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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

16/8 h day/night regimen (light intensity of 8000 lux, temperature of 24/20 °C, relative humidity of 70%). The water content of the quartz sand was maintained at 80% of the maximum water-holding capacity by daily weighing of the flask.

2.1.3. Collection of plant root exudates

Before the collection of root exudates, the rhizosphere solution from each flask with plants was checked for sterility by seeding of a 0.1-mL aliquot on nutrient agar. Thereafter, the sand in each flask was flooded with sterile tap water (~25 mL) so that the entire root system of the plants was dipped in the solution. In this way, the plants were cultured for 2 days under the same conditions. After cultivation, the plants were lifted out of the flask and the exudates-containing rhizosphere solution was collected. Mechanical impurities were removed by centrifuging the rhizosphere solution at 8000 g for 10 min. Before assaying the enzyme activity, the root exudates were concentrated 8–10 fold by using an 8200 Millipore ultrafiltration cell and an Ultracel® 10-kDa membrane (Millipore, USA).

2.1.4. Measurement of the enzymatic activities of root exudates

The protein content in root exudate samples was determined according to Bradford (1976). Enzymatic activities of the root exudates were detected spectrophotometrically by the oxidation rate for various substrates, which was measured by using an Evolution 60 spectrometer (Thermo Scientific, USA). In a total of 2.0 mL, the reaction suspensions contained 0.2 mL of the root exudates and 0.2 mL of the substrates.

Oxidase activity was measured by using 50 mM sodium citrate buffer at pH 3.5, 4.5, 5.5, 6.5 and 7.5 with 30 μM syringaldazine (SGZ, Leonowicz and Grzywnowicz, 1981) at 525 nm, or 0.5 mM 2,6-dimethoxyphenol (DMOP, Slomczynski et al.,1995) at 468 nm, or 1 mM 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (ABTS, Niku-Paavola et al., 1988) at 436 nm, or 23 μM 2,7-diaminofluorene (DAF, Criquet et al., 2000) at 600 nm.

The activity of peroxidase was measured by monitoring the oxidation rate of the same substrates and 0.3 mM *o*-dianisidin (DAZ, at 460 nm) at the same pH values in the presence of 0.1 mM H_2O_2. The activity of monophenol monooxygenase (tyrosinase) was measured in 4 mM 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) and 50 mM sodium–potassium phosphate buffer at pH 6.0 (Criquet et al., 2000). The oxidation rate of the substrate was monitored at 475 nm.

All the activities are expressed in enzyme units (U), defined as μmol oxidized substrate min^{-1} mg^{-1} protein.

2.1.5. Electrophoretic analysis

Alfalfa root extracts and exudates were subjected to electrophoretic analysis to determine the enzyme present in the different treatments. Plant root extract samples were obtained as described previously (Muratova et al., 2009).

Electrophoretic analyses of concentrated root exudates were performed by nondenaturing (without SDS or β-mercaptoethanol) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). For the analysis of peroxidase isoenzymes, polyacrylamide gels (12%) layered with 4% stacking gels were used. To detect peroxidase activity, we stained the gels with 4 mmol L^{-1} *o*-dianisidine in the presence of 0.1 mmol L^{-1} H_2O_2.

2.1.6. Enzymatic oxidation of phenanthrene and its microbial metabolites by root exudates

The enzymatic oxidation of phenanthrene and its derivatives was performed according to Günther et al. (1998). Concentrated root exudate samples were used as a crude enzyme preparation. Enzymatic reactions were run with ABTS (final concentration of 0.3 mM) added as a mediating agent to the reaction suspensions (Günther et al., 1998; Johannes and Majcherczyk, 2000).

Phenanthrene, 9,10-phenanthrenequinone, 9-phenanthrol, 1-naphthol, 2-carboxybenzaldehyde, 1-hydroxy-2-naphthoic acid, salicylic acid, *o*-phthalic acid, and diphenic acid were used as substrates [dissolved in acetonitrile (0.2 g L^{-1})] and were added to the reaction suspensions to a final concentration of 10 mg L^{-1}.

