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EXPERIMENTAL ARTICLES

Biochemical and Physiological Peculiarities of the Interactions between *Sinorhizobium meliloti* and *Sorghum bicolor* in the Presence of Phenanthrene

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Abstract—The effect of phenanthrene, a polycyclic aromatic hydrocarbon (PAH) at concentrations of 0, 10, and 100 mg/kg and the bacterium *Sinorhizobium meliloti* P221 on root exudation of *Sorghum bicolor* L. Moench was studied in laboratory vegetative experiments. Inoculation of the bacterium promoted plant resistance to the pollutant stress and increased their acclimation rate and biomass formation. The ability of this microorganism to produce a phytohormone, indolyl-3-acetic acid, and to degrade phenanthrene, resulted in morphological changes of the plant root system and in the changed intensity of root exudation. In root exudates of sorghum, enzyme activities towards the metabolites formed during microbial degradation of PAH were revealed, which is indicative of a direct involvement of plants in PAH degradation in the rhizosphere as well as of the coupled plant–microbial metabolism in the course of xenobiotic degradation in the root zone. In phenanthrene-contaminated soil, sorghum was found to support selectively the development of the *S. meliloti* P221 population.

Key words: Sinorhizobium meliloti, Sorghum bicolor, plant–microbial interactions, rhizosphere, root exudates.

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Plant–microbial associations and symbioses have great advantages for survival under unfavorable environmental conditions due to their high native adaptive potential and the evolutionarily formed mutually beneficial coexistence of the partners.

Bacteria of the genus *Sinorhizobium* together with representatives of the genera *Rhizobium, Bradirhizobium*, and *Azorhizobium* were evolutionarily associated with the plant root systems through symbiotic or associative interactions. The physiological, biochemical, and genetic characteristics of rhizobia, including *Sinorhizobium meliloti,* which are responsible for their symbiotic properties and capability of root nodule formation in leguminous plants are well studied [1], whereas nonspecific associative interactions of rhizobia with roots of nonleguminous plants are less well understood [2]. Rhizobia were shown to produce the regulators of plant growth; therefore, they can be considered plant-growth-promoting rhizobacteria (PGPR) [3].

Under contamination, rhizobia gain an advantage over nonsymbiotic microorganisms due to their capability to enrich soil with mineral nitrogen, stimulate plant growth, and form efficient plant-microbial associations with both leguminous and nonleguminous plants (e.g., cereals) [4, 5]. However, experimental data on the survival of rhizobia in the presence of organic contaminants, their capability of the pollutant degradation, and the plant–bacterial interactions under these conditions are scarce.

A prerequisite for this study was the isolation of strain *Sinorhizobium meliloti* P221 from the root zone of *Phragmites australis* L., which was able to degrade phenanthrene [6] and to produce large amounts of a phytohormone, indolyl-3-acetic acid (IAA), in the presence of tryptophan [7].

The aim of this work was to study the interactions between the symbiotic microorganism *Sinorhizobium meliloti* P221 and *Sorghum bicolor* L. Moench, a plant which is efficiently applied for the removal of hydrocarbons including PAHs from the environment [8, 9], in the presence of a three-ring PAH, phenanthrene. The studied parameters included growth characteristics of the plants, such as plant establishment and biomass formation, the content and composition of the root exudates, concentrations of the IAA phytohormone and of its precursor, tryptophan, as well as the cell number of this microorganism in the sorghum rhizosphere. Moreover, we studied the enzyme activities of the exudates of the sorghum root towards a number of PAHs and their hydroxylated derivatives.

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MATERIALS AND METHODS

In vegetative experiments, the sorghum plants were grown in 1-l vessels with quartz sand, which was preliminary washed with concentrated HCl and dried at 100°C. In experimental variants, phenanthrene was added to the sand up to the final concentrations of 10 or 100 mg/kg. In the control variant, no pollutant was added. Each vessel was sown with ten sorghum seeds obtained from the Southeast Agricultural Research Institute (Saratov). The seeds were preliminary calibrated and surface-sterilized. Seeds were grown in a Percival Scientific AR-75 (United States) climatic chamber for 1 month under the following conditions: light and dark periods of 14 and 10 h, respectively; illuminance of 450 μmol quanta/(m^2 s); temperature of 24/20°C; and 70% relative air humidity. The watering of the plants was performed, as required (1–2 times a day) up to 80% of the total moisture capacity. The volume of the Ruakura solution [10] used for plant watering was determined by weighing of the vessels with plants.

