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Pyrene removal and transformation by joint application of alfalfa and exogenous microorganisms and their influence on soil microbial community

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ABSTRACT

Phytoremediation is an attractive approach for the cleanup of polycyclic aromatic hydrocarbons-contaminated soil. The joint effect of alfalfa and microorganisms, including *Arthrobacter oxydans*, *Staphylococcus auricularis* and *Stenotrophomonas maltophilia*, on pyrene removal was investigated. The results showed that the joint effect primarily contributed to pyrene removal, and the concentration of residual pyrene in rhizosphere soil was lower than that in non-rhizosphere soil. After joint treatment for 45 d, pyrene in rhizosphere soils decreased from 11.3, 52.5 and 106.0 mg/kg to 2.0–3.0, 15.0–18.7, and 41.2–44.8 mg/kg, respectively. These bacteria significantly enhanced pyrene accumulation and microbial community diversity, and increased soil dehydrogenase and polyphenol oxidase activities. Pyrene was initially degraded through ring cleavage. One of the main metabolites 4-dihydroxy-phenanthrene was transformed into naphthol and 1,2-dihydroxynaphthalene, which were further degraded through salicylic acid pathway and phthalic acid pathway, separately.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) have received significant environmental concerns due to their persistence, toxicity, bioaccumulation activity, and carcinogenic potential (Melymuk et al., 2014; Yu et al., 2011). So far, many studies have indicated that phytoremediation is an attractive alternative with environmental-friendly properties and low cost compared to traditional approaches to extract, sequester and detoxify existing PAHs for the cleanup of contaminated soil (Chigbo and Batty, 2013).

Some effective plants, such as *Lolium perenne* (Rentz et al., 2005), *Medicago sativa* (Rentz et al., 2005), *Helianthus annuus* (Tejeda-Agredano et al., 2013) and *Phragmites australis* (Toyama et al., 2011), with significantly large root surface area and good adaptability to different conditions of the soil have been selected to remove PAHs from the contaminated soils. However, inhibition of seed germination, root elongation, root exudation and plant growth in the presence of PAHs hampers the ability of these plants to decontaminate polluted soils efficiently. As a result, research focuses have been recently shifted to the synergistic interaction between plants and rhizosphere microorganisms for the better PAH accessibility and bioremediation (Khan et al., 2013; Toyama et al., 2011; Yousaf et al., 2011).

Some problems pertaining to the exposure of plants to PAHs can be moderated through the plant–microorganism interactions (Gerhardt et al., 2009). For example, root exudates can be utilized as a primary carbon and energy source in PAH degradation by microorganisms, producing dissolved metabolites of PAHs for plant transfer (Rentz et al., 2005; Wang et al., 2012). To further improve the associations of plant–microorganism and accelerate PAH removal in soil, effective microorganisms can be inoculated in this bioremediation process.

Alfalfa, a legume distributed extensively in the global scope, is a potential and valuable plant in restoring the soils contaminated by organic compounds and heavy metals. The removal of polychlorinated biphenyl and organochloride insecticide in soil by the combined use of alfalfa and microorganisms was investigated by some researchers (Kirk et al., 2005; Xu et al., 2010). However, the treatment of PAHs in soil by combination of alfalfa and effective microorganisms has rarely been reported, and among the few contributions, researches mainly focused on the enhancement of PAH dissipation and degradation by exogenous microorganisms,

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such as arbuscular mycorrhizal fungus (Teng et al., 2011). To further enhance PAH bioremoval, attention needs to be paid to the joint effect of plant and exogenous microorganisms, and the efficiency and pathways of PAH degradation.

The potentials of *Arthrobacter oxydans*, *Staphylococcus auricularis* and *Stenotrophomonas maltophilia* to enhance pyrene degradation with alfalfa were studied in the present work. The contributions of abiotic factors, plant accumulation, biological metabolism, and joint effects of alfalfa and these exogenous microorganisms on pyrene degradation in rhizosphere and non-rhizosphere soils were investigated. To determine the positive effects of these microorganisms on pyrene removal, soil dehydrogenase, polyphenol oxidase and microorganism activities, and microbial community diversity were analyzed. Pyrene metabolites were identified to reveal the pathways of pyrene biodegradation.