In a total of 2.0 mL, the reaction suspensions contained 1.5 mL of 50 mM Na K-phosphate buffer (pH 6.0); 50 μL of acetonitrile solution of substrate; 50 μL of 20 mM ABTS; 200 μL of the concentrated root exudates; 200 μL of 5 mM H_2O_2. Controls were run by using the same reaction mixtures and conditions but with boiled root exudates or deionized water instead of the active enzyme preparations. The enzymatic reactions were run in triplicate in 10-ml tubes closed with sterile plugs in a thermostat at 24 °C in the dark. After 24 h, the reactions were stopped by acidification (pH 2.0) with HCl and addition of 1 mL of ethyl acetate (or carbon tetrachloride for phenanthrene), and the substrates were extracted twice for 5 min. The extracts were collected, evaporated, and redissolved in acetonitrile. The PAH concentrations were determined by HPLC as described above.

2.1.7. Chemicals

Phenanthrene (>98% purity, Fluka) was used in all experiments. Several metabolites were used as standards, including 9-phenanthrol (>97% purity, Aldrich), 9,10-phenanthrenequinone (>99% purity, Aldrich), 2-carboxybenzaldehyde (97% purity, Aldrich), 1-hydroxy-2-naphthoic acid (>97% purity, Fluka), 2,2′-diphenic acid (>95% purity, Fluka), salicylic acid (99% purity, Reakhim, Russia), phthalic acid (99% purity, Reakhim, Russia), and 1-naphthol (99% purity, Reakhim, Russia). For enzymatic analyses ABTS (>99% purity, Sigma–Aldrich), syringaldazine (Sigma), 2,7-diaminofluorene (>97% purity, Fluka), 2,6-dimethoxyphenol (>99% purity, Acros Organics), and *o*-dianisidin (>95% purity, Fluka) were used.

2.1.8. Statistics

All experiments had three replications. Analysis of variance (ANOVA) was used to estimate statistically significant differences between groups of samples. The experimental results for the quantitative estimation of phenanthrene degradation were statistically processed by calculating the means, which were compared by using LSD ($P = 0.05$) Fisher's test. Statistica software version 7 (StatSoft Russia) and Microsoft Excel 2003 were used for statistical analysis.

3. Results

3.1. Microbial degradation of phenanthrene by rhizobacteria

The characteristics of the rhizobacteria used in this study are given in Table 1. All these strains are capable, to varying degrees, of degrading phenanthrene as a sole carbon and energy source.

The summarized data on the microbial metabolites arising from the conversion of phenanthrene by the rhizosphere strains are presented in Table 2. Phenanthrenequinone, 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, *o*-phthalic acid, and salicylic acid were identified as the key metabolites produced by the rhizobacteria in the course of biotransformation of phenanthrene.

It was found that the metabolic pathways for phenanthrene in these rhizobacteria were different: Putative schemes for phenanthrene conversion by the rhizobacteria examined are given in Fig. 1.

Within five days, *E. meliloti* P221 was able to degrade up to 60% of the phenanthrene added to the cultivation medium as a sole carbon and energy source at a concentration of 0.3 g kg^{-1}. The bioconversion of phenanthrene by *E. meliloti* P221, occurring via two pathways (Fig 1a and c), was studied in detail and reported

Table 1
PAH-degrading rhizosphere bacteria used in this study.

Strain	Plant rhizosphere from which the strains have been isolated	PAH substrates	Degradation of phenanthrene ($0.4\,g\,L^{-1}$) within 5 days (%)
Ensifer meliloti P221 (IBPPM 383)	*Phragmites australis*	Flu, Phe	62.8 ± 3.8
Rhizobium petrolearium Rsf11 (IBPPM 350)	*Medicago sativa*	Flu, Phe	59.5 ± 2.6
Pseudomonas kunmingensis L3 (IBPPM 333)	*Medicago sativa*	Ant, Nap, Phe	52.2 ± 4.8
Stenotrophomonas sp. P422 (IBPPM 347)	*Convolvulus arvensis*	Flu, Phe	20.3 ± 1.9

Ant—anthracene, Flu—flurene, Nap—naphthalene, Phe—phenanthrene.

Table 2
Characteristics of the metabolites identified in extracts of the medium after cultivation of rhizobacteria in the presence of phenanthrene as a sole carbon and energy source.

