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Original Research

Impact of Four Plant Species and Arbuscular Mycorrhizal (AM) Fungi on Polycyclic Aromatic Hydrocarbon (PAH) Dissipation in Spiked Soil

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Abstract

Alfalfa, tall fescue, ryegrass, and celery, some of which were inoculated with the AM fungus *Glomus intraradices*, were compared for their contributions to phenanthrene (PHE), pyrene (PYR), and dibenzo(a,h)anthracene (DBA) dissipation in spiked soil. A pot experiment was conducted in which PAHs extracted from soil and plant, quantity of PAH degraders, and plant biomass were evaluated. The results showed that biodegradation was the dominant removal mechanism for PAHs from soil while PAH accumulation in the plant tissue was negligible. PAH dissipation varied with the plant species and decreased with the increase of PAH molecular weight. The four plant species displayed a positive effect on PHE dissipation, and alfalfa improved PYR and DBA dissipation. AM fungi significantly increased plant biomass, phosphorus uptake, and PHE removal rate in planted treatments. Plant biomass and PAH degraders showed a weak linear relationship with PAH dissipation, indicating that there might be other important factors influencing PAH dissipation.

Keywords: AM fungi, phytoremediation, PAHs

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread soil contaminants, and they have caused serious concerns due to their bioaccumulation property, toxicity, and mutagenicity [1]. Up to now, lots of techniques have been used to detoxify PAH-contaminated soils, in which phytoremediation using plants and associated microorganisms to enhance the pollutant dissipation is considered an ecologically and economically attractive remediation technique.

Successful applications of the plant-microorganisms system for the remediation of PAH-contaminated soils and sediments have been well documented [2, 3]. Yet phytoremediation efficacy varies greatly among plant species and varieties. For instance, among 9 plant species tested by Liste and Alexander, the pyrene dissipation varied from 22 (pepper, *Capsicum annuum*) to 60 (red pine, *Pinus resinosa*) mg·kg^{-1} soil (the initial concentration of pyrene was 100 mg·kg^{-1}) after 28 days of cultivation [4]. The different capabilities of the plants for phytoremediation are mainly attributed to the facts that:

(1) plants (seed germination, plant survival, and shoot yields) respond differently to PAH contamination due to their different tolerances against acute toxicity and unfavorable soil conditions of PAH-contaminated sites [5, 6]
(2) plants differ in root and exudate (such as carbon resource, and enzymes) characteristics, which affect PAH degradation and detoxification [3]

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(3) plants vary in their abilities to take up and stabilize PAHs according to their different root lipid concentrations and root surface characters [7, 8].

Therefore, PAH-tolerant and PAH removal efficient plant species should be selected for PAH phytoremediation.

Another approach toward increasing plant fitness and PAH phytoremediation is to inoculate AM fungi in contaminated soil. As ubiquitous symbionts, AM fungi are dependent on plants for their carbon nutrition, and they also can help plants capture nutrients such as phosphorus and micronutrients in soil [9]. Recent investigations showed that AM fungi can generally facilitate plant establishment and survival in PAH-contaminated soil [10, 11], and they also can increase PAH uptake and biodegradation in the plant rhizosphere [12-15]. In a previous study we showed that PAH dissipation in the PAH-spiked soil planted with mycorrhizal alfalfa and tall fescue was affected by water and phosphorus content in the soil as well as mycorrhizal root colonization [16]. The aim of the current study was to investigate the effects of the four different plant species (alfalfa, tall fescue, ryegrass, and celery), some of which were inoculated with the AM fungus *Glomus intraradices* on the dissipation of PAHs with different molecular weights. Phenanthrene (PHE), pyrene (PYR), and dibenzo(a,h)anthracene (DBA) were used to represent the three, four, and five-ring PAHs. Plant growth, AM fungi colonization, microorganism density, and plant uptake and plant-promoted biodegradation of PAHs were evaluated after 6 weeks of plant growth in the PAH-spiked soil.

