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Original Research

The Efficiency of Rhizosphere Bioremediation with *Azospirillum* sp. and *Pseudomonas stutzeri* in Soils Freshly Contaminated with PAHs and Diesel Fuel

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Abstract

In this study the effects of meadow fescue (*Festuca pratensis*) inoculated (I+) and non-inoculated (I-) by diazothrophic species of bacteria (*Azospirillum* spp. and *Pseudomonas stutzeri*) on the degradation of polycyclic aromatic hydrocarbons in soils freshly contaminated with a mixture of PAHs and diesel fuel was investigated. Plants were grown in three different soils (chernozem, rendzina, lessives) for 4 weeks and unplanted soils considered as control. At the end of the experiment, physical properties of soils, dry masses of plants, and Σ 15PAH contents were measured in the soils. The results demonstrated that I (+) plants contained more root and shoot biomass than I (-) plants. Planting stimulated the bioremediation process in contaminated soils. The differences in concentration between the inoculated or non-inoculated soils indicate that the presence of plant roots, in addition to the passage of time, contributes, to reduction in the bioavailability of a mixture of PAHs and diesel fuel. The choice of soils, contamination, and inoculation has a significant effect of PAH degradation (mixture 1: in chernozem 24.8-59.7%, in rendzina 15.4-41.4%, in lessives 48.4-71,4%). It was revealed that they were more degraded in the rhizosphere of (I+) plants compared to I (-) ones. Obtained results suggests that inoculation of plants with *Azospirillum* spp. and *P. stutzeri* looks promising as a low-cost treatment method for PAH-contaminated soil.

Keywords: contaminated soil, phytoremediation, polycyclic aromatic hydrocarbons (PAHs), diesel fuel, *Azospirillum*, *Pseudomonas stutzeri*

Introduction

Some organic contaminants can persist in the environment for a long time and threaten human health. They mainly include: total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) coming from the exploration and consumption of fossil fuel, polychlorinated biphenyls (PCBs) widely used in the industrial process (the most degradation-resistant), and other chlorinated aromatics used as PCB replacements such as polychlorinated terphenyls (PCTs), halogenated compounds like perchloroethylene (PCE) and trichloroethylene (TCE), and pesticides like atrazine and bentazon [1].

Polycyclic aromatic hydrocarbons (PAHs) belong to the group of persistent organic pollutants that are relatively resistant to biodegradation and can remain in the environ-

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ment for a long time. PAHs present in soils may exhibit a toxic activity toward different biological elements of the environment such as plants and microorganisms. Microorganisms, being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. In general, they are very sensitive to low concentrations of contaminants and rapidly respond to soil perturbation [1-3].

Bioremediation is a technology of pollutant removal that uses living microorganisms for destruction catalyzation or transformation of various sorts of pollutants in less harmful forms. Microorganisms are characterized by an exceptional ability in comparison with other organisms to adapt to new environmental conditions, which leads to the acceleration of processes that no longer naturally occur in the soil [4, 5].

Phytoremediation, or the use of plants and/or associated rhizosphere to decontaminate polluted sites, is considered today to be a realistic, low-cost alternative to treating extensive areas of pollution by organic chemicals [6, 7]. This technology is based on the catabolic potential of root-associated microorganisms that 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 with well-designed controls, have specifically shown higher rates of PAH biodegradation in whole soils planted with a variety of species. There is limited knowledge regarding plants and microbes active in phytoremediation of PAH-polluted sites. Biodegradation of PAHs in soils is often limited by the slow mass transfer of these hydrophobic compounds toward 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 pollutants, and production of biosurfactants by bacteria or by plants [1, 7].

Bioaugmentation involves the introduction of microorganisms into contaminated media to promote the degradation of contaminants. Though viewed with skepticism in the past, the use of bioaugmentation has increased significantly in recent years, with mounting evidence that it can be helpful for improving the bioremediation of some contaminants under some site conditions. Bioaugmentation has become particularly popular for increasing the rate and extent of reductive PAH in soil [8, 9].

