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ORIGINAL PAPER

Phytoremediation of Polycyclic Aromatic Hydrocarbons in Soils Artificially Polluted Using Plant-Associated-Endophytic Bacteria and *Dactylis glomerata* as the Bioremediation Plant

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Abstract

The reaction of soil microorganisms to the contamination of soil artificially polluted with polycyclic aromatic hydrocarbons (PAHs) was evaluated in pot experiments. The plant used in the tests was cock's foot (*Dactylis glomerata*). Three different soils artificially contaminated with PAHs were applied in the studies. Three selected PAHs (anthracene, phenanthrene, and pyrene) were used at the doses of 100, 500, and 1000 mg/kg d.m. of soil and diesel fuel at the doses of 100, 500, and 1000 mg/kg d.m. of soil. For evaluation of the synergistic effect of nitrogen fixing bacteria, the following strains were selected: associative *Azospirillum* spp. and *Pseudomonas stutzerii*. Additionally, in the bioremediation process, the inoculation of plants with a mixture of the bacterial strains in the amount of 1 ml suspension per 500 g of soil was used. Chamber pot-tests were carried out in controlled conditions during four weeks of plant growth period. The basic physical, microbiological and biochemical properties in contaminated soils were determined. The obtained results showed a statistically important increase in the physical properties of soils polluted with PAHs and diesel fuel compared with the control and also an important decrease in the content of PAHs and heavy metals in soils inoculated with *Azospirillum* spp. and *P. stutzeri* after cock's foot grass growth. The bioremediation processes were especially intensive in calcareous rendzina soil artificially polluted with PAHs.

Key words: *Azospirillum* spp., *Dactylis glomerata, Pseudomonas stutzeri,* diesel fuel phytoremediation, polycyclic aromatic hydrocarbons (PAHs)

Introduction

Persistent organic pollutants, including petroleum derivatives, are emitted to the environment mainly from anthropogenic sources and are characterized by high toxicity and power for bioaccumulation. More than 90% of global PAH's pollution that is coming from the combustion of organic matter is accumulated in the surface layer of soil (Anderson *et al*., 1993; Andreoni and Gianfreda, 2007). Biological degradation of petroleum derivatives by microorganisms and decline of metals often show a synergistic effect and is one of the most effective and most secure ways to remove them from the environment but the process is lengthy and multistage (Cerniglia, 1992). As a result of the metabolic activity of microorganisms, hydrocarbons are partially or completely turned into bacterial mass and stable, non-toxic end products. The effectiveness of the microbiological decomposition of PAHs in soil, requires the use of strains not only capable of catabolic degradation of pollutants and their usage as the only source of carbon and energy, but also a number of other features allowing adaptation to contaminated conditions and cometabolic degradation of organic compounds (Siciliano and Germida, 1998; Chauhan *et al*., 2008; Lee, 2013).

Phytoremediation, or use of plants and associated rhizosphere to decontaminate polluted sites, is considered today, as a realistic, low-cost alternative for treatment of extensive areas of pollution by organic chemicals (Dominguez-Rosado and Pitchel, 2004; Ma *et al*., 2010). This technology is based on the catabolic potential of root-associated microorganisms, which are supported by the organic substrates in root excretions and by a favorable micro-environment in the rhizosphere. Soils polluted by polycyclic aromatic hydrocarbons (PAHs) are suitable for treatment by phytoremediation, since several scientific studies, performed

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with well-designed controls, have specifically shown higher rates of PAH biodegradation in whole soils planted with a variety of species. Biodegradation of PAHs in soils is often limited by the slow mass transfer of these hydrophobic compounds towards degrading microbes. This slow process may lead to bioavailability restrictions, even in the conditions of massive contamination often faced by bioremediation technologies. Little is known about bioavailability in phytoremediation systems. Specific bioavailability-promoting mechanisms, operating in soils with PAH-degrading populations, may be responsible for increased rates of pollutant transformation. These include an increased bacterial adherence to the pollutants, and production of biosurfactants by bacteria or by plants (Gunther *et al*., 1996; Huang *et al*., 2004).

There are many methods for removing PAH's contamination from natural environment. The elaboration of the most effective method of bioremediation is one of the most important problems related to the protection. Such methods make use of microorganisms inhabiting the natural environment or genetically changed microorganisms which utilize hydrocarbons as a only source of energy and carbon (Liste and Felgentreu, 2006; Liang *et al*., 2011).

The most important of the mentioned transformations are those that involve microbiological processes. The main organisms contributing to the degradation of hydrocarbons in the soil environment are bacteria and fungi. However, it is thought that the dominating role in this process is played by bacteria. Bacteria carrying out the degradation of hydrocarbons belong to the genera: *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Micrococcus*, *Mycobacterium*, *Pseudomonas* (Gogoi *et al*., 2003; Liste and Felgentreu, 2006; Gałązka, 2008; Mahmoud *et al*., 2011; Abhilash *et al*., 2011; Gałązka *et al*., 2012; Tejeda-Agredano *et al*., 2013). The effectiveness of the bioremediation of soils contaminated with PAHs was also confirmed in experiments on soils contaminated with a mixture of PAH and heavy metals (Ciesielczuk *et al*., 2014). It is also known that the use in inoculated plants of a mixture of bacterial strains and in particular bacteria of the genus *Azospirillum* significantly improves plant growth (Naiman *et al*., 2009; Couilleror *et al*., 2013).

Evidence for biological nitrogen fixation in grasses was reported in many publications (Hung *et al*., 2004; Joner *et al*., 2007). Studies on long-term N-balance and 15N isotope dilution technique have shown that some plants may actually obtain up to 70% of their N requirements by nitrogen fixation. In this process both rhizosphere and endophytic diazotrophs seem to participate. Nitrogen fixing bacteria such as *Azospirillum* spp. and *Pseudomonas stutzeri* colonize the plant and its tissues. *Azospirillum* species belong to the facultative endophytic diazotrophs group which colonize the surface and the interior of roots, this kind of association being considered as the starting point of most ongoing BNF programs with non-legume plants worldwide (Muratova *et al*., 2003; Król *et al*., 2007). These bacteria are microaerophilic, nitrogen-fixing, Gram-negative rods and often associated with the roots of cereals and grasses (Muratova *et al*., 2003). However, obligate endophytes such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. seem to be the promissory group in relation to nitrogen fixation associated with grasses. These bacteria have an advantage over root-associated diazotrophs, as *Beijerinckia* spp. and *Azotobacter* spp., they colonize the interior rather than the surface of plants, hence have better possibilities to exploit carbon substrates supplied by the plant (Steenhoudt *et al*., 2000). *Azospirillum* spp. and *P. stutzeri* are capable of creating permanent associations with the roots of most cereals and grasses, and use PAHs as the only carbon and energy source, as well as produce biosurfactants (Okon and Vanderleyden, 1997). Bacteria from the genus *Pseudomonas* are microorganisms that effectively decompose organic pollutants through cometabolism in natural water and soil environment. In the available literature, there is a lack of data on the participation of bacteria from the genus *Azospirillum* in the bioremediation processes. Free-living bacteria that fix nitrogen, namely *Azospirillum* spp. and *P. stutzeri*, may create permanent associations with the roots of most cereals and grasses used in plant production (Król *et al*., 2007; Gałązka *et al*., 2012).