Earlier, we isolated and characterized strain *S. meliloti* P221 [6, 7], which was used in this study. The strain was deposited in the All-Russian Collection of Industrial Microorganisms (Moscow) (no. B-9442).

Inoculation of 4-day-old sorghum seedlings with *S. meliloti* P221 was carried out by watering them with the cell suspension in Ruakura solution up to the final concentration in the soil of 10^6 CFU/g. To prepare microbial suspensions, a 3-day culture grown on LB agar was washed with phosphate buffer (pH 7.2) and resuspended in the medium used for plant watering. In the control variant, the seedlings were treated with the same volume of Ruakura solution without microbial cells.

At the end of cultivation, the plant root system together with the soil was removed from the vessel and washed with 1 l of deionized water for 2 min. The solution was filtered through a glass filter, frozen in liquid nitrogen, lyophilized, and stored at –20°C until required for analysis. The root exudates were obtained according to the method described in [11]; the plant's root system was immersed into 0.05 mM solution of $CaCl_2$ supplemented with the Micropur® antimicrobial preparation and allowed to stand in a climatic chamber for 4 h. The roots were then removed; the solution containing the root exudates was filtered, frozen in liquid nitrogen, lyophilized, and stored at –20°C until required for analysis.

To determine the plant biomass, the shoots were separated from the roots and dried at 70°C for 7–9 hours; the weight of both the above-ground and underground plant mass was measured. The area of the root surface was determined with methylene blue [12].

The exudates of the plant root were divided into acid, neutral, and alkaline fractions by ion-exchange chromatography [13]. The lyophilized samples (10 mg) were dissolved in 2 ml of 50% ethanol; the acid fraction containing carboxylic acids was separated on an Aminex HPX-87H column (300 × 7.8 mm) with a BioRad, Hercules cartridge (United States) [14]. The neutral fraction containing free carbohydrates was separated on a Rezex RCM-Monosaccharide column (300 × 7.8 mm) with a Phenomenex, United States, CA cartridge (United States) according to [14]. The amount of free amino acids in the alkaline fraction was analyzed by high-performance liquid chromatography (HPLC) [15].

The IAA concentration in the solution was measured by HPLC [16]. To determine tryptophan content, free amino acids were extracted with 50% ethanol from the solution containing 10 mg of lyophilized preparation, dried, dissolved in 0.5 ml of Na-citrate buffer (pH 2.2), and analyzed on a T339 amino acid analyzer (Microtechna, Czech Republic).

To determine the protein content and enzyme activities of the exudates of the sorghum root, lyophilized preparations (10 mg) were dissolved in 1 ml of distilled water and dialyzed against 2 l of distilled water. Protein content was determined by the Bradford method [17].

Enzyme activities of the root exudates were determined spectrophotometrically. Laccase activity was determined as formation of the products of oxidation of syringaldazine [18] and ammonium 2,2-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonate (ABTS) [19]; the rate of substrate oxidation was measured on a Specord M40 spectrophotometer (Carl Zeiss, Germany) at 525 and 436 nm, respectively. The assay mixture contained 50 mM Na-tartrate buffer (pH 3.5), 50 mM Na-acetate buffer (pH 5.0), 50 mM Na–K-phosphate buffer (pH 6.0), and 50 mM Tris–HCl buffer (pH 7.5). Peroxidase activity was determined by using the same substrates and buffers in the presence of 0.1 mM hydrogen peroxide. Peroxidase activity was also measured with 2,7-diaminofluorene [20] in 50 mM Na–K-phosphate buffer (pH 6.0) at 600 nm. Tyrosinase activity was determined from the oxidation rate of 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) [20] in 50 mM Na–K-phosphate buffer (pH 7.5) at 475 nm. The volume of the assay mixture in all the variants was 2 ml. One unit of enzyme activity (U) was defined as the amount of product (μmol) formed in 1 min per 1 mg of protein (μmol/min/mg protein).