Materials and Methods

Soil

The soil used was a mixture of a silty clay loam (Bouzule, collected in northeastern France, 2 mm sieved) and sand (1:1, wt/wt). The characteristics of the silty clay loam were as follows: pH, 7.4; organic carbon, 16 $g \cdot kg^{-1}$; total nitrogen, 1.7 $g \cdot kg^{-1}$; Olsen P, 117 $mg \cdot kg^{-1}$; PHE, PYR, and DBA, below detection limit. The silty clay loam was heated at 90ºC for one hour to eliminate any indigenous AM fungi. The sand was 2 mm sieved, acid washed, rinsed, and autoclaved. PHE, PYR, and DBA were separately dissolved in 300 ml of acetone and thoroughly mixed with 10% of the sand. After evaporation of the solvent the spiked sand was mixed with the rest of the sand and the silty clay loam, and then homogenized to reach a final concentration of PHE = 500 $mg \cdot kg^{-1}$, PYR = 500 $mg \cdot kg^{-1}$, and DBA = 50 $mg \cdot kg^{-1}$. A lower DBA concentration was added to reflect their relative concentrations in PAH-contaminated industrial soils [17]. The spiked soil was weighed and loaded in light-tight pots (250 g/pot), and was reinoculated with indigenous soil microflora (except mycorrhizal fungi) by adding 8 ml of a 5 μm-filtered non-sterile soil suspension to each pot [18].

Treatments

Nine treatments, including the four plant species (alfalfa (*Medicago sativa* cv. Europe), tall fescue (*Festuca arundinacea* cv. Bariane), annual ryegrass (*Lolium multiflorum* cv. Barclay), and celery (*Apium graveolen*)) some of which were inoculated with AM fungus and unplanted control were conducted. Four seeds were sown in each planted pot. The AM fungi-inoculated plants received 10 g of commercial *Glomus intraradices* inoculum supplied by the Institüt für Pflanzenkultur (Solkau, Germany) as a mixture of propagules in a lava substrate [19]. The non-AM fungi treatments and unplanted control pots received an equivalent amount of autoclaved AM inoculum. The soil was covered with a layer of coarse sand to minimize PAH volatilization. Three replicates of each treatment were randomized in the growth chamber (Conviron, 24/20ºC day/night, 16 h day, 80% RH, 250 $\mu mol \cdot s^{-1} \cdot m^{-2}$ PAE) and re-randomized weekly. 25 ml of P-deficient Hewitt nutrient solution was added weekly, and the humidity of the soil was maintained to 60-80% of the water-holding capacity by regular weighing [16, 20]. The seedlings in each pot were thinned to 3 on the 7th day after germination.

Plants were harvested after 6 weeks. Pots were left unwatered for 2 days prior to the harvest. The shoots and roots were washed carefully in 300 ml of deionized water to remove the adhering soil particles. Shoots were analyzed for dry weight, phosphorus concentration, and PAH uptake, and roots for dry weight, AM fungi colonization, and PAH sorption. The soil from pots was carefully collected and homogenized. 0.5 g of soil was kept at -20ºC for DNA extraction, and the rest was stored at 4ºC for the residual PAH concentration measurement.

Analysis

The frequency of mycorrhizal roots and arbuscular abundance in root systems were estimated using the trypan blue staining Koske and Gemma [21] and Trouvelot et al. [22] notation methods. Phosphorus concentrations in shoots were measured by ICP-OES after digestion of 0.2 g (0.1 g for celery) of dry shoots in HNO_3 65% (4 ml) and H_2O_2 30% (2 ml) at high temperature and pressure (170ºC, 2 MPa) in a microwave digesting system (MARS 5). The PAHs adsorbed on the root surface were extracted following the method of Binet et al. [10]. Because of the limited plant biomass, root and shoot samples collected from all three replicates of each treatment were mixed for extraction. Briefly, the root samples (0.1 g, dry weight) were successively extracted with chloroform for 4 times (with 8 ml of chloroform and lasted for 25 min each time) at room temperature. The chloroform solutions were collected and adjusted to 50 ml. The PAHs absorbed in the root and shoot tissues were extracted following the method of Binet et al. [10]. The root (after adsorption extraction) or shoot samples (0.1 g dry weight), were ground and extracted with 150 ml of chloroform in a Soxhlet for 4 h. The extracts

Table 1. AM fungi colonization and shoot phosphorus concentrations in planted treatments. Arbuscular mycorrizal colonization is expressed as frequency of mycorrhizal roots (F%) and arbuscular abundance in the root systems (A%).