Dactylis glomerata popularly called cock's foot grass is a very persistent plant in bioremediation studies. It does not require high temperatures for active growth, and is very winterhardy. It appreciates a high soil moisture content. *D. glomerata* can be grown successfully on a wide range of soils. This grass has an early spring growth, with a regrowth consisting mainly of leafy shoots. It is suitable for both cutting and grazing (Muratova *et al*., 2003).

The aim of the work was to estimate the effect of plant inoculation with the bacteria *Azospirillum* spp. and *P. stutzeri* on PAH degradation in soils artificially polluted with the use of cock's foot grass (*D. glomerata*) as a bioremediation plant.

Experimental

Materials and Methods

Soil samples and plant. Soil material, uptaken from the plough – humus horizon (0–20 cm) of arable land, distant from PAH emission sources, from various regions of Poland, was used for the studies

The selected soils are the most common in Lublin Province. The effect of soil (chernozem, calcareous rendzina, and lessives) pollution was studied, artificially polluted with polycyclic aromatic hydrocarbons (PAHs) and diesel fuel (DF) in the phytoremediation process. Agricultural areas from which the soil material for the studies was uptaken were distant from the sources of PAH emissions, and the content of Σ15 PAHs in the soils corresponded to the average content of those compounds in agriculturally used soils:

- charnozem generated from loess silty loam; Kułakowice near Hrubieszów
- calcareous rendzina light loamy sand; Mięćmierz near Kazimierz Dolny
- lessives generated from loess dusty loam, Las Stocki near Wąwolnica.

Plant used in the tests was cock's foot grass (*D. glomerata*), potting used about 50 seeds per pot.

Microorganisms – bacterial inoculants. Three bacterial strains were used in the study: (1) strain Aa1 isolated from endorhizosphere of barley (*Hordeum sativum*), identified by RFLP-PCR and 16S-23S rDNA methods as *Azospirillum amasoense*, (2) strain Ab2 isolated from endorhizosphere maize (*Zea mays* L.), identified by RFLP-PCR and 16S-23S rDNA methods as *Azospirillum brasilense*, (3) strain Ps, identified by RFLP-PCR and 16S-23S rDNA methods as *P. stutzeri*. This bacteria was isolated from the endorhizosphere grass *Leymus arenarius* (Król *et al*., 2007).

The physiological properties of *Azospirillum* spp. were determined with the use of BIOLOG test (Król *et al.*, 2007; Gałązka, 2008). Strains Aa1 and Ab2 have genes of the catabolic pathway of PAH degradation: catechol 2,3-dioxygenase (C2,3-DO), and naphtalene dioxygenase (NDO) (Gałązka, 2008). In the bioremediation process, plant inoculation with bacteria mixtures *Azospirillum* spp. and *P. stutzeri* was additionally applied on *D. glomerata* seeds inoculation in amount of 1 ml per 500 g of soil in proportion 1:1:1 for strains (Aa1:Ab2:Ps). An inoculum with approximate density of 0.5×10^7 cfu/ml was used for the experiments.

Bacteria strains (Aa1, Ab2) *Azospirillum* spp. and *P. stutzeri* (Ps), originated from the collection of the Department of Agricultural Microbiology, Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy, Poland. Measuring the density of pure strains and tested by plating serial dilutions on nutrient agar plates. The plates were incubated in a thermostat at 28°C on PDA medium (*Azospirillum*) and Nfb medium for *P. stutzeri*. In order to verify the purity of the strains a single colony was viewed under a microscope.

Pot experiment. Pot-tests were carried out in controlled conditions in a climatic chamber during a four-week-long plant growth period with 16-hour lighting (light intensity 240 E/ms). Tests were carried out at the temperature of 24°C during the day and for 8 hours at night at the temperature of 18°C. In the pots, 500 g of air-dry, sieved soil were placed. Hydrocarbons were added as a solution in dichloromethane, reaching concentrations of 100, 500, and 1000 mg/kg d.m. of soil. Dichloromethane was also added to control soil for every pollution level, at the concentration equal to the polluted samples. Samples were left for 48 hours for the solvent to evaporate. Subsequently, the soil was thoroughly stirred and moistened to 60% of full water capacity. After soil moistening, in the pots pre-sprouted cock's foot grass seeds were sown (50 plants per pot). After the completion of the four-week plant growth cycle in the particular experiment combinations, basic physical properties of the soils were determined, as well as anthracene, phenanthrene, and pyrene content in artificially polluted soils and Σ15 PAHs in the case of soil pollution with diesel fuel.

Determination of physical and chemical properties of the soils. Basic physical properties of the soils were marked: soil texture (Casagrande'a method), pH (PN-ISO 10390:1997), total organic carbon (C_{org}-using the Tiurin's method) and total Kjeldahl nitrogen content (N_{total}-using flow spectrometry, wet sample mineralization).

Determination of biochemical and microbiological properties of soils. Soil microbial properties were evaluated on the basis of six parameters on different functional levels: three on the population level [total bacteria number (Wallace and Lockhead, 1950), total fungi number (Martin, 1950) and total number of microorganisms capable of degrading PAH (Jones and Edington, 1968)] and three on the activity level [dehydrogenase (Caside *et al*., 1964) and acid and alkaline phosphatase activities (Tabatabai and Bremner, 1969)]. Microorganisms were enumerated in triplicate using the plate-count techniques. Aqueous suspensions of the microbial population in 10 g of soil sample were serially diluted. Plates were inoculated at 28°C for 3 or 5 days prior to counting colony forming units (cfu). After the vegetation period the total number of microorganisms in the soil samples capable of degrading PAH as the sole source of carbon and energy was determined.