Metabolite	Compound identified	R_f (TLC)	R_t (HPLC)	Confirmation of identification
1	1-hydroxy-2-naphthoic acid	0.52	3.37	R_f (TLC) and R_t (HPLC) are identical to those of the standard sample; UV spectrum is identical to that of the standard sample: two maxima at 250 and 340 nm
2	phenanthrenequinone	0.35	2.83	R_f (TLC) and R_t (HPLC) are identical to those of the standard sample
3	2-carboxybenzaldehyde	0.20	1.12	R_f (TLC) and R_t (HPLC) are identical to those of the standard sample
4	o-phthalic acid	0.13	1.01	R_f (TLC) and R_t (HPLC) are identical to those of the standard sample; UV-spectrum is identical to those of the standard sample: maximum at 290 nm
5	salicylic acid	0.50	1.45	R_f (TLC) and R_t (HPLC) are identical to those of the standard sample. UV-spectrum is identical to those of the standard sample: three maxima at 210, 235, and 300 nm

The values represent the averages for the strains studied: *Ensifer meliloti* P221, *Pseudomonas kunmingensis* L3, *Rhizobium petrolearium* Rsf 11, and *Stenotrophomonas* sp. P422; The concentration of phenanthrene in the medium was $0.3\,g\,l^{-1}$; the analyses were made after 0, 1, 3, 7, and 14 days of cultivation; TLC (thin-layer chromatography) was performed on Silufol UV-254 plates in hexane:ethyl acetate:acetic acid (10:30:1); HPLC (high-performance liquid chromatography) was performed on a Thermo Scientific Dionex Ultimate 3000 chromatograph with a diode array detector at 252 nm and with an Acclaim® 300 wide pore (300 Å) column; the solvent system was 40:60 acetonitrile:water plus 50% phosphorous acid (pH 2.5).

previously (Muratova et al., 2014). Briefly, the first, major pathway is through terminal aromatic ring cleavage (presumably at the C3—C4 bond), producing benzocoumarin and 1-hydroxy-2-naphthoic acid, whose further degradation with the formation of salicylic acid is difficult or is very slow. The second pathway is through the oxidation of the central aromatic ring at the C9—C10 bond, producing 9,10-dihydro-9,10-dihydroxyphenanthrene, 9,10-phenanthrenequinone, and 2,2′-diphenic acid.

The rhizobacterial strain *R. petrolearium* Rsf 11 transformed about 60% of phenanthrene (Table 1). In the culture liquid, phenanthrenequinone and 1-hydroxy-2-naphthoic acid were identified as the key metabolites. Neither salicylic nor *o*-phthalic acid was formed in any significant quantities in the medium after cultivation of this microorganism. We supposed that *R. petrolearium* Rsf 11 was capable of incomplete degradation of phenanthrene.

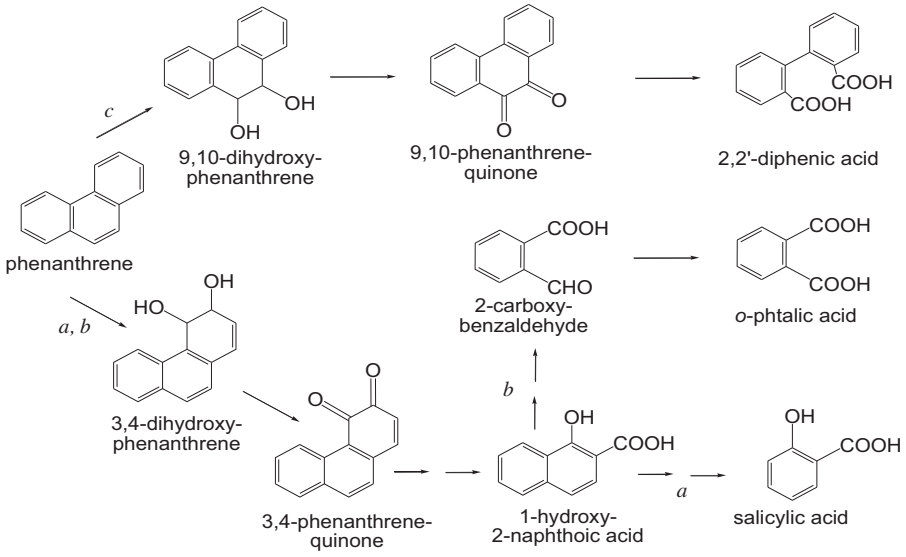

Fig. 1. Proposed schemes for the conversion of phenanthrene by the rhizobacteria studied: *a, c—Ensifer meliloti* P221; *b—Pseudomonas kunmingensis* L3 and *Stenotrophomonas* sp. P422; *Rhizobium petrolearium* Rsf 11 degraded phenanthrene to 1-hydroxy-2-naphthoic acid only.