Treatments		AM fungi colonization		Shoot phosphorus	
		F%	A%	Concentration (mg·kg^{-1})	Quantity (μg/pot)
Alfalfa	AMF	21.42±17.26	1.32±2.10	8.36±0.91 ^{b}	2.92±0.61 ^{a}
	Non AMF	0.00±0.00	0.00±0.00	8.36±0.90 ^{b}	1.87±0.72 ^{a}
Tall fescue	AMF	7.77±5.07	0.04±0.05	18.71±0.74 ^{cd}	7.97±0.59 ^{bc}
	Non AMF	0.00±0.00	0.00±0.00	15.57±0.68 ^{c}	5.57±0.55 ^{b}
Ryegrass	AMF	22.22±15.03	0.17±0.16	21.81±5.86 ^{cd}	9.97±2.80 ^{c}
	Non AMF	0.00±0.00	0.00±0.00	24.75±1.45 ^{d}	7.43±1.90 ^{bc}
Celery	AMF	24.45±13.47	1.05±1.56	3.39±0.36 ^{a}	0.29±0.03 ^{a}
	Non AMF	0.00±0.00	0.00±0.00	3.10±0.18 ^{a}	0.10±0.02 ^{a}
ANOVA	Plant	-	-	***	***
	AMF	-	-	n.s.	**
	Plant × AMF	-	-	n.s.	n.s.

Mean±SD (n=3). Different letters indicate significant differences between treatments with: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. "n.s." no significant difference. "-" not evaluated.

were concentrated to a volume of 50 ml. The bioavailable PAH concentration was estimated using butanol extraction [23]: 2 g of soil and 25 ml of 1-butanol (BuOH) were shaken in 50 ml Teflon centrifuge tubes at room temperature for 2 h, centrifuged at 10,000 g for 10 min, and the supernatant was filtered with a 22 μm filter. 1 ml of BuOH solution was transferred into the injection vials and dried under the nitrogen flow. The PAHs in the vials were redissolved with 1 ml of acetonitrile, and samples were analyzed with reverse-phase chromatography using a Dionex HPLC system (Dionex pumps GP40) equipped with a UV-vis detector and a reverse-phase polymeric C-18 bonding column (250 mm, 4.6 mm, 5 μm). The mobile phase was a mixture of water/acetonitrile (20:80, v/v), with a flow rate of 2.0 ml·min^{-1}. The wavelength used for detection was 254 nm. PAH concentrations were quantified with external standards. Total DNA was extracted from soil-sand mixture samples using a bead beating based method as described in Cébron et al. [24]. The copy number of 16S rDNA and 18S rDNA genes [25] as well as Gram positive and Gram negative PAH-ring dioxygenase (RHDα) genes were estimated by a SYBR Green based real-time PCR quantification using iCycler iQ (Bio-Rad) as described in Cébron et al. [24].

Statistical Analysis

Statistical analysis of the data was performed using one- and two-way ANOVA followed by a Newman-Keuls (SNK) test on xlstat 2009 to determine the significant differences between treatments (P<0.05). Percentage data were arcsine transformed prior to ANOVA analyses.

Results and Discussion

Effect of Plant Species and AM Fungi Colonization on Plant Biomass and Phosphorus Uptake

The biomass of shoot and root was significantly influenced by the plant species (Fig. 1). As shown in Fig. 1, the plants with fibrous roots (ryegrass and tall fescue) had the largest root and shoot biomass followed by alfalfa, while celery had the minimum yield, which was less than 10% of the root biomass of ryegrass and tall fescue. Grass (tall fescue and ryegrass) had a much higher concentration of phosphorus in plant shoots than the other plants (Table 1).

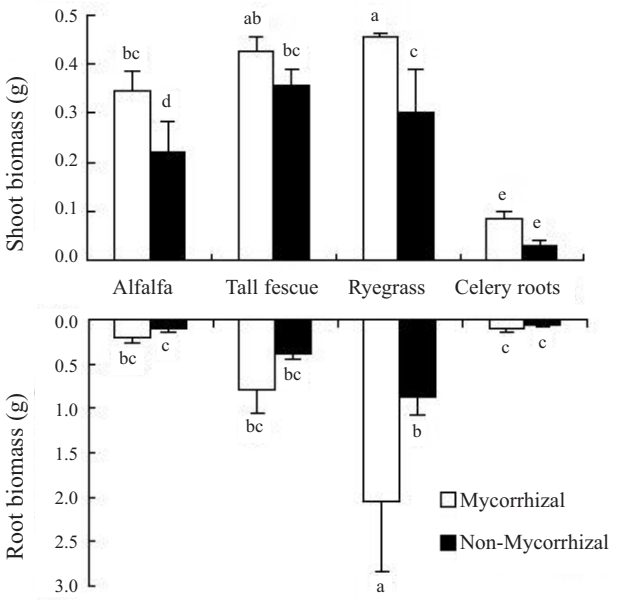
No mycorrhizal colonization was observed in the roots of uninoculated plants, while all the AM fungi-inoculated plants were well colonized. Mycorrhizal frequency of the roots of inoculated plants ranged from 7% to 25%, and arbuscular abundance ranged from 0.1% to 1.5%. The relatively low colonization rate, as well as the large variations among replicates, could be attributed to the toxicity and water repellence of PAHs [26, 27]. In spite of the low colonization, AM inoculation significantly (P<0.05) increased the shoot biomass of ryegrass and alfalfa as well as the root biomass of ryegrass (Fig. 1). AM fungi failed to increase the phosphorus concentration in the shoots, but it significantly increased the total quantity of phosphorus in shoots due to the increased biomass of shoots (Table 1). The fact that AM fungi improved plant fitness in PAH-contaminated soil has been observed in previous studies [12, 18], which may be attributed to the improvement of plant nutrition and water uptake by AM fungi [10].

