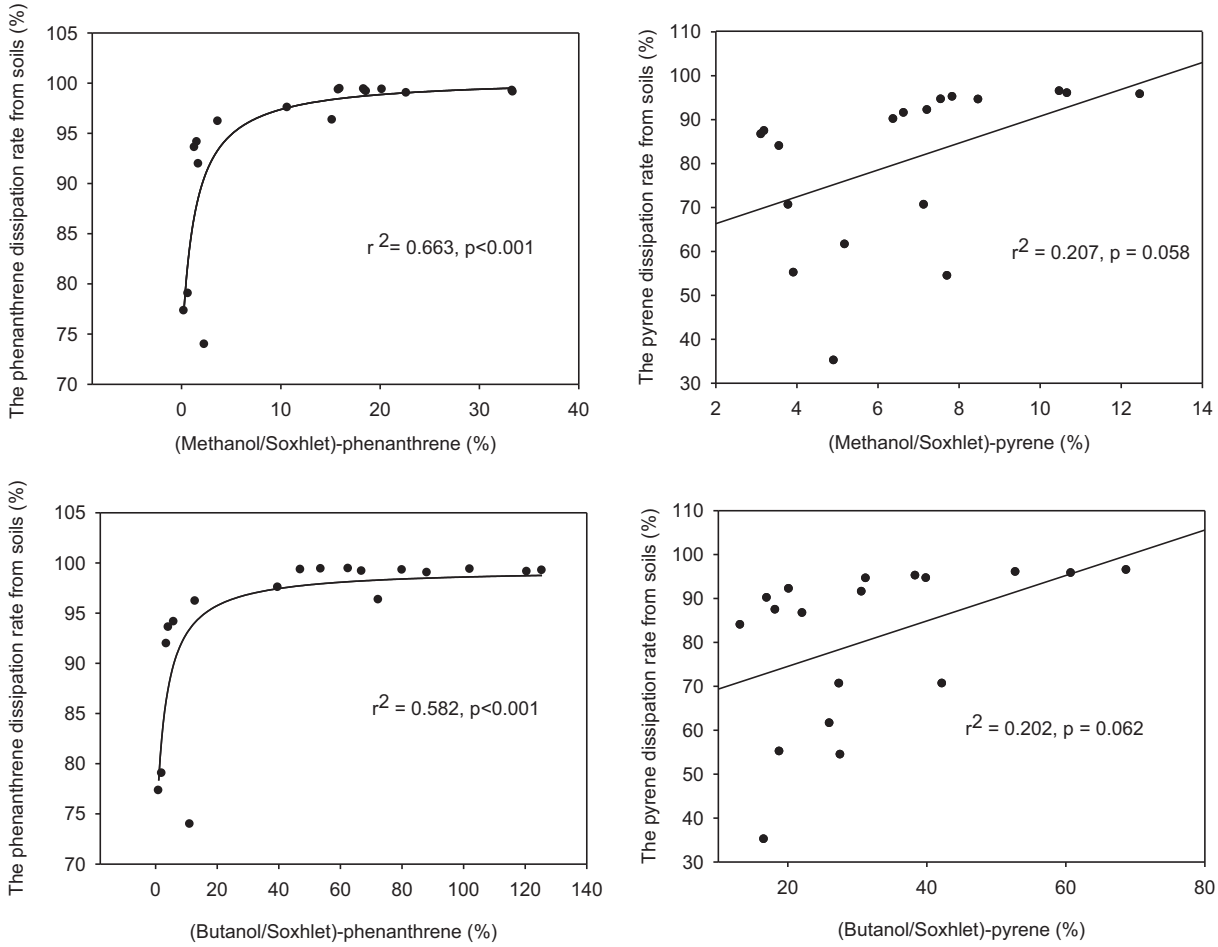

Fig. 6. Correlations between dissipation rate of PAHs and proportions of mild solvent extractable PAHs in soil.

4.3. PAH dissipation under the influence of ryegrass, AMF and Acinetobacter sp.

The present experiment has shown that ryegrass or *Acinetobacter* sp. alone significantly ($p < 0.05$) increased the dissipation of PHE and PYR from soil when compared with control. Plants (including ryegrass) have been demonstrated to enhance the dissipation of PAHs from sterilized or natural soil [11,43,44]. Their interactions with bacteria or AMF may further enhance the dissipation of PAHs from soil [45,46]. In this study, the highest dissipation rates (PHE: >99%; PYR: 90–96%) were noted in treatments of BR, MR and BMR, indicating significant interactions between ryegrass and *Acinetobacter* sp. or AMF on promoting PHE and PYR dissipation. It has been shown that wheat and PAH-degrading bacteria *Pseudomonas* sp. GF3 markedly enhanced the dissipation of PHE [44]. Our previous study also demonstrated that rice and PAH-degrading bacteria *Acinetobacter* sp. significantly accelerated the dissipation of PHE and PYR from waterlogged soil [22]. Other studies also showed that the dissipation of PAHs, DDT or atrazine from soil in the presence of mycorrhizal plants was more dramatic than that of non-mycorrhizal plants [9,47,48]. However, there was no synergistic effect observed between AMF and PAH-degrading bacteria on PAH dissipation because the dissipation rate of PYR in BMR treatment was similar with those in BR and MR treatments.