2. Materials and methods

2.1. Materials

Pyrene was purchased from American USDS Company with a purity of 97%. Alfalfa was obtained from Guangzhou Institute of Agricultural Science, China. *Arthrobacter oxydans*, *S. auricularis* and *S. maltophilia* were isolated from PAHs-contaminated sediments collected at an e-waste processing and recycling town Guiyu, Guangdong, China (Chen et al., 2014; Peng et al., 2012; Ye et al., 2013).

The paddy soil was collected from an experimental field (0–20 cm in depth) at South China Agricultural University, Guangzhou, China. The pH value, organic matter, cation exchange capacity, total nitrogen, total phosphorus and total potassium of soil were 5.86, 11.7 g/kg, 21.6 cmol/kg, 0.7 g/kg, 10.3 mg/kg and 89.1 mg/kg, respectively. There was no detected pyrene in this soil samples. These soil parameters were measured according to the farmland environmental quality evaluation standards of China for edible agricultural products (http://kjs.mep.gov.cn/hjbhbz/bzwb/stzl/%20200611/t20061122_96418.htm), published by Ministry of Environmental Protection of China.

2.2. Strain and plant culture

Strains were grown in medium containing (in g/L) 3 beef extract, 10 peptone and 5 NaCl at 30 °C on a rotary shaker at 120 r/min for 24 h. Subsequently, biomass was separated by centrifugation at 3500 *g* for 5 min, and washed three times with sterile distilled water (Chen et al., 2014). Seeds were surface sterilized in 10% H_2O_2 solution for 10 min and rinsed with sterile distilled water. Then the seeds were germinated in Hoagland solution at 25 °C for 14 d.

2.3. Pyrene degradation experiment

The soil samples were sterilized at 121 °C for 30 min. Pyrene dissolved in distilled acetone was added into the sterile soils at 0 (T0), 11.3 ± 1.0 (T1), 52.5 ± 1.7 (T2) and 106.0 ± 8.8 mg/kg (T3), respectively. After balancing for 14 d with soil moisture of 70%, and fertilizing with 60 mg/kg urea and 120 mg/kg $(NH_4)_2HPO_4$, 600 g soil was put into pot. Alfalfa seedlings were thinned out after 7 d of growth to leave 10 plants each pot. The exogenous bacteria suspended in sterile distilled water were added into the soil. Subsequently, the pots were put in greenhouse in which the day and night temperature, illumination time and intensity were set at 28 ± 2 °C, 24 ± 2 °C, 14 h/d and 5000–6500 lx, separately.

Treatments in this trial were set as follows: (1) alfalfa-planted soil without pyrene (CK); (2) pyrene-contaminated soil without alfalfa (Control); (3) pyrene-contaminated soil with alfalfa (B0); (4) pyrene-contaminated soil with alfalfa and *A. oxydans* (B1); (5) pyrene-contaminated soil with alfalfa and *S. auricularis* (B2); (6) pyrene-contaminated soil with alfalfa and *S. maltophilia* (B3). Biomass of exogenous bacteria was 50 mg/kg and all of the experiments were performed in triplicate.

The transfer coefficients (TCs) and biological accumulation coefficients (BACs) of pyrene are calculated as follows:

TCs = Pyrene concentration in shoot of alfalfa/Pyrene concentration in root of alfalfa.

BACs = Pyrene concentration in biomass/Residual pyrene concentration in soil.

2.4. Preparation of samples

After 45 d, the alfalfa roots and shoots were separated, washed with deionized water, dried by freeze-drying, and then milled. Rhizosphere soil was obtained by collecting soil adhering to root surface after shaking off the excess soil on it, and the non-rhizosphere soil was attained by sampling the mixture of pot soil. All soil samples were used to determine residual pyrene, metabolites, soil enzyme and microbial activity.