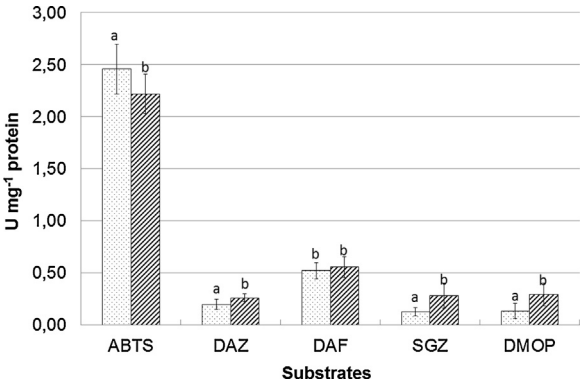

Fig. 2. Peroxidase activity of the alfalfa root exudates collected from untreated ( ) and phenanthrene-treated ( ) sand. ABTS—2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt; DAZ—*o*-dianisidine; DAF—2,7-diaminofluorene; SGZ—syringaldazine; DMOP—2,6-dimethoxyphenol. Enzymatic activities were detected spectrophotometrically by the oxidation rate for substrates as described in Section 2. A 2.0 mL portion of the reaction suspension contained 1.4 mL of 50 mM sodium citrate buffer at optimal pH (3.5–7.5), 200 μL of the concentrated root exudates, 200 μL of the substrates, and 200 μL of 0.1 mM H_2O_2. The values represent the averages of three experiments, and bars represent standard errors of means ($n = 3$); the different letters indicate statistically significant differences between root exudates from untreated and phenanthrene-treated sand.

The strains *P. kunmingensis* L3 and *Stenotrophomonas* sp. P422 degraded 52 and 20% of phenanthrene, respectively. In the course of biotransformation, colored metabolites accumulated in the medium. They were identified as phenanthrenequinone, 1-hydroxy-2-naphthoic acid and *o*-phthalic acid. Thus, the bioconversion of phenanthrene by these strains followed the *o*-phthalate pathway (Fig 1b).

3.2. Plant root exudate enzymes

Study of the enzymatic activity of the alfalfa root exudates revealed a significant peroxidase activity, whereas the presence of oxidase and tyrosinase was detected on the verge of the assay sensitivity.

Evaluation of the enzymatic oxidation of the five test substrates (ABTS, *o*-dianisidine, DAF, DMOP, and syringaldazine) demonstrated that peroxidase activity in the root exudates of alfalfa grown in untreated sand ranged from 0.13 to 2.46 U mg^{-1} protein and from 0.28 to 2.22 U mg^{-1} protein in sand contaminated with phenanthrene (Fig. 2). The optimal substrates for the detection of peroxidase activity of the alfalfa root exudates were ABTS and DAF. The presence of phenanthrene at 0.03 g kg^{-1} in the sand insignificantly reduced ABTS peroxidase activity of the exudates, but significantly stimulated peroxidase activity measured with other substrates. The syringaldazine and 2,6-dimethoxyphenol peroxidase activities were more clearly expressed in the exudates of alfalfa grown in contaminated soil.

Nondenaturing PAGE of the peroxidases detected in root exudates of alfalfa grown in untreated sand revealed the presence of five peroxidase isoenzymes with R_f values of 0.06, 0.14, 0.32, 0.38, and 0.44, using *o*-dianisidine as a substrate (Fig. 3a). Electrophoretic analysis of root exudates of plants grown in phenanthrene-treated sand revealed the presence of eight isoenzymes with R_f values of 0.06, 0.14, 0.29, 0.32, 0.38, 0.44, 0.92, and 0.96 (Fig. 3b). The patterns were similar to that of the root extracts.

The optimum pH values for the manifestation of root exudate peroxidase activity were in the range 4.5–5.5 for ABTS, DAZ, and DAF for root exudate samples from both untreated and phenanthrene-treated sand (Fig. 4). For syringaldazine, the optimum pH values were about 6.0 for samples from both untreated and phenanthrene-treated sand. The DMOP-peroxidase activity was the highest at pH 3.5 and 6.5 for samples from both untreated and phenanthrene-treated sand, respectively.