Azospirillum spp and *Pseudomonas stutzeri* belong to the plant growth-promoting rhizobacteria (PGPR), and they are bacteria capable of promoting plant growth by colonizing the plant root [10]. They belong to the group of diazotrophic bacteria that fix free nitrogen, 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 [11]. Bacteria from genus *Pseudomonas* are microorganisms that effectively decompose organic pollutants through cometabolism in natural water and soil environments [8, 10]. In the available literature, there is a lack of data on the participation of bacteria from genus *Azospirillum* in the bioremediation processes. Free-living bacteria that fix nitrogen, namely *Azospirillum* spp. and *Pseudomonas stutzeri*, may create permanent associations with the roots of most cereals and grasses used in plant production. Rhizodegradation is a biological process of organic pollutant removal from the soil with the participation of microorganisms capable of their degradation in plant root areas. Plant root secretions – sugars, alcohols, organic acids – become a source of carbon for soil microorganisms, due to which they increase their activity, growth, and biodegradation effectiveness [10].

The aim of our work was to estimate the effect of plant inoculation with *Azospirillum* spp. and *Pseudomonas stutzeri* bacteria on the remediation process and degradation of PAHs in mixture with diesel fuel in soils freshly contaminated with the use meadow fescue (*Festuca pratensis*) as a bioremediation plant.

Experimental Procedures

The effect of soil (chernozem, calcareous rendzina, and lessives) pollution was studied, and artificially polluted with a mixture of polycyclic aromatic hydrocarbons (PAHs) and diesel fuel (ON) in the phytoremediation process (Table 1). Agricultural areas from which the soil material for the studies was taken up 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 [12]. The plant used in the tests was meadow fescue (*Festuca pratensis*). The grass was selected because it grows relatively fast, endophytic bacteria can exist in their tissues, and they are natural rangeland plants of Poland. For the experiment, the following compounds were chosen: anthracene, phenanthrene, and pyrene, which were applied in doses of 100, 500, and 1000 $mg \cdot kg^{-1}$ d.m. of soil and diesel fuel (Multi Motor Oil Jasol 12 SG/CE 5W/4 originating from Jaslo Refinery, JSC, Poland) at the concentration of 0.1%, 0.5%, and 1% (v/v) d.m. of soil. And also a mixture of PAHs and diesel fuel:

- mixture 1 – Σ 3WWA (at 100 $mg \cdot kg^{-1}$ every, anthracene, phenanthrene, pyrene,) and diesel fuel 0.1% (v/v)
- mixture 2 – Σ 3WWA (at 500 $mg \cdot kg^{-1}$ every) and diesel fuel 0.5% (v/v)
- mixture 3 – Σ 3WWA (at 1000 $mg \cdot kg^{-1}$ every) and diesel fuel 1% (v/v).

In the bioremediation process, plant inoculation with bacteria mixture *Azospirillum* and *Pseudomonas stutzeri* was additionally applied in the amount of 1 ml per 500 g of soil. Bacteria strains *Azospirillum* spp. (12/6, 15/7, and 77Bb1) and *Pseudomonas stutzeri* (5_3, 5_7, and $40T_4$) originated from the collection of the Department of Agricultural Microbiology, Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy. Those bacteria were isolated from the endorhyzospheres of oat

Table 1. Origin and soil texture of soils chosen for the experiment.

No.	Soil	Uptake location	Percentage content of mechanical fractions, [mm]		
			1.0-0.1	0.1-0.02	< 0.02
1	Chernozem generated from loess silty loam (płg)	Kułakowice near Hrubieszów	17	59	24
2	Calcareous rendzina light loamy sand (pgl)	Mięćmierz near Kazimierz Dolny	65	23	12
3	Lessives generated from loess dusty loam	Las Stocki near Wąwolnica	5	26	69

Soil texture – using the Casagrande's method in Prószyński's modification according to norm PN-R-04032:

(*Hordeum sativum*), maize (*Zea mays* L.), and grass *Elymus arenarius* [11].