The enzyme activities of the exudates of the sorghum root towards PAHs and their derivatives were studied according to the method [21]. A sample of the lyophilized preparation of the root's exudates (20 mg) was dissolved in 1.5 ml of distilled water, dialyzed against 2 l of distilled water, and filter-sterilized (0.2 μm). The substrates were dissolved in dimethylformamide (0.2 g/l) and added into the assay mixture up to the final concentration of 10 μg/ml. The substrates used included PAHs (phenanthrene, anthracene, fluorene, fluoranthene, pyrene, and chrysene) and their derivatives (1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, 1,2-dihydroxynaphthalene, and 1- and 2-naphthols). The enzymatic reactions were performed with and without mediators. The mediators were

Table 1. Effect of phenanthrene and plant inoculation with strain *Sinorhizobium meliloti* P221 on the sorghum development (calculations were made per one plant)

Parameters	Phenanthrene, mg/kg					
	0		10		100	
	–P221	+P221	–P221	+P221	–P221	+P221
Plant acclimation, %	93.3aA	100.0aA	100.0aA	96.6aA	56.7cC	70.0bB
Shoot mass, g	0.311aA	0.245bA	0.286abA	0.287bA	0.119cC	0.171cD
Root mass, g	0.147aA	0.131bB	0.127bB	0.115bC	0.052cD	0.058cF
The area of the root surface, dm^2	1.04aA	1.07aA	1.04aA	1.01aA	0.49bB	0.91aA
Root exudates, mg/h	1.32bB	1.73aA	1.20bB	1.25bB	0.47dD	0.73cC
Intensity of exudation, calculated per the root surface, mg/(dm^2 h)	1.28bB	1.60aA	1.16bBC	1.23bBC	1.03bcC	0.80cD

Note: The values in a line marked with the same lowercase letters were not reliably different ($P = 0.05$) when the pollutant effect was estimated; the values in a line marked with the same capital letters were not reliably different ($P = 0.05$) when the effect of cell inoculation was estimated.

0.3 mM ABTS (for oxidase and peroxidase reactions) and 0.3 mM DL-DOPA (for tyrosinase reaction). In all experiments, the assay mixtures of the total volume of 2 ml included 50 µl of the enzyme-containing sample; 100 µl of the substrate solution; and 100 µl of mediator solution in the buffer; in the case of analyzing the peroxidase activity, 100 µl of H_2O_2 was also added into the assay mixture. The experiments were performed in triplicates. Boiled samples of the filter-sterilized root exudates were used in the control variants. The enzymatic reactions were carried out for 18–24 hours at 27°C. The reaction was terminated by addition of 1 ml of carbon tetrachloride into the assay mixture; the substrates were twice extracted with carbon tetrachloride for 5 min. The extracts were combined and evaporated; dry residue was dissolved in acetonitrile and analyzed by HPLC on a HPC 500 chromatograph (Czech Republic) equipped with a Supelcosil LC-PAH column (United States) and a UV-detector (254 nm). HPLC was operated at the flow rate of the eluent (acetonitrile : water, 70 : 30) 0.2–0.3 ml/min; the cell temperature 24–26°C; inlet column pressure 1.4 MPa; and sample volume 20 µl. Pure substrates were used as standards.

The total number of rhizospheric microorganisms was determined by plating serial dilutions on nutrient agar [22]. The number of the *S. meliloti* P221 cells in the soil and in the zone of the plant root was determined by plating serial dilutions on the medium for PAH-destructors with subsequent treatment of the grown culture with phenanthrene [6]. The colonies of PAH-destructors were tested for their identity with strain *S. meliloti* P221 by immunodiffusion analysis with the use of previously obtained strain-specific rabbit antibodies [23].

The results were statistically processed using the SAS (version 9.1.3) software package (United States). The mean values were compared by using the least significant difference index at $P = 0.05$.