The dissipation of PHE from soil was more dramatic than PYR, mainly due to the higher volatility and biodegradability of PHE than PYR [49]. At the end of the experiment, the amount of available PHE in soil was exhausted, leading to extremely low concentrations of mild solvent extractable PHE detected in all treatments (Fig. 3). The dissipation of PHE and PYR from sterilized control soil was mainly due to volatilization and photooxidation as well as enzymatic activities where 58–71% of PHE and 35–55% of PYR disappeared at the end of 60 days, without considering the un-extractable PAHs bound to clay and organic matter in soil.

The rates of biodegradation of PAHs are highly variable and are dependent not only on PAH structure, but also on the physicochemical parameters such as the content and types of organic matter present, which sorb PAHs in soils and sediments, and the rate of their desorption strongly influences the rate at which microorganisms can degrade the pollutant [50,51]. However, the contents of organic matter (1.26%) or clay (4.4%) of the tested soil were relatively low, thus their effects on sequestration of PAHs might be neglected.

4.4. Accumulation of PAHs by ryegrass

PHE and PYR were mainly accumulated in ryegrass roots, with concentrations in shoots much lower than those in roots. Other studies also revealed that PAHs were mainly accumulated by plant roots and rarely transported to above ground plant parts [22,52]. Nevertheless we detected PHE and PYR accumulated in shoots, with rather low concentrations in non-spiked treatments and marginal higher concentrations in spiked ones, probably indicating a slight translocation of PHE and PYR from roots to shoots. The present results consistently showed that ryegrass roots accumulated much more PYR than PHE. This was probably due to the higher K_{ow} (the