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 $E \cdot m^{-1} \cdot s^{-1}$). Tests were carried out at 24ºC during the day, and for 8 hours at night at 18ºC. In the pots, 500 g of air-dried, sifted soil were placed. Hydrocarbons were added in the form of solution in dichloromethane, reaching in soil concentrations 100, 500, and 1000 $mg \cdot kg^{-1}$ d.m. of soil and in the mixture of PAHs and diesel fuel. For every pollution level, the control was soils without PAHs with dichloromethane added (such an amount of CH_2Cl_2 was added as was put in with the PAHs dose). 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, pre-sprouted plants seeds were sown in the pots.

At the end of the experiment, plant samples were collected and washed with deionized water before being separated into shoots and roots. Then all samples were oven-dried (65ºC) for 48 h to reach a constant weight, and dry weights of shoots and roots were determined. Soil was sampled at the start and end of the experiment. Soil samples in planted pots were collected from the rhizosphere area, After the end of vegetation, plants were gently removed from the pot and the soil surrounding the plant's roots was shaken and after sieving it was stored for further examination. All the samples were kept at 4ºC until analysis. Besides the above experiment, germination of the plants was monitored daily for 4 weeks. The seeds of each plant (I+ and I-) were grown in the contaminated soil. The experimental conditions were the same as mentioned above. The number of germinated seeds in each pot was recorded and expressed as a percentage of the number of planted seeds.

After the completion of the four-week plant growth cycle in the particular experiment combinations, plant growth, total number of bacteria, and dehydrogenase activity of the soils were marked, as well as Σ15 PAH content in freshly polluted soils and Σ15 PAHs in the case of soil pollution with diesel fuel.

Marking the content of PAHs in the soils was carried out at the Oil and Gas Institute in Cracow (at the Laboratory of Analytics and Physical Chemistry of Sewage and Waste and the Laboratory of the Analytics and Physical Chemistry of Hydrocarbon Fuels). Σ15 PAH content was analyzed using the HPLC (high performance liquid chromatography) method, accepted for marking in environmental samples by the United States Environmental Protection Agency, excluding the most volatile hydrocarbons and the ones that rarely occur in soils. A rapid method for the determination of PAHs in soil samples based on their extraction with methylene chloride by sonication and subsequent separation by HPLC with fluorimetric detection is proposed. A Li Chro CART® 250-4 column was used with a gradient of acetonitrile/water as the mobile phase, together with a program of nine excitation and emission wavelength pairs. Recoveries were in the range 70-98%, except for acenaphthene and naphthalene, at concentration levels 1.08-442 $\mu g \cdot kg^{-1}$ with relative standard deviations in the range 2-15% (n=4). Total PAHs found in soil samples were in the range 15-282 $\mu g \cdot kg^{-1}$. The results were compared with those obtained by applying the 3540 EPA method for two samples [13].

Analysis of PAHs and diesel fuel chemical composition was carried out according to the Order by the Ministry of the Environment from September 9, 2002 concerning soil quality standards and ground quality standards [14]. Markings were made in chernozem, calcareous rendzina, and lessives with the highest doses of PAHs and diesel fuel (soil freshly polluted). Dose of 1000 $mg \cdot kg^{-1}$ PAHs, 1% 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 [12].

A randomized complete design in a factorial scheme was implemented with one plant, three soils, two levels of endophytes (I+, I-), and three replications. Analysis of variance procedure (one way ANOVA) for all treatments was conducted using the program packet STATISTICA.PL (7) (Stat. Soft. Inc., 95% significance level). The difference between specific pairs of mean was identified using Tukey test ($P<0.05$).

Results

For the studies, soils material uptaken from the plough-humus horizon (0-20 cm) of soils used agriculturally was used distant from PAH emission sources from various regions of Poland. Chosen soils were those that occur most

Table 2. Correlation coefficient values (r) between physical and biological properties of soils and the dry mass of the above-ground plant parts in soils polluted with PAHs (1000 mg·kg^{-1}) and diesel fuel (1%).