RESULTS AND DISCUSSION

As seen from Table 1, phenanthrene at the concentration of 10 mg/kg had no marked effect on the plant's growth and development. At a tenfold increased concentration of phenanthrene (100 mg/kg), its toxic effect was pronounced: seedling acclimation decreased almost twofold, growth of the above-ground and root biomass was inhibited by 2.6 and 2.8 times, respectively; moreover, the area of root surface was twofold less than that in the control.

The effect of *S. meliloti* P221 on the plant growth depended on the presence of the pollutant. In the variant without the pollutant, after bacterial inoculation the mass of shoots and roots decreased by 21 and 11%, respectively; however, the area of the root system remained almost unchanged, indicating the formation of more thin roots. They possibly excreted the exudates more actively, since the root exudation in this case increased by 25%. In contaminated soil, strain *S. meliloti* P221 promoted plant resistance against the pollutant stress: the sorghum acclimation increased by 13%; mass of shoots and roots also increased compared to that in the uninoculated variant. Bacterial inoculation had the most pronounced effect on the area of the root surface, which increased by 86%. In this case, the weight of dry roots was only slightly higher than in the control. These data may indicate the formation of thinner (branched) roots under the contamination conditions; as a result, the amount of root exudates in the rhizosphere (calculated per one plant) increased by 55%. However, the calculation of intensity of root exudation per unit of area of the root surface showed slight inhibition of this process in the presence of bacteria. The presence of microorganisms is known to affect exudation of the plant root [24] by changing the permeability of the plant cell membranes due to the action of microbial metabolites and enzymes. We suggest that the changes in the exudation of the plant root observed

Table 2. Effect of phenanthrene and plant inoculation with strain *Sinorhizobium meliloti* P221 on the area of the root surface and concentrations of the phytohormone IAA and tryptophan in sorghum rhizosphere (calculations were made per volume of soil in a vegetative vessel)

Parameters	Phenanthrene, mg/kg					
	0		10		100	
	–P221	+P221	–P221	+P221	–P221	+P221
Area of the root surface, dm^2/l	9.70aA	10.72aA	10.35aA	9.77aA	2.75cC	6.29bB
IAA concentration, pmol/l	44.64abB	51.70aA	40.08bcBC	36.87cC	25.66dD	35.89cC
Tryptophan concentration, μmol/l	3.90aA	3.03bcB	3.42abB	3.48abAB	2.66cC	1.81dD

Note: The values in a line marked with the same lowercase letters were not reliably different ($P = 0.05$) when the pollutant effect was estimated; the values in a line marked with the same capital letters were not reliably different ($P = 0.05$) when the effect of cell inoculation was estimated.

Table 3. Effect of phenanthrene and plant inoculation with strain *Sinorhizobium meliloti* P221 on the exudation of carboxylic acids, free carbohydrates, and amino acids by sorghum roots (μg/(dm^2 h))

Components	Phenanthrene, mg/kg					
	0		10		100	
	–P221	+P221	–P221	+P221	–P221	+P221
Carboxylic acids	227.5abA	327.7aA	203.8abA	243.0abA	187.4bA	48.7cB
Free carbohydrates	42.4bcB	90.3aA	67.1abAB	68.3abAB	25.2cBC	17.1cC
Free amino acids	9.5bB	16.8aA	9.1bBC	10.0bB	7.6bBC	4.7bcC
Proteins	20.7aA	13.5bB	11.7bB	5.4cC	5.3cC	5.4cC

Note: The values in a line marked with the same lowercase letters were not reliably different ($P = 0.05$) when the pollutant effect was estimated; the values in a line marked with the same capital letters were not reliably different ($P = 0.05$) when the effect of cell inoculation was estimated.

in our experiment were associated with the metabolic activity of the bacterial strain, which in turn depended on the presence of phenanthrene.

An increase in the area of the root surface correlated with an increase in IAA concentration in the sorghum rhizosphere (Table 2). This phytohormone is known to affect both elongation and *de novo* formation of roots and root hairs [25]. The phytohormone concentration in the soil under the plants decreased in the presence of the pollutant. However, after bacterial inoculation, IAA concentration increased both in uncontaminated soil and in the presence of phenanthrene (100 mg/kg). This increase correlated with a decrease in concentration of the IAA precursor, tryptophan, probably, because of utilization of this amino acid in microbial metabolism, including IAA synthesis [25]. These results suggest that the changes in root morphology observed in plants inoculated with bacteria were due to the phytohormonal activity of *S. meliloti* P221 in the sorghum rhizosphere both in uncontaminated and contaminated soil.