Table 2. PAH (PHE, PYE, and DBA) sorption and root concentration factors of plants in different treatments. Root concentration factor = PAH concentration in root tissue/PAH concentration in soil.

Treatments		PAH sorption (mg PAH / g root)			Root concentration factor		
		PHE	PYR	DBA	PHE	PYR	DBA
Alfalfa	AMF	0.002	0.091	0.102	0.92	5.05	25.89
	Non AMF	0.003	0.365	0.179	1.08	33.66	54.98
Tall fescue	AMF	0.000	0.008	0.091	0.00	0.41	20.27
	Non AMF	0.000	0.017	0.070	0.00	0.72	15.75
Ryegrass	AMF	0.001	0.062	0.069	0.26	2.51	17.43
	Non AMF	0.000	0.075	0.072	0.00	2.46	17.04
Celery	AMF	0.012	0.633	0.081	3.16	23.15	16.22
	Non AMF	0.009	0.102	0.092	1.40	3.72	18.94

Effect of PAH Molecular Weight on PAH Dissipation

The percentage of dissipation decreased with the increase of PAH molecular weight and it followed the order: PHE (the mean value of all samples was 89%) > PYR (55%) > DBA (15%). This result was in agreement with the previous findings of Juhasz and Naidu [1] showing that low molecular weight (LMW) PAHs composed of two or three benzene rings are less resistant to microbial degradation compared to high molecular weight (HMW) PAHs with more than four rings, which hardly provide the carbon and energy source for microorganisms. Large variations in the remaining concentration of PAHs (especially PYR with ryegrass-AM) were observed, which could be due to the soil/sand spiking and to the toxicity of the pollutants affecting plant biomass and rhizosphere processes.



ANOVA	Shoot biomass	Root biomass
Plant	***	***
AMF	***	**
Plant × AMF	n.s	*

Fig. 1. Shoot and root biomass in AM-inoculated (+M) and uninoculated (-M) planted pots. (Mean and SD, n=3). Significant differences (P<0.05) between the AM inoculated and uninoculated groups are indicated by different letters. ***P<0.001, **P<0.01, *P<0.05.

PAHs adsorbed by plant roots were lower than 0.5% of the total PAH dissipation in all the treatments (Table 2). Since there was no leakage and limited volatilization of PAHs in our experiment, biodegradation was supposed to be the dominant mechanism in PAH dissipation in the rhizosphere [10]. The root concentration factor (the ratio of PAH concentration in root-to-PAH concentration in soil) increased with the molecular weight of PAHs, with an average value of 0.8, 8.9, and 23.3 for PHE, PYR, and DBA, respectively. The higher HMW PAH sorption may be due to the easily binding of HMW PAHs to root lipids, as HMW PAHs are highly lipophilic [28].

The PAH concentration in the shoot tissue of the four plants was too low to be detected. The absence of significant PAH transfer in plant tissues was consistent with previous studies, and confirmed that highly hydrophobic organic compounds (with log Kow > 3.5, such as 4.6, 5.2, and 6.8 for PHE, PYR, and DBA, respectively) strongly bind to the soil or root surfaces.