PAHs/diesel fuel	pH	$C_{total} - C_{og.}$%	$N_{total} - N_{og.}$%	P_2O_5	K_2O	Mg	N-NH_4	N-NO_3	TN	DH
Chernozem										
Non-inoculated meadow fescue										
Mixture 1	0.391	0.421	0.321	0.451	0.532	0.361	0.391	0.421	0.678	0.687
Mixture 2	0.815*	0.831*	0.861*	0.831*	0.781*	0.891*	0.761*	0.691*	0.547	0.684*
Mixture 3	0.215	0.412	0.384	0.284	0.187	0.361	0.421	0.451	0.789*	0.541
Meadow feascue inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*										
Mixture 1	0.815*	0.784*	0.786*	0.452	0.567	0.394	0.875*	0.951*	0.875*	0.874*
Mixture 2	0.942*	0.697*	0.859*	0.875*	0.964*	0.984*	0.978*	0.964*	0.749*	0.984*
Mixture 3	0.961*	0.791*	0.915*	0.421	0.367	0.481	0.964*	0.987*	0.861*	0.874*
Calcareous										
Non-inoculated meadow feascue										
Mixture 1	0.875*	0.742*	0.421	0.461	0.317	0.512	0.748*	0.796*	0.584	0.684*
Mixture 2	0.945*	0.814*	0.871*	0.315	0.471	0.876*	0.948*	0.967*	0.784*	0.445
Mixture 3	0.475	0.347	0.367	0.486	0.477	0.475	0.615	0.518	0.789*	0.542
Meadow feascue inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*										
Mixture 1	0.987*	0.998*	0.745*	0.789*	0.874*	0.878*	0.990*	0.945*	0.984*	0.895*
Mixture 2	0.957*	0.914*	0.987*	0.678*	0.974*	0.987*	0.987*	0.912*	0.875*	0.899*
Mixture 3	0.879*	0.745*	0.684*	0.789*	0.847*	0.789*	0.897*	0.872*	0.942*	0.945*
Lessives										
Non-inoculated meadow feascue										
Mixture 1	0.927*	0.877	0.785	0.324	0.452	0.361	0.215	0.451	0.367	0.268
Mixture 2	0.924*	0.854*	0.791	0.521	0.526	0.589	0.785*	0.812*	0.268	0.451
Mixture 3	0.696	0.921*	0.656	0.624	0.418	0.384	0.397	0.458	0.316	0.364
Meadow feascue inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*										
Mixture 1	0.992*	0.912*	0.845*	0.689*	0.748*	0.815*	0.748*	0.815*	0.689*	0.784*
Mixture 2	0.987*	0.891*	0.869*	0.728*	0.915*	0.874*	0.815*	0.912*	0.742*	0.845*
Mixture 3	0.981*	0.951*	0.967*	0.948*	0.877*	0.809*	0.943*	0.972*	0.845*	0.742*

*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

pH – using the potentiometric method [PN – ISO 10390:1997]

$C_{total} - C_{og.}$ – using Tiurin's method [PB 20.1 Ed. I – 20.05.1999]

$N_{total} - N_{og.}$ – using flow spectrometry, wet sample mineralization [PB 16.3 Ed. I – 14.10.2002]

assimilable P_2O_5 using the Egner-Riehm colorimetric method [PN – R – 04023:1996]

assimilable K_2O, Mg using Egner-Riehm flame emission spectrometry [PN – R – 04022:1996]

ammonium and nitrate using flow spectrometry, after 1% K_2SO_4 extraction [PB 8.1 Ed. III – 08.09.2004]

TN – total number of bacteria [40].

DH – dehydrogenase activity [41].

frequently in Lublin Province (Table 1). They varied in origin, mechanical composition, and physicochemical properties. The following mineral soil textures were taken into account: sand, clay, dust, and loam, with a diversified content of organic carbon (C_{org}) and various pH values (Tables 1, 2).

Table 2 presents data concerning the effect of mixtures of PAHs and diesel fuel on basic physical and biological properties of soil involving meadow feascue inoculation applied in the studies with bacteria *Azospirillum* spp. and *Pseudomonas stutzeri* suspensions. It was found that soil

Table 3. One-way ANOVA (P≤0.05).

Variable	Soil		Pollution		Inoculation	
	F value	α	F value	α	F value	α
Dry mass [mg]						
Above-ground parts	2.992	0.05093	5.147	0.00044	24.5118	0.00000
Underground parts	13.139	0.00000	2.829	0.02412	10.9128	0.00101

pollution indeed contributed to a deterioration in the studied physical and biological indicator. Statistically significant improvement was also found in both parameters (physical and biological) of the studied soils after plant inoculation with bacteria *Azospirillum* spp. and *Pseudomonas stutzeri* during four-week plant growth.