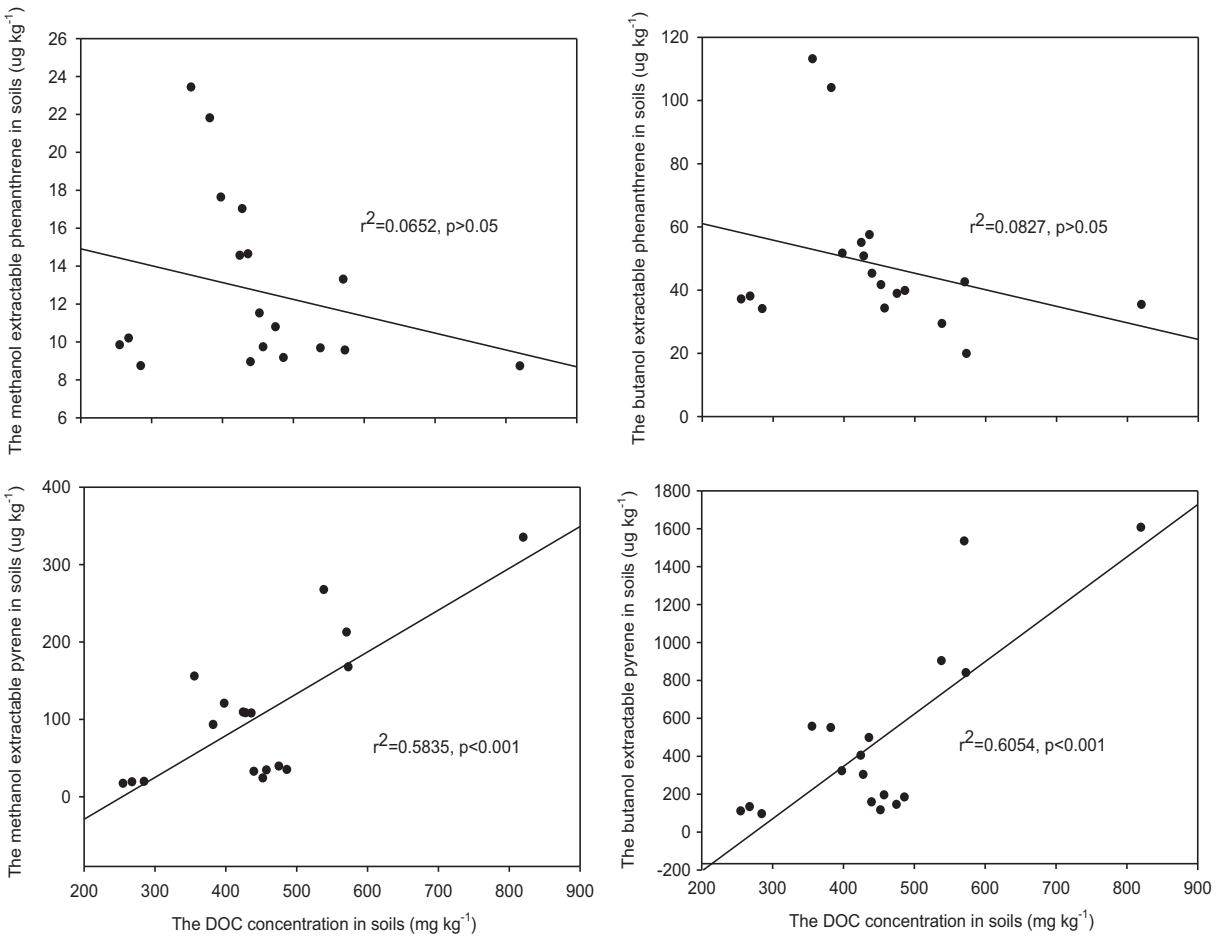

Fig. 7. Correlations between DOC content of soil and mild solvent extractable PAHs.

partition coefficient in octanol/water system) values of PYR compared with PHE. In general, higher K_{ow} values result in higher concentrations of organic compounds in plant roots [53].

Low concentrations of PHE and PYR were detected in ryegrass shoots, even in those grown in un-spiked soil, but no PHE and PYR were detected in the roots of the latter experiment. This indicates that PHE and PYR accumulation in shoots may most likely derive from air uptake, regardless of the soil PAHs level (0–200 mg kg^{-1}). Air samples above soil surface were detected with PHE and PYR in a green house study [54], and direct uptake of PAHs from air by above ground part was demonstrated [54,55]. Some field studies also showed that uptake of PAHs from ambient air was an important pathway for PAHs intake by aboveground plant parts [56,57].

Enhanced uptake or accumulation of PHE and PYR by ryegrass roots was observed in the presence of AMF. Inoculation with AMF alone or together with *Acinetobacter* sp. significantly ($p < 0.05$) enhanced PYR accumulation by ryegrass roots. Binet et al. [58] also found that PAH extracted from root tissue was significantly higher in mycorrhizal than non-mycorrhizal plant, which could be attributed to the invasion of fungi into the plant root. Similar mycorrhiza effects have been reported for atrazine taken up by maize root [47], DDT accumulated by alfalfa (*Medicago sativa*) roots [48], and p,p′-DDE accumulated by zucchini (*Cucurbita pepo*) [59]. A more recent study revealed that the partition coefficients (K_d) of two PAHs (fluorene (Flu) and PHE) by mycorrhizal hyphae were 270–356% greater than those by roots, suggesting the great potential of hyphae to facilitate plant absorbing PAHs [12]. In addition, the PAH with a lower molecular weight and higher water solubility seemed to be much easily taken up and translocated into plant root via hyphae [12,13].

The interactions between AMF and bacteria on translocations of PAHs in plant tissues have not been fully investigated yet. In the present study, it was observed that PYR concentrations of ryegrass shoots in spiked soil co-cultivated with *Acinetobacter* sp. and AMF were significantly ($p < 0.05$) higher than those in other treatments (except for MR treatment at 100 + 100 mg kg^{-1} level). It implies that the translocation of PYR to shoots was promoted by AMF – *Acinetobacter* sp. to some extent. However, further research is needed to reveal the mechanisms involved.

4.5. Soil enzyme activities

In general, PAHs present in soil may exert toxic effects on some soil enzymes [60]. However, in this study only PYR exerted inhibitory effect on the enzymatic activity, but not PHE, which implies that PYR is more toxic than PHE to soil microorganisms. The present results showed that the presence of ryegrass significantly increased ($p < 0.05$) peroxidase activities in soil, when compared with unplanted treatments. Root secretion of oxidative enzymes may be one of the mechanisms responsible to the enhanced dissipation of PAHs in the presence of plants [4,61]. The release of peroxidases by plant roots into soil has been identified [62,63], and their activity may have a direct effect on degradation of aromatics in the external medium [64]. In an attempt to remediate soils contaminated by agrochemicals, petroleum spills and highly hazardous organo-pollutants, the use of plant materials containing

peroxidase has been suggested [61,65]. The elevated peroxidase activity may partly explain the enhanced dissipation of PHE and PYR in the presence of ryegrass. Inoculation of AMF in soil planted with ryegrass significantly ($p < 0.05$) increased peroxidase activities under lower PAH levels (0 and 50 + 50 mg kg^{-1}). Stimulated peroxidase expression after AMF colonization has been demonstrated in lucerne (*M. sativa*) roots and its rhizospheric soil [66]. Elevated non-specific peroxidase and manganese peroxidase activities have also been recorded in soils colonized by ectomycorrhizal fungal mycelia, when compared with uncolonized soil [67,68].

