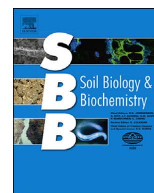
Soil Biology & Biochemistry 113 (2017) 130–142

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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



Microbial mechanisms controlling the rhizosphere effect of ryegrass on degradation of polycyclic aromatic hydrocarbons in an aged-contaminated agricultural soil

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ARTICLE INFO

Article history:
Received 8 February 2017
Received in revised form
21 May 2017
Accepted 4 June 2017
Available online 12 June 2017

Keywords:
Biodegradation
Phytoremediation
Ryegrass
Dioxygenase
DNA/RNA
Rhizosphere effect

ABSTRACT

The phytoremediation of polycyclic aromatic hydrocarbons (PAHs) depends on plant-microbe interactions in the rhizosphere, but the extent and mechanisms of these effects are still unclear. The aim of this study was to investigate the effects of plants on PAHs degradation and on associated changes in transcriptionally active bacterial community structures, degrading bacteria, and especially functional gene expression in a PAH-contaminated agricultural soil. We conducted a rhizo-box experiment planted with ryegrass for 60 days under glasshouse conditions. The degradation of 12PAHs in rhizosphere and non-rhizosphere soils during the experiment was quantified using HPLC analysis. The dynamics of active bacterial community structure in soil were assessed using the molecular tool of cDNA-based denaturing gradient gel electrophoresis (DGGE). Functional PAH-ring hydroxylating dioxygenase (PAH-RHDα) genes were cloned using the Gram-positive (GP) and Gram-negative (GN) primers. The abundance and activity of total bacteria and GP/GN PAH-degraders were monitored by real-time PCR. Results demonstrate that the agricultural soil contained known PAH degraders and RHDα genes, such as *nid*A3, *pdo*A, *nah*Ac and *phn*Ac. The presence of ryegrass promoted the dissipation of PAHs and changed the structures of active bacterial communities in soil. In general, the abundance of total bacteria and GP PAH-degrader increased during the inoculation time. The ryegrass root enhanced the functional bacterial diversity in the early stages (0–10 days). However, the effect of ryegrass roots may stimulate the expression of GP and GN PAH-RHDα genes after 40 days. Our results indicate that ryegrass increased the degradation of PAHs by promoting bacteria diversity, increasing the abundance of total bacteria and PAH degraders, and stimulating RHDα gene expression.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous and ubiquitous pollutants in the environment which are mainly produced by incomplete combustion and pyrolysis of organic matter, as well as spills of crude oil and other petroleum products (van der Heijden and Jonker, 2009; Cvancarova et al., 2013). In recent years, increased amounts of residual PAHs have been detected in agricultural soils, due to industrial processes and wastewater irrigation (He et al., 2015; Guo et al., 2016). The high persistence of PAHs and their toxic, mutagenic, and carcinogenic properties pose high risks to human health and the environment (Haritash and Kaushik, 2009; Fernandez-Luqueno et al., 2011). To date, different strategies have been successfully applied to remediate PAH-contaminated soils, including physical, chemical, and biological techniques (Gan et al., 2009; Haritash and Kaushik, 2009). However, they have different advantages and disadvantages in terms of efficiency, cost, and operation.

Phytoremediation, as a low-cost, green technique, is defined as the use of plants and associated microorganisms to clean up contaminants in soils (Phillips et al., 2012; Shahsavari et al., 2015). Plant

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http://dx.doi.org/10.1016/j.soilbio.2017.06.006
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root-associated microorganisms in the rhizosphere have been exploited for the removal of PAHs (D'Orazio et al., 2013; Khan et al., 2013; Tejeda-Agredano et al., 2013; Liu et al., 2015). The positive effect of rhizosphere on PAH removal is mainly attributed to the enhancement of microbial biomass, activity, and diversity as a result of release of nutrients, exudates, and oxygen into the soil (Cébron et al., 2011; Hamdi et al., 2012; Martin et al., 2014). Improvement of soil aeration and permeability by plant roots is another mechanism that influences the bioavailability and decontamination of soil PAHs (Hamdi et al., 2012).

The assessment of genes key to PAHs biodegradation can provide mechanistic information on microbial processes occurring in response to PAHs contamination. Alpha subunit genes of ring-hydroxylating-dioxygenase (RHDα), involved in the degradation of PAHs (Cébron et al., 2008; Ding et al., 2010), have been used as target genes for detecting PAH-degrading strains and for describing degradation capacities in different environments (Paisseé et al., 2012; Thomas and Cébron, 2016). Plants may increase the abundance of bacteria and induce the expression of microbial RHDα genes (Louvel et al., 2011). Thus far, most studies focused on the abundance and diversity of hydrocarbon-degrading microbial communities in PAH-contaminated soils (Sawulski et al., 2014; Li et al., 2015a; Muangchinda et al., 2015). However, few studies have attempted to link PAH degradation to the rhizosphere effects of plant on the diversity, community composition, and RHDα gene expression of soil microorganisms (Louvel et al., 2011; Thomas and Cébron, 2016). The behavior of microbial communities in the rhizosphere during degradation of PAHs is not clearly understood (Storey et al., 2014; Chen et al., 2016a). Thomas and Cébron (2016) investigated the short-term (2–10 days) effect of the ryegrass rhizosphere on phenanthrene degradation and active microbiome in an aged contaminated industrial soil, and found that ryegrass induced an early shift in the identity of potential phenanthrene degraders. The long-term rhizosphere effects on aged PAH-contaminated agricultural soils still, however, remain unknown.