Determination of polycyclic aromatic hydrocarbons. For the experiment with artificial soil pollution the following compounds were chosen: anthracene, phenanthrene, and pyrene, which were applied in 100, 500, and 1000 mg/kg d.m. of soil doses and diesel fuel (Multi Motor Oil Jasol 12 SG/CE 5W/4 originating from Jasło Refinery, JSC, Poland) at the concentration of 100, 500, and 1000 mg/kg d.m. of soil. PAHs used in the soil samples were determined by HPLC: 20 µl of the extract was injected onto a reverse phase HPLC column (Li Chro CART® 250–4) using water and acetonitrile gradient with a flow rate of 1 ml/min.

PAHs in the samples were detected using UV (254 nm) absorbance detection and degradation was quantified against a negative control. The Σ15 PAHs were analysed, accepted for determination in environmental samples by the United States Environmental Protection Agency, excluding the most volatile hydrocarbons and those that rarely occur in soils.

Analysis of diesel fuel chemical composition was carried our according to the Decree by the Ministry of the Environment from September 9, 2002 concerning soil quality standards and ground quality standards. Determinations were made in chernozem, calcareous rendzina, and lessives with the highest doses of PAHs and diesel fuel. A dose of 1000 mg/kg PAHs, 1000 mg/kg of diesel fuel is equivalent to the border content of PAHs for soil used in agriculture and recreational areas. The scope of the applied PAH levels was equivalent to the content of these compounds that occur in soils in non-polluted areas, as well as from industrial areas (Kabata-Pendias *et al.*,1995).

Determination of heavy metals in soil samples. Microwave digestion of soil with the use of aqua regia in middle pressure (32 bars) digestion vessels coupled with ICP-MS (inductively coupled plasma mass spectrometry) technique was used for quantitative analysis of metals. Mars Xpress by CEM microwave digestion system was used for accelerated pressurized digestion of soil samples, 0.5 g of air dried soils was used with 10 ml of aqua regia prepared from Instra-analyzed grade nitric and hydrochloric acids by J.T. Baker. The setup of the digestion system was as follows: Power: 1600 W, temperature: 170°C, ramping time: 25 minutes, holding time: 20 minutes, cool down time: 20 minutes. Then the analyte was transferred to falcon vials and diluted to 50 ml with distilled water (0.05 μS/cm). The samples were additionally diluted 1:10 directly before the analysis. The same procedure was carried out for blank samples and to ensure quality control, for certified reference materials.

The quantitative analysis was conducted on Agilent 7500ce ICP-MS. This instrument is fitted with a micro mist nebulizer, Peltier cooled double pass spray chamber, peristaltic pump. Argon 5.0 (99.999% purity) was used as the carrier gas. The instrument was also fitted with a torch with "shield torch" system reducing so called "secondary discharge", off-axis ion lenses that prevent photons from entering the reaction cell and quadrupole, reaction/collision chamber with hydrogen 6.0 and helium 6.0 (purity 99.9999%) as reaction/collision gasses for the elimination of interferences. The vacuum system consisted of a rotary and turbomolecular pump. Quadrupol with hyperbolic rods is the mass separator that separates ions on the basis of their mass to charge (m/z) ratio. The detector has the ability to work in two modes: digital and analog that makes measurement through nine orders of magnitude possible. All determination were made in the presence of ^{45}Sc, ^{89}Y, ^{159}Tb as internal standards to minimize the matrix effect and ensure long term stability. The quantification limits were set at 0.01 mg/kg and the accuracy was 10%.

Statistical analysis. A randomized complete design in a factorial scheme was implemented with one plant. Three soils, two patterns of plants: (+) – inoculated, (–) – noninoculated and three replications. Analysis of variance procedure (one way ANOVA) for all treatments was conducted using the programme packet STATISTICA.PL (7) (Stat. Soft. Inc., 95% significance level). The difference between specific pairs of means was identified using Tukey test ($P < 0.05$).

Results and Discussion

Polycyclic aromatic hydrocarbons (PAHs) are compounds whose presence in contaminated soils and sediments poses a significant risk to the environment, and they have cytotoxic, mutagenic, and in some cases carcinogenic effects on human tissue (Parales *et al.*, 2002; Yu *et al.*, 2013).

The data concerning the effect of PAH's and diesel fuel on basic physical properties and biological indicators of the soil involving grass inoculation applied in the studies with bacteria *Azospirillum* spp. and *P. stutzeri* suspensions are presented in this work (Table I). It was found that soil pollution indeed contributed to a deterioration in the studied physical indicator. Statistically significant improvement was also found in the physical parameters and biological activity of the studied soils after grass inoculation with bacteria *Azospirillum* spp. and *P. stutzeri* during four-week plant growth. A statistically significant decrease in the content higher values of such parameters as: pH, total carbon was found in soils after bioaugmentation of plants with nitrogen fixing bacteria. The highest dehydrogenase activity and total number of bacteria were found in chernozem after growth of grasses inoculated with nitrogen fixing bacteria. Also soil contaminated with diesel fuel stimulated the enzymatic activity. Dehydrogenase, alkaline phosphatase and acidic phosphatase activities in chernozem and rendzina polluted with PAHs after the growth of plants was always higher after bioaugmentation of the plants. Relatively, the highest enzymatic activities and total number of bacteria were noted for samples inoculated with *Azospirillum* spp. and *P. stutzeri* – almost 20–40% more than for the control. The dehydrogenase activity appeared to be the most sensitive parameter of all six biological indexes tested. High applicability of this parameter for soil ecotoxicological testing was pointed out by other authors (Gogoi *et al.*, 2003; Parrish

Table I
Physical and biological properties of soils polluted with PAHs (1000 mg·kg^{-1}) and diesel fuel (PAHs (1000 mg·kg^{-1}).