Effect of Plant Species on PAH Dissipation

Four plant species (alfalfa, tall fescue, ryegrass, and celery) were selected based on their distinct morphological and physiological characteristics, including the large root biomass of ryegrass and tall fescue, nitrogen fixation capability of alfalfa, and abundant root-derived linoleic acid of celery [11, 29, 30]. All four plant species displayed a positive effect on PHE dissipation, but their effects on HMW

PAH dissipation were highly variable, ranging from stimulatory effect to negative or no effects (Fig. 2). Alfalfa led to higher PYR and DBA dissipation in comparison to the other treatments (Fig. 2). Previous studies showed that alfalfa was an effective plant for HMW PAH phytoremediation. Fan et al. found that after 60 days of remediation, PYR dissipation in alfalfa rhizosphere was higher than that in non-rhizosphere soils [31]. Su et al. compared the effects of different plants with equal planted densities on PAH remediation, and showed that the efficiency of PYR degradation in alfalfa rhizosphere (28%) was higher than that of tall fescue (9.9%) [32]. The capability of forming nitrogen fixing nodules could be an advantage for alfalfa in PAH-contaminated soil, although it has not been proved yet. Adding nitrogen nutrient may increase microorganism activity and PAH dissipation [33, 34].

We hardly detected any DBA dissipation in the celery planted treatment, and the average value of DBA dissipation in the celery planted treatment (0.74 $mg \cdot kg^{-1}$) was lower than that in non-planted control treatment (7.12 $mg \cdot kg^{-1}$). Celery was previously suggested as an efficient plant for improving HMW PAH bioavailability and biodegradation through large amounts of linoleic acid secretion [30, 35]. In the experiment of Yi and Crowley, over 90% of 100 $mg \cdot kg^{-1}$ of PYR and the same amount of 100 $mg \cdot kg^{-1}$ of benzo(a)pyrene were degraded in the celery planted treatment within 40 and 60 days, respectively [30]. Differences in plant size, growth phase (the celery was younger and its biomass was smaller in our experiment than in the one of Yi and Crowley [30]), and experimental conditions could affect root exudate quantity and composition [36], such as the release of linoleic acid. Different soil properties, PAH concentrations, and nutrient and water regimes could affect the growth and activities of plant and PAH degraders [16, 26, 37]. Under harsh environmental conditions plants may secrete plant protection chemicals such as terpenes, which are antibacterial and may reduce the quantity and activities of PAH degraders [30]. The different results of phytoremediation with celery underline the complexity of the processes controlling the fate of PAHs in the plant rhizosphere. Considering the poor growth and performance of celery in our experiment, it deserves more investigation before being used for the phytoremediation of PAH-contaminated soil.

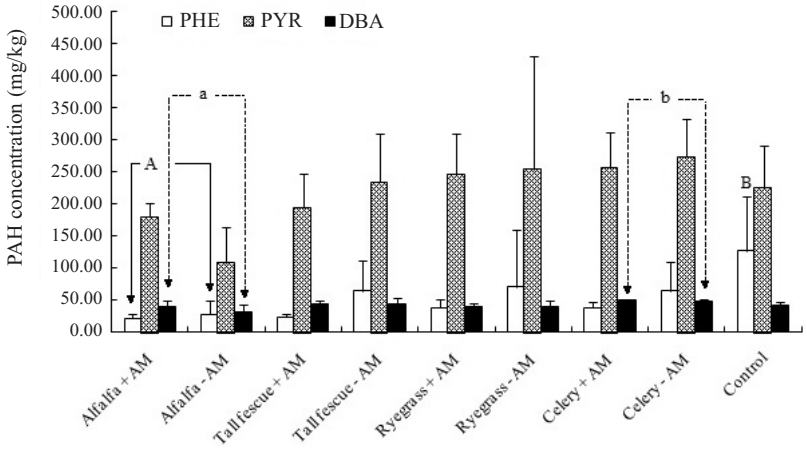
Effect of AM Fungi on PAH Phytoremediation

AM fungi colonization promoted PHE dissipation in planted treatments (Fig. 2). The positive effect of AM fungi on PHE dissipation also was observed in the investigation of Wu et al. [11]. AM inoculation did not significantly increase the number or the percentage of PAH-RHDα genes (Table 3). The increase of PHE dissipation might be caused by an increase of the activities of the PAH degraders [11, 12].

Previous studies reported a positive effect of AM fungi on HMW PAH dissipation [12, 16]. However, *G. intraradices* used in our experiment failed to facilitate PYR and DBA phytoremediation. In a previous experiment with the same PAH-spiked soil, the mixture of mycorrhizal alfalfa and tall fescue improved DBA dissipation in comparison to unplanted controls [16]. But the increase of DBA dissipation was not observed when alfalfa and tall fescue were grown separately in the current study. Xu et al. [38] and Meng et al. [35] reported that a multispecies mixture could enhance the efficiency of PAH phytoremediation compared to the monoculture.

