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The coupling of the plant and microbial catabolisms of phenanthrene in the rhizosphere of *Medicago sativa*

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ABSTRACT

We studied the catabolism of the polycyclic aromatic hydrocarbon phenanthrene by four rhizobacterial strains and the possibility of enzymatic oxidation of this compound and its microbial metabolites by the root exudates of alfalfa (*Medicago sativa* L.) in order to detect the possible coupling of the plant and microbial metabolisms under the rhizospheric degradation of the organic pollutant. A comparative study of phenanthrene degradation pathways in the PAH-degrading rhizobacteria *Ensifer meliloti*, *Pseudomonas kunmingensis*, *Rhizobium petrolearium*, and *Stenotrophomonas* sp. allowed us to identify the key metabolites from the microbial transformation of phenanthrene, including 9,10-phenanthrenequinone, 2-carboxybenzaldehyde, and 1-hydroxy-2-naphthoic, salicylic, and *o*-phthalic acids. Sterile alfalfa plants were grown in the presence and absence of phenanthrene (0.03 g kg^{-1}) in quartz sand under controlled environmental conditions to obtain plant root exudates. The root exudates were collected, concentrated by ultrafiltration, and the activity of oxidoreductases was detected spectrophotometrically by the oxidation rate for various substrates. The most marked activity was that of peroxidase, whereas the presence of oxidase and tyrosinase was detected on the verge of the assay sensitivity. Using alfalfa root exudates as a crude enzyme preparation, we found that in the presence of the synthetic mediator, the plant peroxidase could oxidize phenanthrene and its microbial metabolites. The results indicate the possibility of active participation of plants in the rhizospheric degradation of polycyclic aromatic hydrocarbons and their microbial metabolites, which makes it possible to speak about the coupling of the plant and microbial catabolisms of these contaminants in the rhizosphere.

1. Introduction

Plant–microbial associations and symbioses, with flexible metabolisms and unique enzyme systems, have great advantages for survival in rugged environments, and their survival is due not only to their increased tolerance to xenobiotics, but also to active removal of toxic substances from the habitat. Despite the increased use of plants and their associated microorganisms in the form of phytoremediation technology, biochemical interactions between partners in the rhizosphere that are aimed at the degradation of organic pollutants are not fully understood.

Polycyclic aromatic hydrocarbons (PAHs) are widespread and hazardous environmental pollutants that are priority control substances. Both natural and anthropogenic sources, such as forest fires, vehicular emissions, and industrial combustion of fossil fuels contribute to the release of PAHs into the environment. Additional sources of these toxic, mutagenic, and carcinogenic substances are wastes from oil-refining and byproduct-coking.

Phytoremediation, an environmentally friendly biotechnology based on the use of plants and associative microorganisms, has demonstrated its success and strength because it is inexpensive, esthetically attractive, and efficient. The accumulated research and field trial experience has led investigators to identify effective remediating plants capable of intensive elimination of pollutants from contaminated soil. One such plant is alfalfa (*Medicago sativa* L.), which is effective for the cleanup of soils polluted with PAHs (Criquet et al., 2000; Schwab et al., 2006; Phillips et al., 2006; Martí et al., 2009).

A principal factor of enhanced degradation of hydrocarbons in the plant rhizosphere during phytoremediation is the rhizosphere effect, i.e., the increased numbers and activity of soil microorganisms in the plant-root zone. Alfalfa can selectively increase the number of PAH degraders in its rhizosphere, thereby intensifying microbial degradation of pollutants in soil (Muratova et al., 2003;

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Kirk et al., 2005; Phillips et al., 2006). The diversity of microorganisms inhabiting the plant rhizosphere implies that there are different methods of microbial degradation of organic pollutants, including PAHs, and that a variety of microbial metabolites of these pollutants are present in the plant root zone. In this context, the study of PAH degradation pathways by typical rhizosphere microorganisms and the identification of their metabolites seems important for our knowledge of plant-promoted microbial degradation.

In addition to microbial degradation, the involvement of plant root-released enzymes in chemical reactions catalyzing the transformation of organic xenobiotics in soil is an important mechanism of PAH degradation in the rhizosphere (Adler et al., 1994; Schnoor et al., 1995; Siciliano et al., 1998). The enzymes released by the plant roots include a large group of oxidoreductases (monophenol monooxygenase, laccases, and peroxidases), certain esterases, proteases, lipases, dehalogenase, nitroreductase, and nitrilase (Schnoor et al., 1995; Gramss and Rudeschko, 1998; Gramss et al., 1999, 2000; Harvey et al., 2002) Gramss and Rudeschko, 1998; Gramss et al., 1999; Gramss et al., 2000 Harvey et al., 2002). Gramss et al. (1999) have shown that the roots of some plants release a sufficient amount of oxidoreductases to participate in the oxidative degradation of soil organic matter. Hence, the involvement of plant extracellular enzymes in the rhizosphere degradation of organic pollutants can be significant. Being the primary oxidizing system in the root exudates of various plants, oxidoreductases can transform pollutants into compounds that are more available to plants and/or rhizosphere microorganisms (Gramss and Rudeschko, 1998; Gramss, 2000; Gianfreda et al., 2006). Thus, the active involvement of plant oxidoreductases in the phytoremediation process has been suggested. This assumption is also based on data showing that the peroxidase activity of some plants, including alfalfa, is high in PAH-contaminated soil (Criquet et al., 2000; Flocco et al., 2002; Muratova et al., 2009). With this in mind, we hypothesized that the efficacy of alfalfa in the phytoremediation of PAH-contaminated soil could be connected not only with the selectively increased numbers of PAH degraders but also with the plant's own enzymatic activity toward aromatic contaminants.