PAHs/diesel fuel	pH	C_{total}%	N_{total}%	B+A	F	DEH	PHO Acid	PHO Alk
Chernozem								
Control	7.48	2.75	0.147	123	42	75	85	36
Non-inoculated grass								
Anthracene	7.25	2.52	0.121	54	36	54	38	15
Phenanthrene	7.15	2.58	0.113	48	28	48	41	21
Pyrene	7.13	2.55	0.128	37	21	52	45	18
Diesel fuel	7.02	2.84	0.135	124*	46	64	56	14
Grass inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*								
Anthracene	7.48*	2.78*	0.142*	110*	52	62	48	25
Phenanthrene	7.48*	2.85*	0.152*	144*	45	56	56	31*
Pyrene	7.35*	2.95*	0.148*	154*	48	78*	57*	36*
Diesel fuel	7.32*	2.96*	0.157*	174*	21*	88*	69*	42*
Calcareous rendzina								
Control	6.75	2.21	0.123	187	37	64	68	42
Grass non-inoculated								
Anthracene	6.64	1.78	0.107	74	23	42	36	26
Phenanthrene	6.72	1.73	0.105	56	31	38	42	28
Pyrene	6.52	1.77	0.110	53	28	36	39	31
Diesel fuel	6.31	1.88	0.092	197*	42	45	31	27
Grass inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*								
Anthracene	6.88*	2.22*	0.134*	98	21*	56	45	36*
Phenanthrene	6.82*	2.21*	0.144*	237*	18*	58	56*	41*
Pyrene	6.85*	2.23*	0.138*	186*	24	66*	43*	33*
Diesel fuel	6.89*	2.32*	0.146*	245*	16*	56	58*	28
Lessives								
Control	5.37	1.21	0.097	42	18	32	46	26
Grass non-inoculated								
Anthracene	5.22	1.32	0.094	34	14	12	15	15
Phenanthrene	4.84	1.33	0.085	24	16	14	21	10
Pyrene	4.67	1.22	0.084	26	12	7	18	8
Diesel fuel	4.52	1.27	0.092	87*	8*	23	16	12
Grass inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*								
Anthracene	5.75*	1.56*	0.099	56	8	34	32	18
Phenanthrene	5.75*	1.63*	0.112*	78*	5*	24	37	15
Pyrene	5.78*	1.62*	0.108	69*	6*	26	25	10
Diesel fuel	5.82*	1.45*	0.115*	120*	5*	42	34	15

* statistically significant decrease in the content ($P \leq 0.05$) in comparison with the control in the particular soils;
data is an arithmetic mean (n = 6) control –soil non-polluted with PAHs and diesel fuel – with no plant
pH – using the potentiometric method
C_{total} – using the Tiurin's method
N_{total} – using flow spectrometry, wet sample mineralisation
B+A – total number of bacteria and Actinomycetes (10^7 cfu·g^{-1} d.m. of soil)
F– total number of fungi (10^4 cfu·g^{-1} d.m. of soil)
DEH – dehydrogenase activity (μg·g^{-1} d.m. of soil)
PHO Acid – acid phosphatase activity (μg·g^{-1} d.m. of soil)
PHO Alk – alkaline phosphatase activity (μg·g^{-1} d.m. of soil)

et al., 2005; Gałązka *et al*., 2012). Muratova *et al*. (2003) contaminated soil with 5 g/kg of diesel oil and observed that the activity of soil dehydrogenase increased immediately after oil introduction. *Azospirillum* spp. and *Pseudomonas* spp. are predominant plant growth-promoting rhizobacteria extensively used as phytostimultory crop inoculants, but mixed inocula involving more than two strains are not very common. The cooperation

of *Azospirillum* and *Pseudomonas* bacterial strains with fungi of the genus Glomus has a significant effect on promoting the growth and yield of maize (Couillerot *et al*., 2013). According to Gunther *et al*. (1996), pyrene had an inhibitive effect on alfalfa growth and the residual concentrations of pyrene in the rhizosphere soil were lower than those in the non-rhizosphere soil. The rhizospheric bacterial counts were 30–50% higher than those in the non-rhizosphere soil, respectively. The effectiveness of the bioremediation of high molecular weight polycyclic aromatic hydrocarbons by *Bacillus thuringiensis* strain NA2 was presented in a work by Maiti *et al*. (2012).

A wide range of different soil microorganisms are able to metabolise, co-metabolize and utilize PAHs as a sole source of carbon and energy. The aerobic catabolism of one-cyclic and two-, three-cyclic aromatic hydrocarbons by bacteria has been extensively studied. Naphthalene has often been selected as a model compound for the study of PAH degradation because of its high aqueous solubility and the ease of isolation of microbes capable of its degradation. Since the first report of a biochemical pathway for naphthalene oxidation by *Pseudomonas* species in 1964 by Davis and Evans, extensive studies have rigorously defined the metabolic pathway genes, and the enzymes involved. In the last decade a number of bacteria that metabolise larger PAHs molecules have been isolated. These include *Azoarcus evansii*, various *Mycobacterium* species and several *Pseudomonas* species (Walton *et al*., 1994; Parrish *et al*., 2005; Pizzul *et al*., 2007).

The presented results are a consequence of research initiated to obtain an answer to the question of the possibility of using the bacterial strains *Azospirillum* spp. and *P. stutzeri* in bioremediation processes, and at the same time to supplement missing data in this field of science. A positive effect of the bacteria *Azospirillum* spp. and *P. stutzeri* on PAH degradation was found in soils artificially polluted with PAHs. The bioremediation process in aged polluted soil was more intense perhaps because in that environment, numerous autochthonous groups of microorganisms capable of pollution degradation are situated and the introduced strains additionally increased the effect (Walton *et al*., 1994; Parrish *et al*., 2005).

Phytoremediation occurs the most intensely in the rhizosphere, so the depth to which the roots grow is one of the most important factors that limit the process (Muratova *et al*., 2003; Hung *et al*., 2004; Gałązka *et al*., 2012). The studies conducted so far demonstrate that the most effective phytoremediation of soil polluted with hydrocarbons is obtained with the sowing of monocotyledonous plants, including grasses (Leigh *et al*., 2002). Good results are given also by legumes, which may be related to root secretions rich in nitrogen compounds (Liste and Felgentreu, 2006; Ouvrard *et al*., 2013).

In order to establish the effect of plant inoculation on the degree of PAH's degradation in the polluted soils, chromatographic determinations of aromatic hydrocarbons were carried out. In the soils artificially polluted with PAHs, a significant degree of anthracene, phenanthrene, and pyrene degradation was noted (dose 1000 mg/kg) after plant inoculation with the bacteria *Azospirillum* spp. and *P. stutzeri*, particularly visible in the case of calcareous chernozem and rendzina pollution (Fig. 1a). The bioremediation process occurred most efficiently in rendzina soil, especially in degradation degree of anthracene, phenanthrene, and pyrene in the three applied doses (100, 500, and 1000 mg/kg) during four-week long grasses growth inoculated and non-inoculated with *Azospirillum* spp. and *P. stutzeri* (Fig. 1b). With the highest PAH doses (1000 mg/kg), a decrease in the content of anthracene in the soil took place – from 96% with no plant inoculation to 24% with inoculation, phenanthrene from 56 to 22%, and pyrene from 42 to 18%.