3.3. Enzymatic peroxidation of PAHs and their microbial metabolites by the plant root exudates

Using alfalfa root exudates as a crude enzyme preparation, we found that in the presence of the synthetic mediator ABTS, plant peroxidase can oxidize phenanthrene and its oxidized derivatives (Fig. 5). About 15 and 16% of the phenanthrene were oxidized in the reaction mixture by root exudates from uncontaminated and phenanthrene-contaminated sand, respectively. The peroxidation of 1-naphthol and 1-hydroxy-2-naphthoic acid was almost complete—92 and 95% by root exudates from uncontaminated sand and 94 and 99% by root exudates from contaminated sand, respectively. Differences in enzyme activity between the root exudates from untreated and phenanthrene-treated sand were observed for several substrates. 2-Carboxybenzaldehyde and *o*-phthalic acid were oxidized only by root exudates from phenanthrene-contaminated sand (by 14 and 15%, respectively). Peroxidation of 9-phenanthrol and salicylic acid by root exudates from phenanthrene-contaminated sand increased eight- and six-fold (from 9 to 68% and from 13 to 78%), respectively, in comparison with the root exudates from untreated sand.

With HPLC, 9,10-phenanthrenequinone was identified as the product of 9-phenanthrol peroxidation. With spectrophotometry, the peroxidation products of 1-naphthol, presumed to be hydroxynaphthoquinone and its conjugated oligomers, were detected. No peroxidation products of 1-hydroxy-2-naphthoic acid and salicylic have been detected.

4. Discussion

Phenanthrene is one of the most common PAHs. Its molecule comprises three fused benzene rings and contains a bay-region and a K-region, participating in the formation of the epoxide, which is believed to be extremely carcinogenic (Samanta et al., 1999; Samanta et al., 2002). Owing to its relatively low toxicity and moderate bioavailability, phenanthrene is often used as a model substrate for studying catabolism of toxic compounds containing bay- and K-regions such as benzo [a] pyrene, benz [a] anthracene, and chrysene (Samanta et al., 1999).

Microbial degradation of phenanthrene has been well studied to date (Seo et al., 2009). Many bacterial species belonging to different genera (*Acidovorax*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Aeromonas*, *Arthrobacter*, *Bacillus*, *Beijernickia*, *Brevibacterium*, *Burkholderia*, *Comamonas*, *Cycloclasticus*, *Gordona*, *Flavobacterium*, *Halomonas*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Rhodotorula*, *Sinorhizobium*, *Sphingomonas*, *Stenotrophomonas*, *Streptomyces*, and *Vibrio*) are capable of utilizing phenanthrene as a sole carbon and energy source (Kanaly and Harayama 2000; Juhasz and Naidu, 2000; Keum et al., 2005; Baboshin et al., 2005; Zhang et al., 2006). The data available in the literature show the diversity of metabolic pathways of phenanthrene degradation by bacteria (Seo et al., 2009).

The results of the present research on microbial degradation of phenanthrene by four rhizobacteria belonging to the *Ensifer*, *Pseudomonas*, *Rhizobium*, and *Stenotrophomonas* genera agree well with known schemes for phenanthrene metabolism reported previously (Seo et al., 2009). With the example of these bacteria, the main degradation pathways for phenanthrene were demonstrated: through the dioxygenation of the terminal rings of phenanthrene and the formation of salicylic acid (*E. meliloti*) or *o*-phthalic acid (other rhizobacteria), and through the dioxygenation of the middle ring of phenanthrene and the formation of 2,2′-diphenic

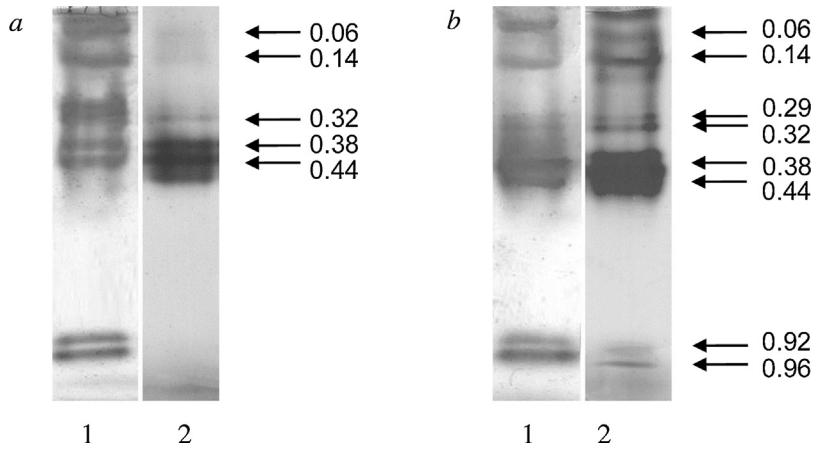

Fig. 3. PAGE profile of peroxidase from the root extracts (1) and root exudates (2) of alfalfa grown in untreated (a) and phenanthrene-treated (b) sand. Nondenaturing (without SDS or β-mercaptoethanol) polyacrylamide gel was used, and activities were detected with 4 mM *o*-dianisidine and 0.1 mM H_2O_2.