Quantity of Total Microorganisms and PAH Degraders

The average quantities of bacteria and fungi in planted treatments were higher than those in non-planted controls (Table 3). The quantity of PAH degraders tended to be higher in the planted (0.9-1.6%) than in the control treatments (0.7%), although the difference was not significant.



ANOVA	PHE	PYR	DBA
Plant	*	n.s.	*
AM fungi	*	n.s.	n.s.

Fig. 2. Remaining concentrations of PHE, PYR, and DBA in spiked soil after 6 weeks' treatment. +AM: mycorrhizal; -AM: non-mycorrhizal. (Mean and SD, n=3). The table shows plant and AM fungus' effect analyzed by two-way ANOVA. Significant differences ($P<0.05$) between treatments are indicated by different capital and lowercase letters. ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Table 3. Number of 16S rDNA, 18S rDNA, and PAH-RHDα gene copies in AM-inoculated and non-inoculated planted and unplanted (control) pots.

		Microorganisms		PAH-RHDα genes	
		16S rDNA (10^9 g^{-1})	18S rDNA (10^5 g^{-1})	Gram positive (10^7 g^{-1})	Gram negative (10^5 g^{-1})
Alfalfa	AMF	3.64±1.30	2.95±1.20	5.19±3.33	0.15±0.08
	Non AMF	3.97±0.65	2.30±0.29	4.48±2.75	0.20±0.20
Tall fescue	AMF	4.85±0.26	5.23±3.13	7.65±2.44	3.91±6.05
	Non AMF	3.85±1.11	3.86±1.25	4.28±3.24	1.52±1.49
Ryegrass	AMF	4.15±0.58	8.56±6.23	5.00±2.31	0.18±0.07
	Non AMF	2.99±3.07	2.56±1.73	2.71±3.11	4.97±3.91
Celery	AMF	2.96±1.11	4.47±3.44	3.03±1.24	3.56±4.85
	Non AMF	3.02±1.99	8.94±8.18	4.74±5.83	1.65±2.59
Control		1.77±1.02	0.91±0.29	1.18±0.82	0.21±0.20

Mean±SD (n=3). ANOVA analyses showed no significant effects of plants and AM inoculation.

Most of the PAH-RHDα genes belonged to Gram-positive bacteria, and the average quantity of the Gram-positive PAH-RHDα gene copies in each gram of soil was around two orders of magnitude higher than that of Gram-negative PAH-RHDα gene copies. Previous studies indicated that Gram-positive PAH degraders were mainly involved in the degradation of HMW PAHs, while Gram-negative ones were mainly involved in the degradation of LMW PAHs [24, 39, 40]. Therefore, the high percentage of Gram-positive bacteria in PAH degraders may be attributed to the high residual concentration of HMW PAHs in the soil after six weeks. In spite of the lower concentration of Gram-negative bacteria genes, a linear relationship between PHE dissipation and Gram-negative PAH-RHDα genes was observed in alfalfa planted pots (R^2>0.8). Cébron et al. also detected a positive linear relationship between Gram-negative PAH-RHDα genes and LMW PAH concentrations in an industrial polluted soil [24]. However, no relationship was detected between PAH-RHDα genes and PAH dissipation, indicating that there might be other important factors that influence PAH dissipation.

There was no linear relationship between plant biomass and the dissipation of PAHs. Previous studies reported that the root exudates and PAH degrader quantity were important factors that influenced PAH dissipation in microplate experiments [30]. However, we failed to find a similar relationship in our pot experiment. Many parameters such as plant species, nutrients, and water regimes could influence PAH phytoremediation [16].

Conclusions

The present study suggests that biodegradation was the major mechanism of phytoremediation of PAH-spiked soil, and plant uptake was the minor one. All four plant species displayed a positive effect on LMW PAH dissipation, and alfalfa was the most efficient plant in our investigation for it increased the dissipation of HMW PAHs as well. *G. intraradices* colonized plants has the potential to contribute to PAH remediation, since AM inoculation increased plant growth and phosphorus uptake, as well as PHE dissipation in PAH-spiked soil. Plant biomass and PAH degraders showed a weak linear relationship with PAH dissipation, indicating that there might be other important factors influencing PAH dissipation.

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