Therefore, this study was conducted to investigate the microbial mechanism controlling PAH degradation in an aged-contaminated agricultural soil following the long-term presence of ryegrass plants. To achieve the objective, we studied the biodegradation of 12 PAHs in ryegrass associated with the rhizosphere and non-rhizosphere component of agricultural contaminated soils. We inoculated a *Mycobacterium* strain into specially constructed rhizo-boxes, and compared the dynamics of the active bacterial community structures using denaturing gradient gel electrophoresis (DGGE). The diversity, abundance and activity of total bacterial, PAH Gram-positive (GP) and Gram-negative (GN) degraders were also monitored based on DNA/RNA analyses.

2. Materials and methods

2.1. Soil and microorganisms

Clay sandy loam agricultural soil was collected from the top 20 cm of a wastewater-irrigated site in the suburbs of Shenyang, China. It was air-dried, ground, sieved to 2 mm and stored in the dark at room temperature (20–25 °C) prior to analysis. The soil was a silty loam, containing 62.2% clay, 26.2% silt, and 11.6% sand. The organic matter content of the soil was 2.2% and the bulk concentration of 12 PAHs was 9.72 mg kg^{-1}. For a summary of the methods used to quantify the individual PAHs in the soil, see Section 2.3.

The PAH-degrading bacterium used in this study was *Mycobacterium gilvum* (Genbank accession number DQ512892), which was described previously (Guo et al., 2016). The strain was grown in LB broth and placed in a shaker at 28 °C for 5 d. The number of *Mycobacterium* sp. in the liquid medium for following inoculation was determined by a most probable number assay. The inoculum suspension (~8×10^8 cells mL^{-1}) was diluted with sterile water to 10^6 cells mL^{-1}. Then, at a ratio of 1:10 (v/w), the diluted suspension (mL) was added to the PAH-contaminated agricultural soil (g) to a final concentration of approximately 10^5 cells g^{-1} soil, and an equivalent volume of autoclaved LB broth (without *Mycobacterium*) diluted with sterile water was added to the other part of the soil to serve as the control.

2.2. Rhizo-box experimental design

A rhizo-box experiment was conducted in a greenhouse for 60 days. In this set-up, a rectangular rhizo-box with dimensions of $200 \times 200 \times 300$ (length × width × height, in mm) was prepared similarly to that described by He et al. (2005). The box was divided by nylon mesh (<25 μm pore size) into three compartments: a root compartment, or a rhizosphere zone in the middle (80 mm in width), and non-rhizosphere zones at the left and right sides (each 60 mm in width). The design successfully constrained root hairs from entering the adjacent non-rhizosphere soil and separated the soil zones. The soil samples, either inoculated with *Mycobacterium* sp. or without inoculation, were added to the rhizo-box, respectively. Three kilograms of soil was placed in the rhizosphere zone, while each of the non-rhizosphere zones received 1.75 kg of soil. Soil moisture content was adjusted to 60% (v/w) of water-holding capacity (WHC) before use.

The ryegrass seeds (*Lolium multiflorum* L., Shenyang Agriculture University, Shengyang, China) were sterilized using 30% (v/v) H_2O_2 for 30 min, sown in the rhizosphere zone (70 seeds per root compartment), and allowed to germinate at room temperature in the dark. After germination, the seedlings were thinned to 50 plants and allowed to grow for two months in an artificially controlled climate chamber, with a day/night (14/8 h) temperature 28/22 °C (10,000 Lux), and a relative humidity of 50%. The plants were watered with sterile distilled water throughout the experiment to keep the soil at approximately 60% of its WHC by periodically weighing the microcosms. Soils from non-planted microcosms were handled in the same way. In total, four treatments were carried out in rhizo-boxes: soil without ryegrass and *Mycobacterium* sp. (control), soil inoculated with *Mycobacterium* sp. (M), soil planted with ryegrass (R), and soil inoculated with *Mycobacterium* sp. and planted with ryegrass (MR).

On days 0, 3, 10, 20, 40 and 60, three randomly chosen rhizo-boxes of each treatment were sampled from different root zones. For R and MR treatments, the soil that strongly adhered to the root surface was separated and regarded as rhizosphere soil (+), and the soils sampled from the left and right compartments of the rhizo-boxes were mixed and were referred to as non-rhizosphere or bulk soil (−). The treatments without plant (control and M) were sampled from different compartments of the rhizo-box and mixed uniformly. The soil samples (control, M, R-, R+, MR- and MR+) were used for PAHs dissipation analysis and DNA/RNA extraction.

2.3. Extraction and analysis of PAHs

The total and individual amounts of PAHs in the contaminated soil samples were measured by high performance liquid chromatography (HPLC) after extraction using a mechanical chemical extraction procedure (Gong et al., 2010; Guo et al., 2016). An external standard mixture (M-8310, AccuStandard, New Haven, Connecticut, USA) was used for PAH calibration and quantification. The detection limits of the methods for PAHs were in the range of 0.2–12 ng L^{-1}. Recovery study was carried out by spiking a mixture of PAHs to soils using the same extraction procedure. Average

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