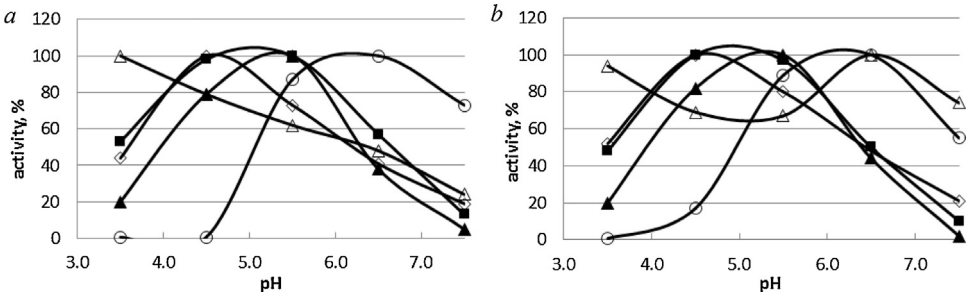

Fig. 4. Dependence of the peroxidase activity of the alfalfa root exudates collected from untreated (a) and phenanthrene-treated (b) sand on the pH of the reaction suspension: ◊—ABTS; ■—DAZ; ▲—DAF; ○—SGZ; Δ —DMOP. A 2.0 mL portion of the reaction suspension contained 1.4 mL of 50 mM sodium citrate buffer at pH (3.5–7.5), 200 μL of the concentrated root exudates, 200 μL of the substrates, and 200 μL of 0.1 mM H_2O_2.

acid (*E. meliloti*). The main phenanthrene metabolites were identified, with 1-hydroxy-2-naphthoic acid being the predominant metabolite in all strains. For *R. petrolearium*, a new recently described phenanthrene-degrading species isolated from oil-contaminated soil (Zhang et al., 2012), 1-hydroxy-2-naphthoic acid has been detected for the first time in this work as a product of phenanthrene catabolism.

In addition to microbial degradation of PAHs in the rhizosphere, the contribution of plant-root extracellular enzymes (mostly oxidoreductases) to the rhizodegradation of organic pollutants seems

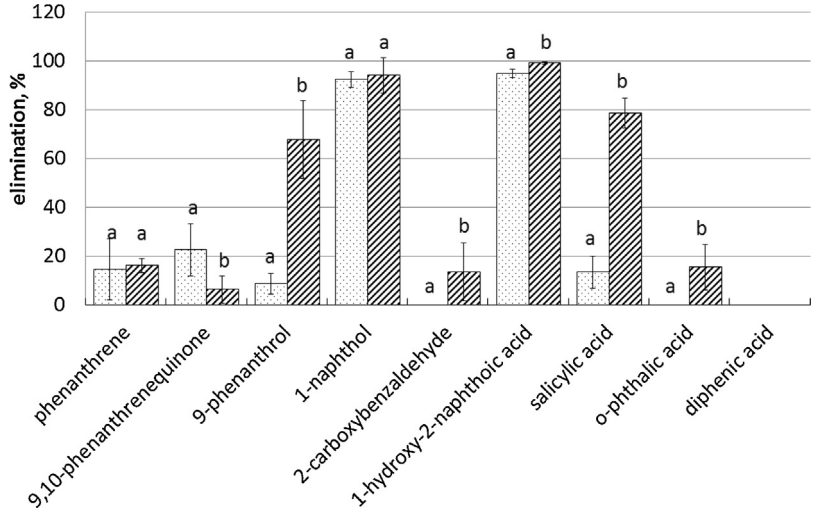

Fig. 5. Peroxidation of phenanthrene and its microbial metabolites by a crude enzyme preparation of alfalfa root exudates collected from untreated ( ) and phenanthrene-treated ( ) sand. Experiments were performed in vitro as described in Materials and Methods. A 2.0 mL portion of the reaction suspension contained 1.5 mL of 50 mM Na/K-phosphate buffer (pH 6.0), 50 μL of the acetonitrile solution of substrate, 50 μL of 20 mM ABTS, 200 μL of the concentrated root exudates, and 200 μL of 5 mM H_2O_2. The values represent the averages and bars represent confidence interval ($n = 3$; $P \leq 0.05$); the different letters indicate statistically significant differences between root exudates from untreated and phenanthrene-treated sand.