Taking into account the important role of root exudates in microbial degradation of the pollutant, we studied the effect of plant inoculation with bacteria on the composition of the exudates of the sorghum root (Table 3). It was revealed that in uncontaminated soil, strain *S. meliloti* P221 promoted root exudation of low-molecular substances, such as carboxylic acids, carbohydrates, and amino acids (by 44, 112, and 77%, respectively) and inhibited excretion of protein compounds (by 75%). In the presence of phenanthrene (100 mg/kg), bacterial inoculation had an opposite effect: it inhibited excretion of carboxylic acids, carbohydrates, and amino acids (by 36, 24, and 40%, respectively); no reliable changes in protein excretion were observed. In this case, proportions of the main compounds in the exudates changed considerably: the level of carboxylic acids decreased and, accordingly, the ratios of amino acids, carbohydrates, and proteins increased, possibly as a result of adaptive changes in plant metabolism in response to the presence of both the pollutant and bacteria.

Under stress conditions caused particularly by the presence of PAHs, the production of peroxide compounds was observed in plants [26]; this process can be accompanied by increased activities of peroxidases, which are possibly involved in PAH degradation [20, 21, 27]. We found three oxidoreductases in the exudates of sorghum root: oxidase, peroxidase, and tyrosinase, among which the peroxidase activity predominated (Table 4). Although laccase is widespread in the root's

Table 4. Effect of phenanthrene and plant inoculation with strain *Sinorhizobium meliloti* P221 on the enzyme activities of the exudates of the sorghum root

Enzymatic activities	Phenanthrene, mg/kg					
	0		10		100	
	–P221	+P221	–P221	+P221	–P221	+P221
Oxidase (ABTS), U/mg	0.058cB	0.118bcB	0.264bB	0.759aA	0.184bcB	0.141bcB
Peroxidase (ABTS), U/mg	0.085cD	0.390bcC	0.485bB	0.277bcCD	0.706aB	0.995aA
Tyrosinase (DL-DOPA), U/mg	0.075eE	0.135dD	0.174cC	0.300aA	0.248bB	0.328aA

Note: The values in a line marked with the same lowercase letters were not reliably different ($P = 0.05$) when the pollutant effect was estimated; the values in a line marked with the same capital letters were not reliably different ($P = 0.05$) when the effect of cell inoculation was estimated.

Table 5. Oxidation of PAHs and their derivatives by the preparations of *Sorghum bicolor* L. Moench root exudates in the presence of mediators

Substrate	Substrate diminution, %		
	+ABTS	+ ABTS + H_2O_2	+ DL-DOPA
Phenanthrene	0	0	0
Anthracene	0	0	0
Fluorene	0	0	13.75 ± 3.58
Fluoranthene	0	0	0
Pyrene	10.92 ± 4.70	0	0
1-Hydroxy-2-naphthoic acid	15.32 ± 11.34	71.02 ± 11.48	0
2-Carboxybenzaldehyde	0	0	9.46 ± 2.65
1,2-Dihydroxynaphthalene	0	32.10 ± 12.10	35.59 ± 6.64
1-Naphthol	26.73 ± 4.90	38.60 ± 3.98	0
2-Naphthol	0	82.38 ± 3.13	33.72 ± 5.43

zone of plants, we did not observe the oxidation of syringaldazine, the test-substrate for laccase; therefore, we cannot explain the oxidase activity in root exudates by the presence of laccase. It was shown that activities of the revealed enzymes increased in the presence of phenanthrene and were additionally stimulated by inoculation with *S. meliloti* P221. Based on these results, we can imply that an increase in the enzyme activities of root exudates of inoculated plants may result from microbial production of the substrates for these enzymes in the plant rhizosphere. It was earlier found [28] that strain *S. meliloti* P221 is able to oxidize phenanthrene with the formation of 1-hydroxy-2-naphthoic acid, which in turn can be oxidized by plant enzymes, e.g., peroxidase as it was shown with the use of a commercial enzyme preparation [21].