In the root area of plants, an increased bioremediation rate of organic pollutants is observed in comparison with non-rhizospheric soil (Liste and Aleksander, 2000). This is related first of all to the metabolic activity

Fig. 1a.

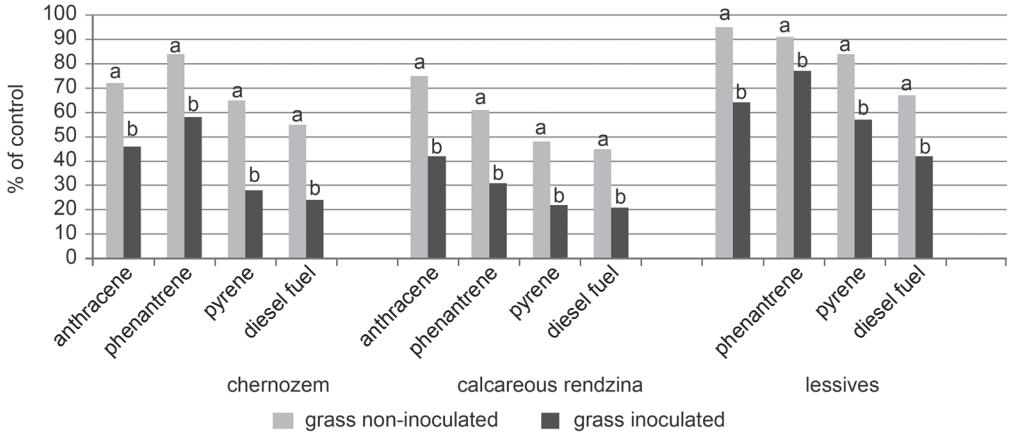

Fig. 1b.

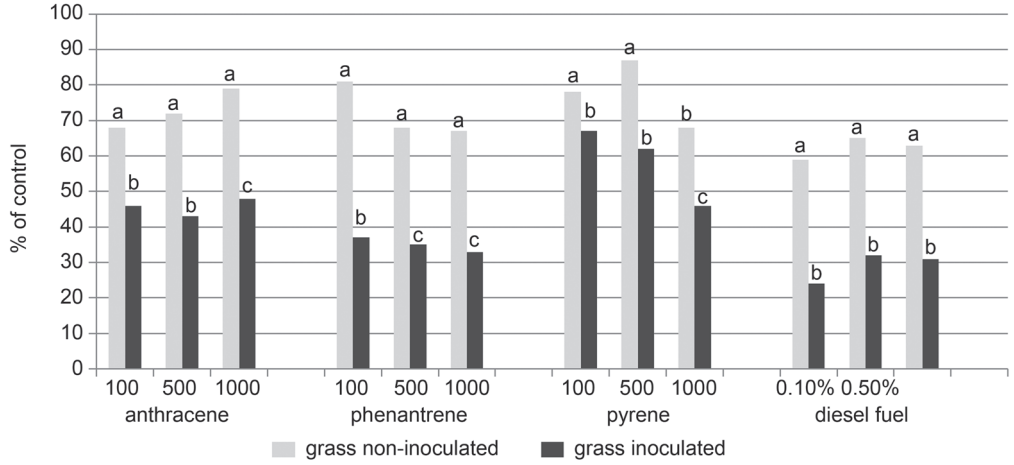

Fig. 1. Degree of PAHs degradation: a) in soils artificially polluted with anthracene, phenanthrene, and pyrene (doses in soil 1000 mg/kg) and diesel fuel (doses in soil 1000 mg/kg); b) in calcareous rendzina artificially polluted with PAHs.

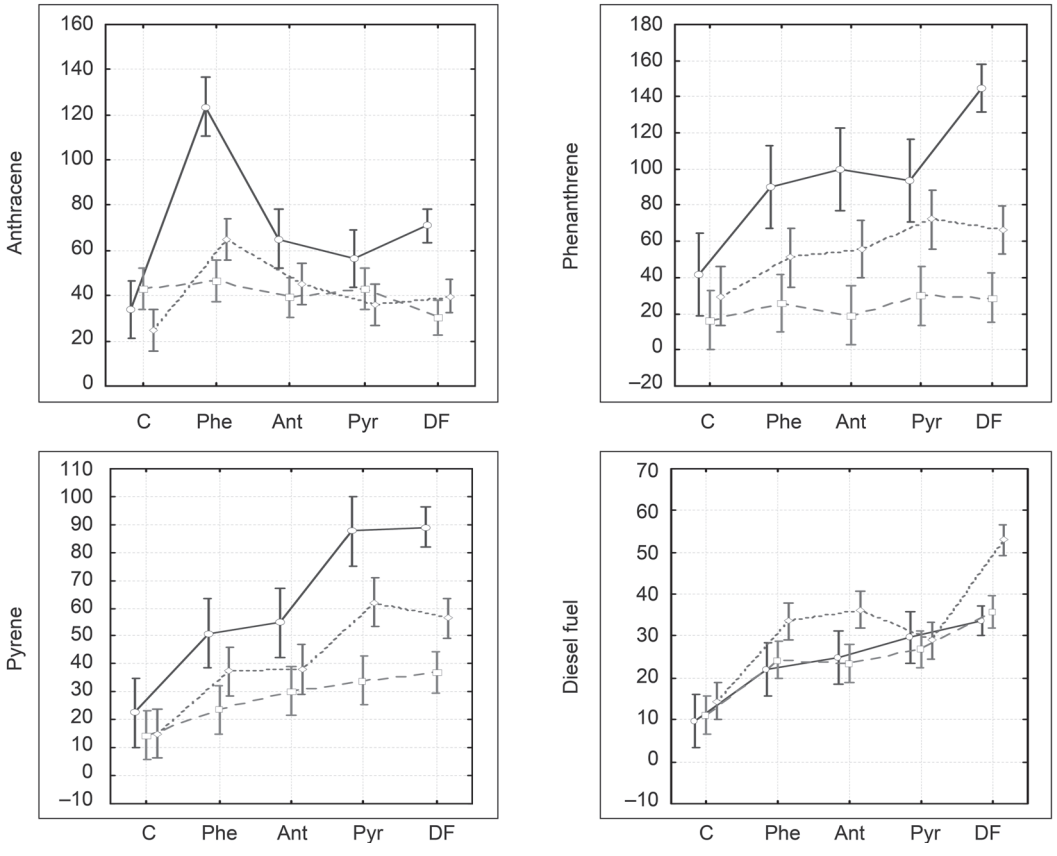

Fig. 2. The total number of microorganisms capable of degrading PAH (doses in soil 1000 mg/kg) and diesel fuel (doses in soil 1000 mg/kg) as a sole source of carbon and energy [10^4 cfu/g d.m. of soil] – two-way analysis of variance.
C – control; Phe – phenantrene; Ant – anthracene; Pyr – pyrene; DF – diesel fuel ——— chernozem – – – – rendzina ----- lesives