The objective of this research was to detect the possible coupling of the plant and microbial catabolisms under the rhizospheric degradation of PAHs. To attain this objective, we studied the catabolism of phenanthrene by several rhizobacterial strains and the possibility of enzymatic oxidation of this compound and its microbial metabolites by the root exudates of alfalfa (*M. sativa* L.).

2. Materials and methods

2.1. Microorganisms

The following microbial strains isolated from the root zone of different plants grown in oil-contaminated soils and maintained in the Collection of Rhizosphere Microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences (IBPPM RAS), were used in this study: *Ensifer meliloti* P221 (IBPPM 383), *Pseudomonas kunmingensis* L3 (IBPPM 333), *Rhizobium petrolearium* Rsf11 (IBPPM 350) and *Stenotrophomonas* sp. P422 (IBPPM 347).

2.1.1. Microbial degradation of phenanthrene

For investigating the metabolism of phenanthrene, microorganisms were incubated in 1.0-L Erlenmeyer flasks containing liquid mineral salt medium (MSM) with phenanthrene. Phenanthrene in an isopropanol solution ($10\,g\,L^{-1}$) was added to empty flasks to achieve final concentrations of $0.3\,g\,L^{-1}$. After evaporation of the solvent, 200 mL of mineral medium was added to the flasks. The MSM contained ($g\,L^{-1}$): K_2HPO_4, 0.5; NH_4Cl, 1.0; Na_2SO_4, 2.0; KNO_3, 2.0; $MgSO_4$, 0.5; traces of $FeSO_4$; micronutrient solution, 1.0 mL. The micronutrient solution contained ($g\,L^{-1}$): H_3BO_3, 0.5; $CuSO_4{\cdot}5H_2O$, 0.04; KI, 0.1; $FeCl_3$, 0.2; $MnSO_4{\cdot}H_2O$, 0.4; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2; $ZnSO_4{\cdot}7H_2O$, 0.4. The growth medium was inoculated with a fresh microbial culture grown in MSM with sodium succinate ($1\,g\,L^{-1}$) to bring the initial absorbance of the culture medium (A_{540}) to about 0.5. Incubation was performed at 29 °C with rotary shaking (130 rpm) for up to 14 days. The control flasks contained the same medium and equal concentrations of phenanthrene without the inocula.

Metabolites were analyzed at 0, 1, 3, 7, and 14 days. After cultivation, phenanthrene and its metabolites were extracted with ethyl acetate (50 mL of ethyl acetate per 200 mL of culture medium, three times for 5 min each), first from the native culture medium and then after acidifying the culture medium to pH 2 by adding 1 M hydrochloric acid. Neutral and acid extracts were dried over sodium sulfate and were concentrated by solvent evaporation at room temperature.

The key metabolites formed in the biotransformation of phenanthrene were identified by different kinds of chromatography and mass spectrometry.

Thin-layer chromatography (TLC) was used for the preparative isolation of metabolites. TLC was performed on Silufol UV-254 plates (Kavalier, Czech Republic), which were developed in hexane:ethyl acetate:acetic acid (10:30:1) or in benzene:dioxane:acetic acid (90:10:2). The spots corresponding to metabolites were marked under ultraviolet light, scraped from the plates, and extracted with methanol.

The residual concentration of phenanthrene in the medium was analyzed by extraction with carbon tetrachloride and by gas chromatography (GC) on a Shimadzu 2010 chromatograph equipped with an Equity-1 (Supelco, USA) nonpolar capillary column, a flame ionization detector, and He as a carrier and makeup gas. A solution of authentic phenanthrene was used as a standard.

The concentration of phenanthrene metabolites were determined by ethyl acetate extraction and by high-performance liquid chromatography (HPLC) on a Thermo Scientific Dionex Ultimate 3000 (USA) chromatograph with a diode array detector (DAD) at 252 nm and with an Acclaim® 300 wide pore (300 Å) column (3 μm × 150 mm × 2.1 mm, Dionex). The solvent system was 40:60 acetonitrile:water plus 50% phosphorous acid (pH 2.5) for all substituted PAHs and methanol:acetonitrile:water (67.5:10:22.5) for the unsubstituted PAHs. Isocratic elution was at a rate of $0.5\,mL\,min^{-1}$, and the temperature was 30 °C.

Some metabolites were also identified by mass spectrometry as described previously (Muratova et al., 2014).

The degradation of phenanthrene and its metabolites was expressed in percent of reduction of their initial concentration.

2.1.2. Plant cultivation

Seeds of alfalfa (*M. sativa* L.) were obtained from the Scientific Research Institute of Agriculture in the South-East (Saratov, Russia). The seeds were calibrated by size, surface sterilized in a sodium hypochlorite solution (active chlorine content of 8–9%) for 30 min, and then washed with sterile tap water for at least three times. For checking sterility, the seeds were germinated on the surface of a doubly diluted nutrient agar medium in Petri dishes for 3 days.

Quartz sand (particle size of 1–2 mm) in 0.3 L Erlenmeyer flasks (150 g per flask) was heat sterilized and sprayed with a 1.5% (w/v) acetonic solution of phenanthrene to a final concentration of the PAH of $0.03\,g\,kg^{-1}$. The control sand substrate was treated with pure acetone of equal volume. After evaporation of the solvent, the sand substrates were moistened to 80% of the maximum water-holding capacity by adding Ruakura nutrient solution (Smith et al., 1983). Ten sterile germinated seeds were placed in each flask and cultivated for three weeks in a growth chamber with a