2.5. Determination of samples

Pyrene and its metabolites in samples were analyzed by gas chromatography–mass spectrometry (GC–MS) (QP2010, Shimadzu) equipped with a type Rxi-5MS GC column (30 m × 0.25 mm × 0.25 μm) (Teng et al., 2011). Briefly, 20 g freeze-dried samples were extracted by the mixture of 40 ml *n*-hexane, 40 ml dichloromethane and 20 ml acetone for 36 h. The organic mixture was collected, concentrated, dissolved and transferred to alumina-silica gel packed column. Subsequently, the eluent was dried, dissolved and analyzed.

BIOLOG ECO microplates were used to analyze substrate utilization patterns of microbial communities in rhizosphere soil of T0, T1, T2 and T3 treatments. These plates contained 96 wells with different carbon sources and a blank well with no substrate. Each well had the redox dye tetrazolium which was reduced by NADH produced by microbial metabolic pathways. The rate of color development in the wells correlated with the rate of cellular metabolism. Briefly, 10 g soil samples were mixed with 100 ml 0.85% sterilized NaCl solution, blended at 120 r/min for 15 min. Then samples of 150 μl were inoculated into each well of the microplate, and incubated at 25 °C in the dark. The optical density at 590 nm of each well was determined every 12 h. Diversity of microbial carbon utilization was studied by means of species richness, Shannon, Simpson and McIntosh (Kirk et al., 2005).

2.6. Statistical analysis

The mean values of triplicate samples were used in the calculations of pyrene degradation, soil enzyme and Biolog data by Origin 7.5 and SPSS 13.0 software. Principal component analysis (PCA) was performed to investigate the differences of the functional diversity in soil microbial communities.

3. Results and discussion

3.1. Residual pyrene in soil under different conditions

With increase in initial concentration of pyrene, the removal efficiencies decreased both in rhizosphere and non-rhizosphere

Table 1
Residual pyrene in soils under different treatments.

Soil samples		Rhizosphere soil				Non-rhizosphere soil			
Treatments		T0	T1	T2	T3	T0	T1	T2	T3
Residual pyrene (mg/kg)	CK	nd	–	–	–	nd	–	–	–
	Control	nd	10.0 ± 0.5[a]	47.5 ± 3.8[a]	98.3 ± 6.8[a]	nd	10.5 ± 1.2[a]	48.8 ± 5.2[a]	99.8 ± 8.5[a]
	B0	nd	6.9 ± 0.9[b]	33.8 ± 3.6[b]	71.3 ± 4.5[b]	nd	7.2 ± 0.2[b]	36.4 ± 2.9[b]	76.3 ± 7.0[b]
	B1	nd	2.0 ± 0.3[c]	15.0 ± 1.5[c]	41.2 ± 1.8[c]	nd	2.7 ± 0.1[c]	17.4 ± 1.1[c]	47.0 ± 2.4[c]
	B2	nd	3.0 ± 0.2[d]	18.7 ± 1.4[d]	44.8 ± 1.0[d]	nd	4.6 ± 0.3[d]	21.8 ± 1.7[d]	51.3 ± 3.7[d]
	B3	nd	2.2 ± 0.3[e]	16.1 ± 1.0[e]	42.3 ± 3.1[c]	nd	2.8 ± 0.2[c]	18.8 ± 1.2[c]	48.2 ± 2.9[c]

[a,b,c,d] Dancan examination was used to determine the difference between data ($P < 0.05$). Data marked with different superscripts mean significant difference, those marked with same superscripts mean non-significant difference. Pyrene concentrations of T0, T1, T2 and T3 were 0, 11.3 ± 1.0, 52.5 ± 1.7 and 106.0 ± 8.8 mg/kg, respectively. CK, Control, B0, B1, B2 and B3 represented alfalfa-planted soil without pyrene, soil without alfalfa and microbes, alfalfa treatment, alfalfa and *A. oxydans* treatment, alfalfa and *S. auricularis* treatment, alfalfa and *S. maltophilia* treatment, separately.