of microflora, which populates the rhizosphere in great numbers. It turns out that of significant importance are also microorganisms directly connected with the plant that live inside root, stem, and leaf tissues. Examples of such microorganisms are strains of *Azospirillum* spp. and *P. stutzeri*. The total number of microorganisms capable of degrading PAHs as a sole source of carbon and energy was found in in each of the studied soils (Fig. 2). The tested soils showed a large number of microorganisms capable of degrading anthracene, phenanthrene, pyrene, and diesel fuel (10^4 cfu/g d.m. of soil). The analysis of variance indicate the statistical important differences of total number of microorganism able to degrade anthracene, phenanthrene

and pyrene in different soils. The highest total number of microorganisms able to degrade diesel fuel was observed in chernozem after grown of cock's food grass. Bacteria are mayor players in the degradation of PAHs, bioremediation is an increasingly popular option for reclamation of oil-contaminated sites. Many bacteria that utilize a polycyclic aromatic hydrocarbon as the source of carbon and energy have been isolated (Pizzul *et al.*, 2007).

A number of different metabolic pathways have been established for the bacterial degradation of PAHs. The genes coding for the enzymes involved in the degradation of alkanes (*alk*), naphthalene (*nah*), benzoate *via* ortho cleavage of catechol (in bacteria) or prothocatechuate (in fungi) by the β-ketoadipate pathway have been extensively characterized. Evaluation of the effect of contaminants on soil microflora strongly depended on the applied parameters. A more detailed discussion of the results regarding the effect of PAH's on soil microorganisms is given elsewhere (Johnsen *et al.*, 2007; Ahammed *et al.*, 2012). In contrast to inorganic compounds, microorganisms can degrade and even mineralize organic compounds in association with plants. Hence the discovery of effective pathways for degradation and mineralization of organic compounds may play an important role in the future. So far, bacteria capable of degrading certain kinds of organic pollutant, such as PAHs have been isolated from a range of sites and the pathways and encoding genes have also been well studied. But most of these bacteria cannot survive in the near-starvation conditions found in soils, including the rhizosphere (Joner *et al.*, 2007). Compared with physical and chemical remediation, phytoremediation has several advantages: it preserves the natural properties of soil; it acquires energy mainly from sunlight; high levels of microbial biomass in the rhizosphere can be achieved; it is low in cost; and it has the potential to be rapid. Although with these advantages, some plants show very low tolerance to soil contaminants, which limits the degradation efficiency to an insufficient level for meaningful soil remediation. As described above, although rhizobacteria may play an important role in the degradation and mineralization of organic compounds, the metabolic efficiency can be very low. Possible causes may be the small microbial biomass or the low solubility and bioavailability under high toxic pressure (Liste and Aleksander, 2000).

Grasses, thanks to a well-developed and dense root system, became an adequate habitat for the applied in the inoculation entophytic bacteria capable of using PAHs as the only source of carbon and energy (Leigh *et al.*, 2002). A statistically significant ($P \leq 0.05$) decrease in aromatic carbon content was obtained in the polluted soils. It cannot be unambiguously stated, however, that the entire amount of PAHs per soil pollution was used by the bacteria in the bioremediation process. In the conducted studies with the use of non-inoculated plants, a decrease in PAH content in the soil was also observed, but it was significantly smaller than in the inoculated combinations. The degree of degradation of the particular aromatic hydrocarbons in the soils polluted with the highest diesel fuel doses (1000 mg/kg) is presented in Fig. 3. The highest degree of their degradation was found in calcareous rendzina (Fig. 3b). Equally intensive was the degradation of those hydrocarbons in chernozem (Fig. 3a), whilst it was significantly weaker in lessives (Fig. 3c).

Liste and Felgentreu (2006) found a decrease in gasoline hydrocarbon content to 68.7% and PAHs to 59% at mustard growth during a 90-day-long bioremediation process with natural plant rhizosphere microflora. In the present studies, with significantly higher soil pollution (1000 mg/kg) at non-inoculated and inoculated maize growth in calcareous rendzina the following results were obtained: decrease in the anthracene content in the soil from 95% with no plant inoculation to 42% with inoculation, phenanthrene from 72% to 36%, whilst pyrene from 58 to 27%. Gałązka *et al.*

Fig. 2a.

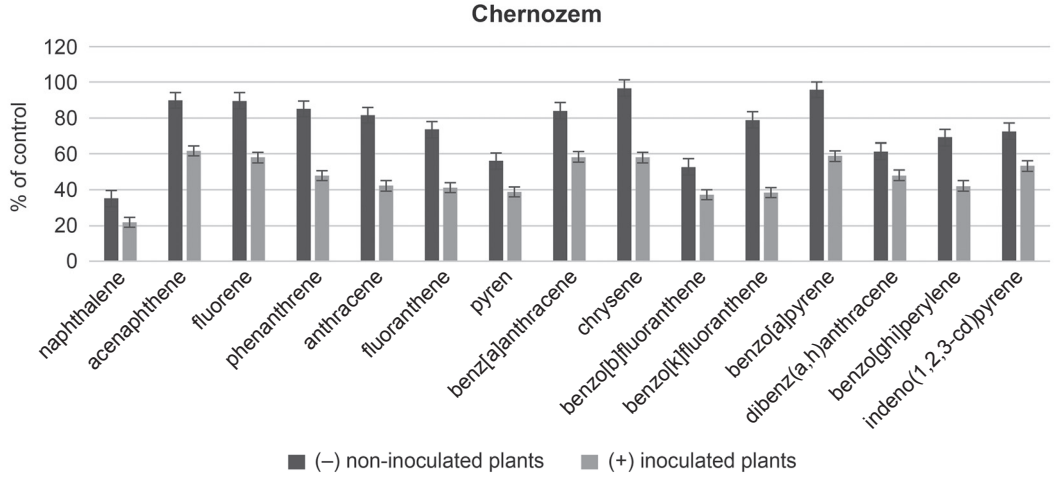

Fig. 2b.