Table 2 presents Pearson's product-moment correlation coefficient of the physical properties of the soils with meadow fescue yield expressed as the sum of the above- and underground plant parts in the phytoremediation process. Strong correlations were found between the particular physical parameters of rendzina and also the total number of bacteria and dehydrogenase activity in the case of plants inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri*.

Effect of inoculation with *Azospirillum* spp. and *Pseudomonas stutzeri* on plant growth in the conditions of soil pollution with PAHs is presented as the analysis of two parameters: the dry mass of the aboveground parts and the dry mass of the underground plant parts. On the basis of the analysis of variance (ANOVA, Table 3), average variables (of the above- and underground plant parts) in the polluted soils group, it can be stated that the mean values did not differ significantly statistically. It was found that in the case of the aboveground parts, the average value for the control differed significantly from all average values except for diesel fuel. The remaining average values did not differ significantly between one another.

In order to establish the effect of plant inoculation on the degree of crude oil derivatives degradation in the polluted soils, chromatographic markings of aromatic hydrocarbons were carried out. In soils freshly polluted by a mixture of PAHs and diesel fuel, a significant degree of Σ 15PAHs degradation was noted after plant inoculation with bacteria *Azospirillum* spp. and *Pseudomonas stutzeri*, particularly visible in the case of calcareous rendzina pollution (Table 4). The choice of soils, contaminants and inoculation has the significant effect of PAH degradation (mixture 1: in chernozem 24.8-59.7%, in rendzina 15.4-41.4%, in lessives 48.4-71.4%). The degradation degree Σ 15PAHs in charnoziem and lessives during a four-week-long meadow feascue growth inoculated and non-inoculated with *Azospirillum* spp. and *Pseudomonas stutzeri* is shown in Table 4. With the highest PAHs and diesel fuel doses (mixture 3), a decrease in the content of Σ 15PAHs in the soil took place – from 65% with no plant inoculation to 15% with inoculation.

In charnoziem freshly polluted with a mixture of PAHs and diesel fuel (mixture 3), a significant decrease of Σ15 PAH content was noted with the application of meadow feascue inoculation with bacteria *Azospirillum* spp. and *Pseudomonas stutzeri* (Table 4). A decrease in hydrocarbon content was noted at meadow fescue growth from 80-91% (non-inoculated plant) to 18-56% (inoculated plant), while it was significantly weaker in lessives (Table 4).

Discussion

The remediation of soil impacted by oil production and transport is not only of importance considering environmental problems, but also for the preservation of agricultural productivity. Chemical and physical methods applied for remediation of PAH-contaminated soils such as thermal treatment, soil washing, solidification and stabilization are expensive, disruptive to the environment, and involve high energy consumption. Therefore, natural remediation techniques have been developed to provide more environmentally friendly and cost-effective cleanup of sites impacted by PAH spills [15].

Phytoremediation is an emerging green technology that uses plants to remediate soil, sediment, surface, and ground water contaminated with organic contaminates. This technique has been shown to be effective for PAH-contaminated soils in several laboratory and in field studies [16].

Biodegradation of polycyclic aromatic hydrocarbons that are associated with oil contamination and are recalcitrant to microbial degradation, can be promoted by rhizosphere effects of plants [16-18]. Although plants with highly branched fine fibrous root systems that have higher total rhizosphere volume have been reported to enhance biodegradation of organic contaminants more than plants with taproot systems, a fine fibrous root system, is not critical for phytoremediation [19].

The plant roots seem to provide an ideal environment for degradation of organic compounds as a result of several mechanisms. The plant root system allows rapid movement of water and gases through soil due to the improvement of soil structure. It also provides a biologically active soil region (i.e. the rhizosphere), which encourages microbial activity and enhances contaminant bioavailability [20, 21]. Hence, the use of plants and their associated microorganisms such as endophytes, is a promising green technology for remediation of contaminated soils. Endophytes are

Table 4. Degradation of PAHs in soils polluted with a mixture of PAHs and diesel fuel planted with meadow feascue (*Festuca pratensis*) inoculated (I+) and non-inoculated (I-) with *Azospirillum* spp. and *Pseudomonas stutzeri*. Data are mean ±standard deviation.