considerable. Previously, it has been demonstrated that peroxidases prevail over the other oxidoreductases in alfalfa root exudates and that the plant roots are the main source of this enzyme in the rhizosphere (Gramss and Rudeschko, 1998; Muratova et al., 2009). Among other tested plant species, alfalfa was notable for its highest root peroxidase activity, especially in contaminated soil (Gramss and Rudeschko, 1998; Dubrovskaya et al., 2010). This study has also shown that peroxidase is the principal oxidizing system in the root exudates of alfalfa. On the one hand, peroxidase is known as a stress enzyme, the biosynthesis and activity of which is increased in plants in response to different stresses. On the other hand, peroxidase has a wide substrate specificity and may be involved in the oxidation of different organic substances. Peroxidase catalyzes a reaction in which substrate oxidation uses the active species of oxygen contained in hydrogen peroxide:

$$RH_2 + H_2O_2 \rightarrow 2H_2O + R$$

The broad substrate specificity of peroxidases can be considered an important tool in the detoxification/degradation of environmental pollutants (Xu, 2005).

It has also been shown that plant peroxidase activity is stimulated by PAHs (Kraus et al., 1999; Criquet et al., 2000). In this study, this was observed also for four of the five substrates (DAZ, SGZ, DAF and DMOP, but not for ABTS). Active involvement of plant peroxidases in the rhizosphere degradation of PAH has been assumed by several researchers (Gunther et al., 1998; Kraus et al., 1999; Criquet et al., 2000; Chroma et al., 2002 Kraus et al., 1999; Criquet et al., 2000; Chroma et al., 2002). For instance, Günther et al. (1998) and Kraus et al. (1999) have shown the possibility in principle of oxidation of PAHs and their derivatives by plant enzymes by using commercial horseradish and soybean peroxidases. Yet, data on the oxidation of PAHs by root exudate enzymes are still limited. There are no data on the ability of alfalfa peroxidases to oxidize PAHs and their derivatives. From this point of view, the results obtained in this study make up a deficiency of such information.

The high peroxidase activity of the alfalfa root exudates found in this study allows us to explain the specific effectiveness of alfalfa for the phytoremediation of PAH-contaminated soil. This explanation is confirmed by the activity of the alfalfa root exudates peroxidase towards phenanthrene and its oxidized derivatives. A previous study of the enzymatic activity of alfalfa peroxidase toward phenanthrene showed that the PAH was not oxidized without a mediator (Muratova et al., 2009). It is known that the substrate specificity of some enzymes can be broadened with the use of redox mediators (Johannes and Majcherczyk, 2000; Günther et al., 1998). Indeed, the use of a mediating agent (ABTS) in the enzymatic reaction allowed us to observe elimination of phenanthrene from the reaction mixture. The oxidation of the PAH in the presence of a synthetic mediator in vitro does not exclude the existence of such reactions in natural environments, in which various aromatic substances can mediate similar enzymatic reactions (Johannes and Majcherczyk, 2000).

The stimulation of alfalfa peroxidase in response to PAH contamination of the substrate can be observed from the results obtained. Electrophoretic analysis of root exudates from phenanthrene-treated sand revealed the presence of a greater number of isoenzymes than that found in untreated sand. The peroxidation of 2-carboxybenzaldehyde and *o*-phthalic acid was observed only when alfalfa root exudates from phenanthrene-contaminated sand were used, and the oxidation of 9-phenanthrol and salicylic acids was increased significantly by the exudate peroxidase from contaminated sand. As a whole, the enzymatic oxidation of the alcohols and acids as phenanthrene derivatives was more active than that of phenanthrene and its corresponding quinine.

5. Conclusions

The results of this study indicate that plants are actively involved in the rhizospheric degradation of PAHs and their microbial metabolites. It may be assumed that rhizosphere microorganisms begin their catabolic attack on the native PAH (e.g., phenanthrene), and the enzymes released from the plant roots are involved in the oxidation of microbial metabolites. Thus, it is possible to speak about the coupling of the plant and microbial catabolisms of PAHs in the rhizosphere.

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