To verify this assumption, we examined the enzymatic activities of the exudates of lyophilized sorghum root towards a number of PAHs and their hydroxylated derivatives, which are often formed during microbial degradation of these hydrocarbons. It was revealed that the exudates of sorghum root did not oxidize native PAHs as the sole substrates. In the presence of a synthetic mediator, only oxidation of fluorene and pyrene was observed, as well as of the PAH derivatives, 1,2-hydroxynaphthoic acid, 2-carboxybenzaldehyde, 1,2-dihydroxynaphthalene, and naphthols. These results may indicate a direct involvement of plants in the degradation of pollutants as well as a coupled plant–microbial metabolism of PAHs in the sorghum rhizosphere (Table 5).

The monitoring of the number of the *S. meliloti* P221 cells in the sorghum root zone was performed by immunodiffusion analysis. In spite of noncompetitive conditions (bacteria were inoculated into sterile soil), we could estimate the ratio of *S. meliloti* P221 cells in the total number of heterotrophic microorganisms both in contaminated and uncontaminated soils with and without plants (Table 6). After four weeks of cultivation, the total number of heterotrophic microorganisms in the rhizosphere decreased in the presence of the pollutant, while the *S. meliloti* P221 population increased and reached the maximum (over 50% of the total number of heterotrophs) in the variant with a low level of contamination, where the root exudates were still present but the pollutant's impact was already

Table 6. Cell number of heterotrophic microorganisms and strain *S. meliloti* P221 after 4 weeks of cultivation in the unplanted soil and in the bulk soil from the vessels planted with sorghum

Phenanthrene, mg/kg	CFU/g soil		Ratio of strain *S. meliloti* P221, %
	Heterotrophs	*S. meliloti* P221	
Without plants			
0	2.5×10^6bcB	2.8×10^5bB	11.2
10	3.6×10^6aA	2.2×10^5bcBC	6.1
100	2.3×10^6cBC	2.1×10^5bcBC	9.2
With plants			
0	2.8×10^6bB	3.0×10^5bcBC	10.7
10	1.9×10^6dC	9.6×10^5aA	50.8
100	1.0×10^6eD	3.3×10^5cC	32.3

Note: The values in a column marked with the same lowercase letters were not reliably different ($P = 0.05$) when the effect of plants was estimated; the values in a column marked with the same capital letters were not reliably different ($P = 0.05$) when the effect of pollutant was estimated.

observed. Comparing these data with the number of microbial cells in the variants, where the growth of microbial populations was stimulated by either root exudates or the organic pollutant (uncontaminated soil with sorghum or contaminated soil without plants), we suggest that sorghum selectively supported growth of the population of this strain under the impact of pollution even in the presence of novel degrader strains. No *S. meliloti* 221 cells were revealed in the sorghum rhizosphere without preliminary inoculation.

Thus, this study gained some insight into the physiological and biochemical interactions between the microorganisms and plants in the presence of PAHs. The inoculation of sorghum with *S. meliloti* P221 promoted plant resistance to the pollutant stress and increased their acclimation in contaminated soil. Due to its ability to produce the phytohormone IAA, this microorganism changed the sorghum root system by promoting formation of thinner (branched) roots that resulted in increased root exudation. Strain *S. meliloti* P221 either stimulated root exudation (in uncontaminated soil) or inhibited this process (in the presence of a high concentration of phenanthrene), possibly depending on the metabolic activity of microbial cells. The revealed ability of the exudates of sorghum root to oxidize the products of microbial metabolism of PAHs indicated a possible direct involvement of plants in phenanthrene degradation in the rhizosphere as well as a coupled plant–microbial metabolism of the xenobiotic in the root zone. In the laboratory vegetative experiments, the sorghum plants were shown to support selectively the growth of the inoculated strain. On the whole, these results demonstrate that the rhizosphere conditions are favorable for the plant–microbial degradation of pollutants.

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