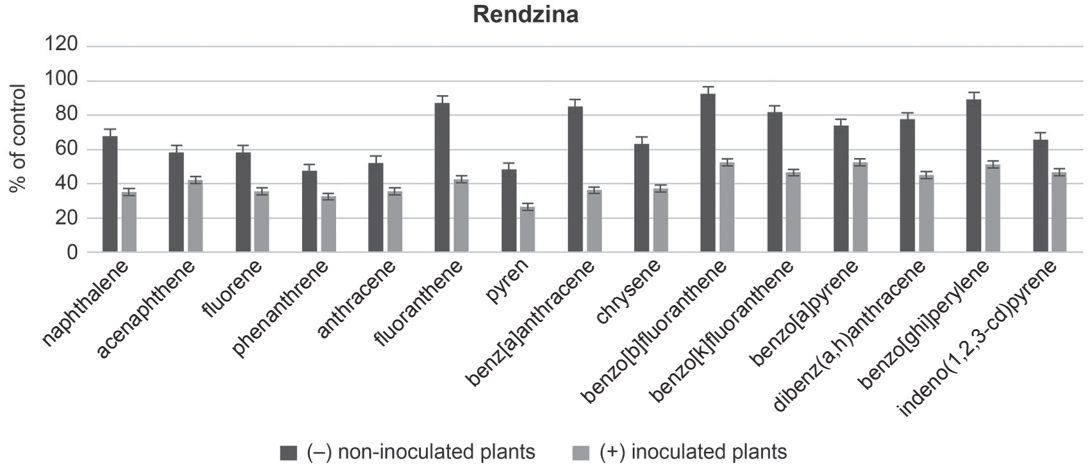

Fig. 2c.

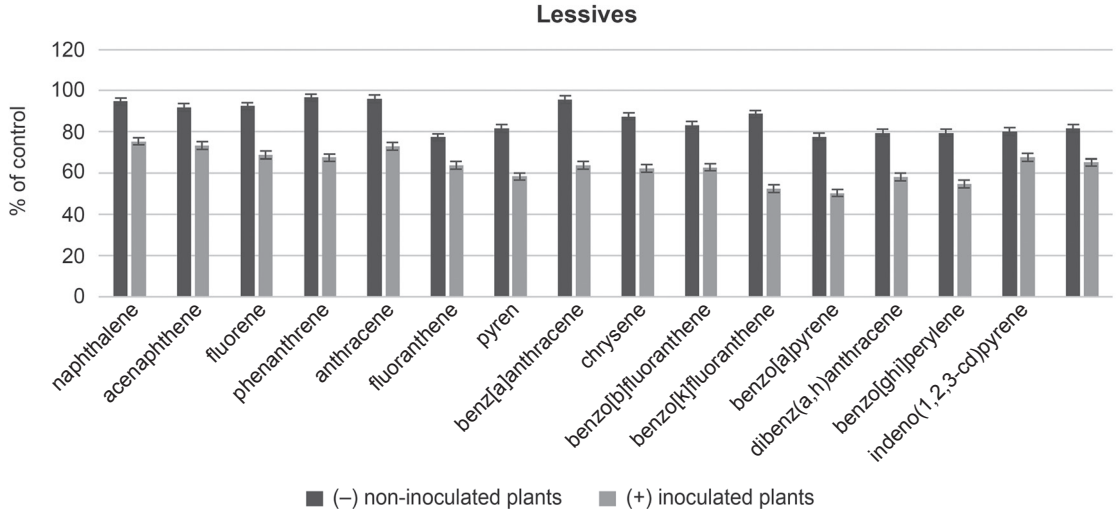

Fig 3. The PAHs content in in soils polluted with 1000 mg/kg diesel fuel.
a) chernozem, b) calcareous rendzina, c) lessives.

(2012) found the degradation degree with the highest PAHs and diesel fuel doses in soils artificially polluted during four-week long meadow fescue growth inoculated and non-inoculated with nitrogen fixing bacteria a decrease in the content of Σ15PAHs in the soil took place – from 65% with no plant inoculation to 15% with inoculation. A decrease in hydrocarbon content was noted at meadow fescue growth from 80–91% (non-inoculated plant) to 18–56% (inoculated plant), whilst it was significantly weaker in lessives.

A plant is capable – through the root system – of absorbing various organic compounds depending on their relative lipophilicity (Kang and Xing, 2006). Compounds uptaken by the plant may accumulate in the root or become permanently built into its structure, for example lignin, which is an example of pollution phytostabilisation (Siciliano and Germida, 1998). However, a significant part of the absorbed organic compound undergoes only translocation along the vascular bundles of the plant and is transpirated through the leaves. This process decreases pollution concentration in the soil but it is not advantageous to the environment because it causes atmosphere pollution. Moreover, the presence of plants in the soil intensifies humification (Liste and Aleksander, 2000; Smith *et al.*, 2006), as the organic compounds of the pollutant are built into humus components. Immobilised in such a way do not pose a significant threat to the environment, but this does not solve the problem of pollution, either. Much better results are obtained during bioaugmentation processes with the use of soil microorganisms capable of pollution degradation (Johnsen *et al.*, 2007).