Compound	% of control					
	Chernozem		Calcareous rendzina		Lessives	
	I (-)	I (+)	I (-)	I (+)	I (-)	I (+)
Mixture 1						
naphthalene	37.8±1.2^{a}	24.8±2.2^{a}	61.9±1.8^{b}	29.2±0.5^{a}	90.8±2.1^{b}	71.4±1.2^{a}
acenaphthene	92.8±0.8^{b}	59.7±1.6^{b}	55.3±1.3^{a}	32.2±0.2^{a}	87.9±1.0^{b}	70.8±0.7^{a}
fluorene	90.6±1.5^{b}	56.1±1.8^{a}	52.4±0.8^{a}	26.7±0.8^{c}	90.5±1.0^{b}	61.8±0.5^{a}
phenanthrene	88.0±0.7^{b}	40.9±1.2^{a}	42.4±1.1^{a}	25.5±1.1^{c}	92.5±0.8^{b}	65.5±1.1^{a}
anthracene	78.5±1.2^{b}	38.3±2.2^{a}	35.2±0.2^{a}	15.4±0.8^{c}	94.1±1.2^{b}	67.9±0.7^{a}
fluoranthene	76.5±2.2^{b}	38.2±2.3^{a}	80.2±0.8^{b}	32.6±2.5^{a}	74.3±0.5^{b}	53.9±0.4^{a}
pyren	50.1±1.6^{a}	31.9±0.4^{c}	35.2±0.7^{a}	16.4±1.4^{c}	76.7±1.1^{b}	48.4±1.2^{a}
benz[a]anthracene	80.0±2.5^{b}	51.4±0.7^{a}	79.1±0.8^{b}	34.6±1.3^{c}	91.6±0.4^{b}	62.8±0.3^{a}
chrysene	94.7±1.2^{b}	53.1±1.6^{a}	56.4±1.1^{a}	25.4±1.1^{c}	81.5±0.3^{b}	52.4±0.6^{c}
benzo[b]fluoranthene	48.8±1.3^{a}	33.3±0.8^{a}	87.5±1.7^{b}	37.6±0.2^{a}	89.3±1.2^{b}	67.9±0.8^{a}
benzo[k]fluoranthene	72.9±1.9^{b}	32.4±1.4^{a}	78.7±0.7^{b}	42.5±0.4^{c}	85.7±1.2^{b}	50.4±0.4^{c}
benzo[a]pyrene	97.8±2.2^{b}	52.9±1.8^{a}	63.9±0.4^{b}	32.4±0.3^{a}	71.6±0.5^{a}	55.4±0.9^{c}
dibenz(a,h)anthracene	61.5±3.2^{a}	48.1±2.2^{a}	47.6±1.2^{a}	35.1±1.2^{a}	72.5±0.4^{b}	55.3±0.3^{c}
benzo[ghi]perylene	73.1±0.8^{b}	35.2±0.8^{a}	79.2±1.1^{b}	41.4±0.4^{a}	80.2±0.6^{b}	54.8±1.4^{a}
indeno(1,2,3-cd)pyrene	57.6±1.7^{a}	56.5±1.2^{a}	60.7±0.5^{b}	36.8±0.7^{a}	76.7±1.0^{b}	64.7±1.1^{a}
Mixture 2						
naphthalene	87.5±0.2^{b}	44.8±1.2^{a}	71.9±1.8^{b}	52.2±0.4^{a}	80.8±1.1^{b}	61.4±0.5^{a}