Polyphenol oxidase is an important oxidoreductase in soil and can catalyze the degradation and transformation of aromatic compounds [69]. Polyphenol oxidase activities in this study were significantly ($p < 0.05$) enhanced by PAH-degrading bacteria *Acinetobacter* sp. Elevation of polyphenol oxidase activities by PAH-degrading bacteria *Pseudomonas* sp. GF3 was also observed in both planted and unplanted soil [44]. However, polyphenol oxidase activities in treatments containing ryegrass (R, BR, MR and BMR) were significantly ($p < 0.05$) lower than those in control soil. As polyphenol oxidase and peroxidase can catalyze the oxidation of the same substrates, plants usually manifest activity of only one of these enzymes [70]. Therefore, it was reasonable that peroxidase activity in soil was enhanced by ryegrass, while polyphenol oxidase activity was not.

It was not surprising that the peroxidase and polyphenol oxidase activities also detected even in control (sterilized soil). During the 60-day experiment, there may be a possibility of re-colonization by airborne microbes in the soil without PAH-degrading bacterial inoculation, although they had been proven not to be able to grow on the medium containing PAHs only as a unique carbon source. In summary, the contribution to soil enzymatic activities from soil bacteria, plant and AMF or the consortia would easily identified based on the result from control, B and R treatments.

4.6. Bioavailability of PAHs, ryegrass uptake and dissipation

Mild solvent extraction of PAHs with methanol or butanol has been used to estimate available fractions of soil PAHs [25,71]. At the end of the experiment, PHE or PYR content of ryegrass roots (R, BR, MR and BMR) was significantly ($p < 0.05$) correlated with methanol or butanol extractable soil PHE or PYR. Ryegrass roots might accumulate PHE and PYR through direct adsorption and absorption of bioavailable PHE and PYR [21]. In an attempt to evaluate the bioavailability of DDT in soil to wheat uptake, a significant positive correlation was observed between the amount of n-hexane-extractable DDT in soil and the amount of DDT accumulated in wheat root [72]. Bogolte et al. [73] also demonstrated a highly significant correlation between plant (*Lepidium sativum*) accumulation of PHE and available PHE in soil extracted by supercritical fluid extraction with carbon dioxide under very mild conditions (fluid density: 0.25 g/ml; temperature: 40 °C). Moreover, uptake of bioavailable PYR by ryegrass was confirmed by the significant ($p < 0.05$) reduction of available PYR extracted with methanol or butanol in soil, when compared with control and B treatment (Fig. 3). Parrish et al. [74] also noted the contribution of plant roots (*Festuca arundinacea*, *L. multiflorum*, *Melilotus officinalis*) to the reduction of available PAH (extracted with an Amberlite XAD-2 macroreticular styrenedivinylbenzene copolymer resin) of soil collected from a manufactured gas plant site.

In addition, a very significant ($p < 0.001$) hyperbola relationship was found between available proportions of PHE and dissipation rates of PHE in corresponding treatments (control, R, B, BR, MR, BMR). This indicated that bioavailable PHE was inclined to be transformed, through several possible pathways including volatilization, degradation and plant accumulation. This was in line with the finding that available fractions of PAHs in soil were more likely to be degraded by bacteria and accumulated by living organisms [75,76]. It has been shown that butanol extractable PAHs approximate the trends in bacterial degradation [25]. In addition, the results in this study indicated that butanol or methanol extractable PAHs approximate the trends in root accumulation of PAHs, although there is a severe lack of information concerning the feasibility of using mild solvent extractable PAH to estimate plant accumulation of PAHs. These evidence also implied that mild solvent extractable PAHs may be suitable for predicting the dissipation of PAHs in a multi-component remediation system. However, further analyses on aged soils should be conducted before a definite conclusion can be drawn.

DOC content of soil was significantly ($p < 0.05$) correlated with methanol or butanol extractable PYR concentrations of soil. Water soluble organic matter has been shown to be closely related to the bioavailability of organic compounds [72,77]. Some studies [78,79] even demonstrated that DOC is associated with solubilized PAHs and increased water solubility of PAHs, which is expected to be biologically available, i.e., bacterial degradation and plant uptake [79,80].