After 30 days of experiment concentrations of PAH's in soils decreased almost 10–60% comparing to control. In other hand content of heavy metals in soils was also lower. The content of heavy metals determined by ICP-MS methods confirmed the statistically significant decrease in their levels if inoculated plants were used (decline in chernozem and rendzina by 20–45% and lessives 15–23%) (Table II). Through the root system

Table II
Degree of PAHs degradation in soils polluted with 1000 mg·kg^{-1} diesel fuel: calcareous rendzina, chernozem, lessives, brown soil aged polluted with crude oil

	Factors of the experiment					
	chernozem		calcareous rendzina		lessives	
	I–	I+	I–	I+	I–	I+
PAHs [% of control]						
naphthalene	35.2 ± 1.2^a	21.8 ± 2.1^a	67.9 ± 1.5^b	35.2 ± 0.5^a	94.8 ± 2.1^b	75.4 ± 1.2^a
acenaphthene	89.8 ± 0.5^b	61.7 ± 1.6^b	58.3 ± 1.3^a	42.2 ± 0.2^a	91.9 ± 1.0^b	73.8 ± 0.7^a
fluorene	89.6 ± 1.5^b	58.1 ± 1.8^a	58.4 ± 0.8^a	35.7 ± 0.2^c	92.5 ± 1.0^b	68.8 ± 0.5^a
phenanthrene	85.1 ± 0.5^b	47.9 ± 1.1^a	47.4 ± 1.1^a	32.5 ± 1.1^c	96.5 ± 0.5^b	67.5 ± 1.1^a
anthracene	81.5 ± 1.2^b	42.3 ± 2.2^a	52.2 ± 0.2^a	35.4 ± 0.8^c	96.1 ± 1.2^b	72.9 ± 0.7^a
fluoranthene	73.5 ± 2.2^b	41.2 ± 2.3^a	87.2 ± 0.5^b	42.6 ± 2.5^a	77.3 ± 0.5^b	63.9 ± 0.4^a
pyren	56.1 ± 1.5^a	38.9 ± 0.4^c	48.2 ± 0.7^a	26.4 ± 1.4^c	81.7 ± 1.1^b	58.4 ± 1.2^a
benz[a]anthracene	84.0 ± 2.5^b	58.4 ± 0.7^a	85.1 ± 0.8^b	38.6 ± 1.3^c	95.6 ± 0.4^b	63.8 ± 0.3^a
chrysene	96.7 ± 1.2^b	58.1 ± 1.5^a	63.4 ± 1.1^a	37.4 ± 1.1^c	87.5 ± 0.3^b	62.4 ± 0.6^c
benzo[b]fluoranthene	52.8 ± 1.3^a	37.3 ± 0.8^a	92.5 ± 1.2^b	52.6 ± 0.2^a	83.3 ± 1.2^b	62.9 ± 0.5^a
benzo[k]fluoranthene	78.9 ± 1.9^b	38.4 ± 1.2^a	81.7 ± 0.7^b	46.5 ± 0.1^c	88.7 ± 1.2^b	52.4 ± 0.4^c
benzo[a]pyrene	95.8 ± 1.2^b	58.9 ± 1.8^a	73.9 ± 0.4^b	52.4 ± 0.3^a	77.6 ± 0.5^a	50.4 ± 0.9^c
dibenz(a, h)anthracene	61.5 ± 1.2^a	48.1 ± 2.2^a	77.6 ± 1.2^a	45.1 ± 1.2^a	79.5 ± 0.4^b	58.3 ± 0.3^c
benzo[ghi]perylene	69.1 ± 0.8^b	42.2 ± 0.5^a	89.2 ± 1.1^b	51.4 ± 0.4^a	80.2 ± 0.5^b	54.8 ± 1.2^a
indeno(1, 2, 3–cd)pyrene	72.6 ± 1.7^a	53.5 ± 1.2^a	65.7 ± 0.5^b	46.8 ± 0.7^a	81.7 ± 1.0^b	67.7 ± 1.1^a
Heavy metal [mg·kg^{-1}]						
Cr	16.4 ± 0.2^a	14.5 ± 0.4^a	15.8 ± 0.5^a	11.5 ± 0.1^b	17.4 ± 0.3^a	16.2 ± 0.2^a
Pb	24.2 ± 1.2^a	11.4 ± 0.8^b	22.4 ± 0.7^a	15.4 ± 0.5^b	21.4 ± 0.7^a	18.4 ± 0.2^a
Cu	11.4 ± 0.4^a	7.4 ± 0.4^b	10.7 ± 0.2^a	8.6 ± 0.3^b	11.6 ± 0.1^a	8.4 ± 0.4^a
Zn	62.4 ± 0.2^a	45.5 ± 0.5^b	58.7 ± 0.5^a	32.5 ± 0.4^b	60.4 ± 0.2^a	55.2 ± 0.3^a
Ni	16.5 ± 0.4^a	9.4 ± 0.2^b	15.7 ± 0.3^a	8.4 ± 0.5^b	14.2 ± 0.2^a	10.4 ± 0.6^c
Co	5.4 ± 0.2^a	3.4 ± 0.3^b	6.2 ± 0.4^a	4.2 ± 0.5^b	6.1 ± 0.6^a	3.1 ± 0.4^b
Cd	0.298 ± 0.02^a	0.098 ± 0.01^b	0.242 ± 0.02^a	0.075 ± 0.01^b	0.187 ± 0.02^a	0.142 ± 0.02^a
Sn	1.2 ± 0.02^a	0.4 ± 0.02^b	1.7 ± 0.01^a	0.3 ± 0.02^b	1.9 ± 0.02^a	0.7 ± 0.01^b
Pb	24.6 ± 0.3^a	11.4 ± 0.2^b	22.6 ± 0.2^a	14.5 ± 0.1^b	28.4 ± 0.2^a	18.7 ± 0.1^b

values marked with different letters (a, b, c) are statistically significantly different ($P < 0.05$)
I+ inoculated plants; I– non inoculated plants

heavy metals were accumulated in the plant tissues. The results obtained show that *D. glomerata* is not only a good bioremediation plant but also effective in soils polluted with heavy metals. Lower concentrations of PAH's and heavy metals were observed in chernozem and rendzina after inoculation grass with *Azospirillum* spp. and *P. stutzeri*.

As described, applied coupling decreased the pollution of the soil environment with PAHs and metals which means it was simultaneously beneficial for the growth of plants in contamination conditions. Under the influence of synergistic reaction of nitrogen fixing bacteria the toxic effect of PAH's content and heavy metals on chosen plant species and their rhizosphere was reduced and the processes of PAH's degradation in soils increased. To conclude, the application of grass inoculation with *Azospirillum* spp. and *P. stutzeri* had a positive effect on the degradation processes of polycyclic aromatic hydrocarbons in soils artificially polluted with PAHs. A detailed understanding of all the mechanisms responsible for physiological and biological interactions during hydrocarbons degradation may be useful for the application of these bacteria in field studies on bioremediation of oil contaminated sites. Therefore, the present inoculation of plants with *Azospirillum* spp. and *P. stutzeri* was effective in promoting the phytoremediation of freshly added PAH's into the soil.

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