acenaphthene	96.8±0.4^{b}	69.7±1.0^{b}	65.3±1.3^{b}	42.2±0.5^{b}	97.9±1.4^{b}	50.8±0.7^{a}
fluorene	94.6±0.8^{b}	66.1±1.0^{a}	58.4±0.8^{b}	36.7±0.9^{c}	94.5±0.4^{b}	61.8±0.7^{a}
phenanthrene	82.0±0.7^{b}	50.9±0.6^{a}	62.4±1.1^{b}	45.5±1.4^{c}	96.5±0.8^{b}	65.5±1.1^{a}
anthracene	74.5±0.6^{b}	48.3±0.3^{a}	52.2±0.2^{b}	32.4±0.8^{c}	82.1±0.4^{b}	57.9±1.2^{a}
fluoranthene	66.5±1.2^{b}	58.2±1.3^{a}	62.2±0.8^{b}	42.6±1.5^{a}	84.3±0.7^{b}	43.9±0.4^{a}
pyren	68.1±1.0^{a}	51.9±0.6^{c}	48.2±0.7^{a}	31.4±1.1^{c}	76.7±1.0^{b}	48.4±0.8^{a}
benz[a]anthracene	83.0±0.5^{b}	61.4±0.6^{a}	69.1±0.9^{b}	24.6±1.0^{c}	71.6±1.4^{b}	62.8±0.3^{a}
chrysene	84.7±1.0^{b}	43.1±1.1^{a}	62.4±1.1^{b}	35.4±1.5^{c}	71.5±0.5^{b}	62.4±0.6^{c}
benzo[b]fluoranthene	78.8±1.3^{a}	53.3±0.8^{a}	72.5±1.4^{b}	47.6±0.8^{a}	89.3±0.8^{b}	57.9±0.6^{a}
benzo[k]fluoranthene	82.9±1.2^{b}	62.4±1.0^{a}	69.7±0.8^{b}	52.5±1.4^{c}	75.7±0.7^{b}	40.4±0.4^{c}
benzo[a]pyrene	91.8±0.8^{b}	72.9±1.1^{a}	58.9±0.4^{b}	32.4±1.3^{a}	81.6±0.5^{b}	55.4±0.5^{c}
dibenz(a,h)anthracene	75.5±1.2^{a}	58.1±1.0^{a}	68.6±1.2^{a}	45.1±1.5^{a}	82.5±0.8^{b}	65.3±0.9^{c}
benzo[ghi]perylene	76.1±0.5^{b}	65.2±0.5^{a}	71.2±1.1^{b}	51.4±0.8^{a}	70.2±0.9^{b}	64.8±1.0^{a}
indeno(1,2,3-cd)pyrene	82.6±1.4^{a}	66.5±1.2^{a}	58.7±0.5^{b}	46.8±1.0^{a}	71.7±1.1^{b}	44.7±0.4^{a}
Mixture 3						
naphthalene	97.5±0.2^{b}	75.8±1.2^{a}	81.9±1.8^{b}	52.2±0.6^{a}	88.8±1.4^{b}	64.4±0.5^{a}
acenaphthene	98.8±0.4^{b}	65.7±1.0^{b}	75.3±1.0^{b}	42.2±0.7^{b}	87.9±1.2^{b}	60.8±0.5^{a}
fluorene	94.6±0.8^{b}	76.1±1.0^{a}	68.4±0.9^{b}	36.7±0.5^{c}	96.5±0.4^{b}	51.8±0.7^{a}
phenanthrene	92.0±0.7^{b}	60.9±0.6^{a}	72.4±1.1^{b}	55.5±1.0^{c}	96.5±0.5^{b}	55.5±1.0^{a}