5. Conclusion

Unexpectedly the highest dissipation rates of PAHs in the treatments of BR, MR and BMR were observed, due to the presence of ryegrass, PAHs-degrading bacteria and AMF or the consortia, which accelerated the dissipation of PHE and PYR from soil. The results reflected the significant contribution of mycorrhizae in cleaning up PAHs contaminated sites. Ryegrass enhanced the dissipation of PHE and PYR through the significant ($p < 0.05$) increase in peroxidase activities in soil. Mild solvent extractable PHE and PYR were significantly ($p < 0.01$) correlated with the concentrations of PHE and PYR in ryegrass root, which confirmed that mild solvent extractable PAH in soil could be used to estimate PAH bioavailability to plant. Ryegrass associated with AMF and/or bacteria significantly ($p < 0.05$) enhanced dissipation of PYR from soil, PYR accumulation by ryegrass roots and peroxidase activities in soil. This has revealed the role of mycorrhiza in the phytoremediation practices at PAH contaminated sites.

Acknowledgements

The authors thank the Research Grants of the University Grants Committee of Hong Kong (Central Allocation, Group Research, HKBU/03C), and the Mini-AoE (Area of Excellence) Fund from Hong Kong Baptist University for financial support.

References

[1] T.J. Cutright, S. Lee, Microorganisms and metabolic pathways for remediation of PAH in contaminated soil, Fresenius Environ. Bull. 3 (1994) 413–421.
[2] E. Colleran, Uses of bacteria in bioremediation, in: D. Sheehan (Ed.), Bioremediation Protocols, Humana Press, Totowa, New Jersey, 1997, pp. 3–5.
[3] J.L. Schnoor, L.A. Licht, S.C. McCutcheon, N.L. Wolfe, L.H. Carreira, Phytoremediation of organic and nutrient contaminants, Environ. Sci. Technol. 29 (1995) 318–323.
[4] S.D. Cunningham, D.W. Ow, Promises and prospects of phytoremediation, Plant Physiol. 110 (1996) 715–719.
[5] B. Suresh, G.A. Ravishankar, Phytoremediation-A novel and promising approach for environmental clean-up, Crit. Rev. Biotechnol. 24 (2004) 97–124.
[6] A.A. Meharg, J.W.G. Cairney, Ectomycorrhizas-extending the capacities of rhizosphere remediation? Soil Biol. Biochem. 32 (2000) 1475–1484.
[7] C. Leyval, P. Binet, Effect of polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants, J. Environ. Qual. 27 (1998) 402–407.
[8] E.J. Joner, C. Leyval, Influence of arbuscular mycorrhiza on clover and ryegrass grown together in a soil spiked with polycyclic aromatic hydrocarbons, Mycorrhiza 10 (2001) 155–159.
[9] E.J. Joner, C. Leyval, Rhizosphere gradients of polycyclic aromatic hydrocarbon 'PAH' dissipation in two industrial soils and the impact of arbuscular mycorrhiza, Environ. Sci. Technol. 37 (2003) 2371–2375.

[10] S.C. Corgie, F. Fons, T. Beguiristain, C. Leyval, Biodegradation of phenanthrene, spatial distribution of bacterial populations and dioxygenase expression in the mycorrhizosphere of *Lolium perenne* inoculated with *Glomus mosseae*, Mycorrhiza 16 (2006) 207–212.
[11] P. Binet, J.M. Portal, C. Leyval, Dissipation of 3-6-ring polycyclic aromatic hydrocarbons in the rhizosphere of ryegrass, Soil Biol. Biochem. 32 (2000) 2011–2017.
[12] Y.Z. Gao, Z.X. Cheng, W.T. Ling, J. Huang, Arbuscular mycorrhizal fungal hyphae contribute to the uptake of polycyclic aromatic hydrocarbons by plant roots, Bioresour. Technol. 101 (2010) 6895–6901.
[13] Y.Z. Gao, Q.L. Li, W.T. Ling, X.Z. Zhu, Arbuscular mycorrhizal phytoremediation of soils contaminated with phenanthrene and pyrene, J. Hazard. Mater. (2010), doi:10.1016/j.jhazmat.2010.09.076.
[14] R.A. Kanaly, S. Harayama, Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria, J. Bacteriol. 182 (2000) 2059–2067.
[15] K. Rehmann, H.P. Noll, C.E.W. Steinberg, A.A. Kettrup, Pyrene degradation by *Mycobacterium* sp. strain KR2, Chemosphere 36 (1998) 2977–2992.
[16] K. Somtrakoon, S. Suanjit, P. Pokethitiyook, M. Kruatrachue, H. Lee, S. Upatham, Phenanthrene stimulates the degradation of pyrene and fluoranthene by *Burkholderia* sp. VUN10013, World J. Microbiol. Biotechnol. 24 (2008) 523–531.
[17] P. Baldrian, C. Wiesche, J. Gabriel, F. Nerud, F. Zadrazil, Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil, Appl. Environ. Microbiol. 66 (2000) 2471–2478.
[18] A.K. Haritash, C.P. Kaushik, Biodegradation aspects of polycyclic aromatic hydrocarbons 'PAHs': a review, J. Hazard. Mater. 169 (2009) 1–15.
[19] R.K. Miya, M.K. Firestone, Phenanthrene biodegradation in soil by slender oar root exudates and root debris, J. Environ. Qual. 30 (2001) 1911–1918.
[20] T.M. Ruuhola, M.R.K. Julkunen-Tiitto, Salicylates of intact Salix myrsinifolia plantlets do not undergo rapid metabolic turnover, Plant Physiol. 122 (2000) 895–905.
[21] F.X. Kang, D.S. Chen, Y.Z. Gao, Y. Zhang, Distribution of polycyclic aromatic hydrocarbons in subcellular root tissues of ryegrass '*Lolium multiflorum* Lam.', BMC Plant Biol. 10 (2010) 210–216.
[22] Y. Gao, X.Z. Yu, S.C. Wu, K.C. Cheung, N.F.Y. Tam, P.Y. Qian, M.H. Wong, Interactions of rice '*Oryza sativa* L.' and PAH-degrading bacteria '*Acinetobacter* sp.' on enhanced dissipation of spiked phenanthrene and pyrene in waterlogged soil, Sci. Total Environ. 372 (2006) 1–11.
[23] X.Z. Yu, Y. Gao, S.C. Wu, H.B. Zhang, K.C. Cheung, M.H. Wong, Distribution of polycyclic aromatic hydrocarbons in soils at Guiyu area of China, affected by recycling of electronic waste using primitive technologies, Chemosphere 65 (2006) 1500–1509.
[24] D.L. Johnson, K.C. Jones, C.J. Langdon, T.G. Piearce, K.T. Semple, Temporal changes in earthworm availability and extractability of polycyclic aromatic hydrocarbons in soil, Soil Biol. Biochem. 34 (2002) 1363–1370.
[25] H.H. Liste, M. Alexander, Butanol extraction to predict bioavailability of PAHs in soil, Chemosphere 46 (2002) 1011–1017.
[26] USEPA, Method 3540C: Soxhlet Extraction, U.S. Environmental Protection Agency, Washington, DC, 1996.
[27] USEPA, Method 3620B: Florisil Cleanup, U.S. Environmental Protection Agency, Washington, DC, 1996.
[28] USEPA, Method 8270C: Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry 'GC/MS', U.S. Environmental Protection Agency, Washington, DC, 1996.
[29] T.P. McGonigle, M.H. Miller, D.G. Evans, G.L. Fairchild, J.A. Swan, A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi, New Phytol. 115 (1990) 495–501.
[30] Y. Chen, C.X. Wang, Z.J. Wang, S.B. Huang, Assessment of the contamination and genotoxicity of soil irrigated with wastewater, Plant Soil 261 (2004) 189–196.
[31] M.L. Gaspar, M.N. Cabello, M.C. Cazau, R.J. Pollero, Effect of phenanthrene and *Rhodotorula glutinis* on arbuscular mycorrhizal fungus colonization of maize roots, Mycorrhiza 12 (2002) 55–59.
[32] M.N. Cabello, Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi 'AMF', FEMS Microbiol. Ecol. 22 (1997) 233–236.
[33] A.H. Fitter, J. Garbaye, Interactions between mycorrhizal fungi and other soil organisms, Plant Soil 159 (1994) 123–132.
[34] J.M. Barea, C. Azcon-Aguilar, R. Azcon, Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil-plant systems, in: A.C. Gange, V.K. Brown (Eds.), Multitrophic Interactions in Terrestrial Systems, Blackwell Science, Cambridge, England, 1997, pp. 65–77.
[35] V. Tandon, A. Prakash, Influence of soil inoculation with VA mycorrhiza and phosphorus solubilising microorganisms on growth and phosphorus uptake in Sesamum indicum, Int. J. Trop. Agric. 16 (1998) 201–209.
[36] G. Kovtunovych, O. Lar, S. Kamalova, V. Kordyum, D. Kleiner, N. Kozyrovska, Correlation between pectate lyase activity and ability of diazotrophic Klebsiella oxytoca VN 13 to penetrate into plant tissues, Plant Soil 215 (1999) 1–6.
[37] S.C. Verma, J.K. Ladha, A.K. Tripathi, Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice, J. Biotechnol. 91 (2001) 127–141.
[38] E.J. Joner, S.C. Corgi, N. Amellal, C. Leyval, Nutritional constraints to degradation of polycyclic aromatic hydrocarbons in a simulated rhizosphere, Soil Biol. Biochem. 34 (2002) 859–864.
[39] M.E. Toal, C. Yeomans, K. Killham, A.A. Meharg, A review of rhizosphere carbon flow modeling, Plant Soil 222 (2000) 263–281.
[40] C. Fang, M. Radosevich, J.J. Fuhrmann, Characterization of rhizosphere microbial community structure in five similar grass species using FAME and BIOLOG analyses, Soil Biol. Biochem. 33 (2001) 679–682.
[41] J.L. Butler, M.A. Williams, P.J. Bottomley, D.D. Myrold, Microbial community dynamics associated with rhizosphere carbon flow, Appl. Environ. Microbiol. 69 (2003) 6793–6800.
[42] J.L. Kirk, J.N. Klironomos, H. Lee, J.T. Trevors, The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil, Environ. Pollut. 133 (2005) 455–465.
[43] H.H. Liste, M. Alexander, Plant-promoted pyrene degradation in soil, Chemosphere 40 (2000) 7–10.
[44] X.F. Sheng, J.X. Gong, Increased degradation of phenanthrene in soil by *Pseudomonas* sp. GF3 in the presence of wheat, Soil Biol. Biochem. 38 (2006) 2587–2592.
[45] C. Mougin, Bioremediation and phytoremediation of industrial PAH-polluted soils, Polycycl. Aromat. Compd. 22 (2002) 1011–1043.
[46] E.J. Joner, C. Leyval, Phytoremediation of organic pollutants using mycorrhizal plants: a new aspect of rhizosphere interactions, Agronomie 23 (2003) 495–502.
[47] H.L. Huang, S.Z. Zhang, X.Q. Shan, B.D. Chen, Y.G. Zhu, J.N.B. Bell, Effect of arbuscular mycorrhizal fungus 'Glomus caledonium' on the accumulation and metabolism of atrazine in maize 'Zea mays L.' and atrazine dissipation in soil, Environ. Pollut. 146 (2007) 452–457.
[48] N.Y. Wu, S.Z. Zhang, H.L. Huang, X.Q. Shan, P. Christie, Y.S. Wang, DDT uptake by arbuscular mycorrhizal alfalfa and depletion in soil as influenced by soil application of a non-ionic surfactant, Environ. Pollut. 151 (2008) 569–575.
[49] A.L. Juhasz, R. Naidu, Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene, Int. Biodeter. Biodegr. 45 (2000) 57–88.
[50] K.L. Shuttleworth, C.E. Cerniglia, Environmental aspects of PAH biodegradation, Appl. Biochem. Biotech. 54 (1995) 291–302.
[51] S.K. Samanta, O.V. Singh, R.K. Jain, Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation, Trends Biotechnol. 20 (2002) 243–248.
[52] S. Tao, X.C. Jiao, S.H. Chen, W.X. Liu, R.M. Coveney Jr., L.Z. Zhu, Y.M. Luo, Accumulation and distribution of polycyclic aromatic hydrocarbons in rice '*Oryza sativa*', Environ. Pollut. 140 (2006) 406–415.
[53] S. Trapp, M. Matthies, I. Scheunert, E.M. Topp, Modeling the bioconcentration of organic chemicals in plants, Environ. Sci. Technol. 24 (1990) 1246–1252.
[54] Y.Z. Gao, L.Z. Zhu, Plant uptake, accumulation and translocation of phenanthrene and pyrene in soils, Chemosphere 55 (2004) 1169–1178.
[55] Q. Zuo, H. Lin, X.L. Zhang, Q.L. Li, S.Z. Liu, S. Tao, A two-compartment exposure device for foliar uptake study, Environ. Pollut. 143 (2006) 126–128.
[56] A.M. Kipopoulou, E.M.C. Samara, Bioconcentration of polycyclic aromatic hydrocarbons in vegetables grown in an industrial area, Environ. Pollut. 106 (1999) 369–380.
[57] M. Howsam, K.C. Jones, P. Ineson, PAHs associated with the leaves of three deciduous tree species II: uptake during a growing season, Chemosphere 44 (2001) 155–164.
[58] P. Binet, J.M. Portal, C. Leyval, Fate of polycyclic aromatic hydrocarbons 'PAH' in the rhizosphere and mycorrhizosphere of ryegrass, Plant Soil 227 (2000) 207–213.
[59] J.C. White, D.W. Ross, M.P.N. Gent, B.D. Eitzer, M.J.I. Mattina, Effect of mycorrhizal fungi on the phytoextraction of weathered p,p-DDE by *Cucurbita pepo*, J. Hazard. Mater. 137 (2006) 1750–1757.
[60] V.L. Andreoni, L. Cavalca, M.A. Rao, G. Nocerino, S. Bernasconi, E. Dell'Amico, M. Colombo, L. Gianfreda, Bacterial communities and enzyme activities of PAHs polluted soils, Chemosphere 57 (2004) 401–412.
[61] T.A. Anderson, J.R. Coats, Bioremediation Through Rhizosphere Technology, ACS Symposium Series 563, American Chemical Society, Washington, DC, 1994.
[62] T.M. Lee, Y.H. Lin, Changes in soluble and cell wall-bound peroxidase activities with growth in anoxia-treated rice '*Oryza sativa* L.' coleoptiles and roots, Plant Sci. 1116 (1995) 1–7.
[63] G. Gramss, K.D. Voigt, B. Kirsche, Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material, Chemosphere 38 (1999) 1481–1494.
[64] J.J. Kraus, I.Z. Munir, J.P. McEldoon, D.S. Clark, J.S. Dordick, Oxidation of polycyclic aromatic hydrocarbons catalyzed by soybean peroxidase, Appl. Biochem. Biotechnol. 80 (1999) 221–230.
[65] J.M. Bollag, Decontaminating soil with enzymes, Environ. Sci. Technol. 26 (1992) 1876–1881.
[66] S. Criquet, E. Joner, P. Leglize, C. Leyval, Anthracene and mycorrhiza affect the activity of oxidoreductases in the roots and the rhizosphere of lucerne '*Medicago sativa* L.', Biotechnol. Lett. 22 (2000) 1733–1737.
[67] R.P. Griffths, B.A. Caldwell, Mycorrhizal mat communities in forest soil, in: D.J. Read, D.H. Lewis, A.H. Fitter, I.J. Alexander (Eds.), Mycorrhizas in Ecosystems, CAB International, Wallingford, 1992, pp. 98–105.
[68] G. Gramss, Activity of oxidative enzymes in fungal mycelia from grassland and forest soils, J. Basic Microbiol. 37 (1997) 407–423.
[69] S.L. Liu, Y.M. Luo, Z.H. Cao, L.H. Wu, K.Q. Ding, P. Christie, Degradation of benzo[a]pyrene in soil with arbuscular mycorrhizal alfalfa, Environ. Geochem. Health 26 (2004) 285–293.
[70] J. Nowak, K. Kaklewski, M. Ligocki, Influence of selenium on oxidoreductive enzymes activity in soil and in plants, Soil Biol. Biochem. 36 (2004) 1553–1558.
[71] J.W. Kelsey, B.D. Kottler, M. Alexander, Selective chemical extractants to predict bioavailability of soil-aged organic chemicals, Environ. Sci. Technol. 31 (1997) 214–217.
[72] S. Tao, L.Q. Guo, X.J. Wang, W.X. Liu, T.Z. Ju, R. Dawson, J. Cao, F.L. Xu, B.G. Li, Use of sequential ASE extraction to evaluate the bioavailability of DDT and its

metabolites to wheat roots in soils with various organic carbon contents, Sci. Total Environ. 320 (2004) 1–9.
[73] B.T. Bogolte, G.A.C. Ehlers, R. Braun, A.P. Loibner, Estimation of PAH bioavailability to *Lepidium sativum* using sequential supercritical fluid extraction-a case study with industrial contaminated soils, Eur. J. Soil Biol. 43 (2007) 242–250.
[74] Z.D. Parrish, M.K. Banks, A.P. Schwab, Assessment of contaminant lability during phytoremediation of polycyclic aromatic hydrocarbon impacted soil, Environ. Pollut. 137 (2005) 187–197.
[75] H. Harms, T.N.P. Bosma, Mass transfer limitation of microbial growth and pollutant degradation, J. Ind. Microbiol. Biotechnol. 18 (1997) 97–105.
[76] M. Krauss, W. Wilckew, W. Zech, Availability of polycyclic aromatic hydrocarbons 'PAHs' and polychlorinated biphenyls 'PCBs' to earthworms in urban soils, Environ. Sci. Technol. 34 (2000) 4335–4340.
[77] G. Cornelissen, P.C.M. van Noort, H.A.J. Govers, Mechanism of slow desorption of organic compounds from sediments: a study using model sorbents, Environ. Sci. Technol. 32 (1998) 3124–3131.
[78] A.A. Mackay, P.M. Gschwend, Enhanced concentrations of PAHs in groundwater at a coal tar site, Environ. Sci. Technol. 35 (2001) 1320–1328.
[79] G. Bengtsson, P. Zerhouni, Effects of carbon substrate enrichment and DOC concentration on biodegradation of PAH in soil, J. Appl. Microbiol. 94 (2003) 608–617.
[80] B.W. Bogan, W.R. Sullivan, Physicochemical soil parameters affecting sequestration and mycobacterial biodegradation of polycyclic aromatic hydrocarbons in soil, Chemosphere 52 (2003) 1717–1726.