Table 4. Continued.

Compound	% of control					
	Chernozem		Calcareous rendzina		Lessives	
	I (-)	I (+)	I (-)	I (+)	I (-)	I (+)
Mixture 3						
anthracene	84.5±0.6^{b}	58.3±0.3^{a}	62.2±0.2^{b}	42.4±0.8^{c}	92.1±0.4^{b}	67.9±1.0^{a}
fluoranthene	86.5±1.2^{b}	58.2±1.3^{a}	62.2±0.8^{b}	42.6±1.5^{a}	84.3±0.7^{b}	73.9±0.4^{a}
pyren	78.1±1.0^{a}	61.9±0.6^{c}	88.2±0.8^{a}	31.4±1.1^{c}	96.7±1.0^{b}	78.4±0.8^{a}
benz[a]anthracene	93.0±0.5^{b}	41.4±0.6^{a}	59.1±0.9^{b}	34.6±1.0^{c}	91.6±1.0^{b}	62.8±0.5^{a}
chrysene	94.7±1.0^{b}	53.1±1.1^{a}	62.4±1.1^{b}	35.4±1.5^{c}	91.5±0.5^{b}	62.4±0.6^{c}
benzo[b]fluoranthene	88.8±1.3^{a}	63.3±0.8^{a}	62.5±1.0^{b}	37.6±0.3^{a}	89.3±0.8^{b}	77.9±0.6^{a}
benzo[k]fluoranthene	83.9±1.2^{b}	72.4±1.0^{a}	59.7±0.5^{b}	32.5±1.0^{c}	95.7±0.7^{b}	70.4±0.5^{c}
benzo[a]pyrene	91.8±0.8^{b}	82.9±1.1^{a}	48.9±0.4^{b}	32.4±1.3^{a}	81.6±0.5^{b}	75.4±0.5^{c}
dibenz(a,h)anthracene	95.5±1.2^{a}	68.1±1.0^{a}	58.6±1.0^{a}	35.1±1.0^{a}	82.5±0.8^{b}	65.3±0.9^{c}
benzo[ghi]perylene	96.1±0.5^{b}	55.2±0.5^{a}	61.2±1.1^{b}	31.4±0.8^{a}	90.2±0.6^{b}	64.8±1.0^{a}
indeno(1,2,3-cd)pyrene	92.6±1.4^{a}	56.5±1.2^{a}	58.7±0.7^{b}	36.8±1.0^{a}	91.7±1.1^{b}	64.7±0.4^{a}

Different letters (a, b, c) in each row represent significant differences with Tukey test ($P<0.05$).

a group of bacteria or fungi that live asymptomatically within a plant and may increase host plant tolerance to biotic and abiotic stresses [11, 22]. During phytoremediation of organic contaminants, plants can further benefit from endophytes possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants. Although phytoremediation of organic contaminated soils using endophytic bacteria have been the subject of several studies [1, 6, 7, 19-21], there is little information about the effect of infected plants with endophytic bacteria on PAHs and diesel fuel-contaminated soils. Therefore, we hypothesized that endophyte inoculation of plants may enhance phytoremediation efficiency of PAHs and diesel fuel-contaminated soils in comparison to non-inoculated plants.

The presented studies were conducted in order to obtain an answer to the question on the possibility of using bacteria strains *Azospirillum* spp. and *Pseudomonas stutzeri* in the bioremediation process, and at the same time to supplement missing data in this field of science. The positive effect of bacteria *Azospirillum* spp. and *Pseudomonas stutzeri* on PAH degradation was found in soils freshly polluted with a mixture of PAHs and diesel fuel.

Phytoremediation occurs most intensely in the rhizosphere, so the depth to which the roots grow is one of the most important factors that limit the process [23]. 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 [3, 16, 24-26]. Good results are given also by legumes, which may be related to root secretions rich in nitrogen compounds [27].

Meadow feascue, thanks to a well-developed and dense root system, became proper habitat for the endophitic bacteria applied in the inoculation capable of using PAHs as the only source of carbon and energy. A statistically significant ($P\leq0.05$) 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.

In the research by Pizzul et al. [28], about 66% phenanthrene degradation was obtained, as well as more than 90% degradation degree of anthracene and pyrene in the soil polluted with these compounds (dose 50 mg·kg^{-1}) during 49 days after soil inoculation with bacteria *Rhodococcus wratislaviensis* with the addition of 1% rapeseed oil to the soil. In the present studies, with a significantly higher soil pollution (1000 mg·kg^{-1}) at non-inoculated and inoculated plants growth in calcareous rendzina, the following results were obtained: a decrease in the anthracene content in the soil from 95% with no plant inoculation to 42% with inoculation, phenanthrene from 72% to 36%, and pyrene from 58 to 27%. On the other hand, Huang et al. [16] during a four-month-long naturally occurring soil bioremediation at *Festuca arabinacea* growth with the participation of rhizosphere microflora noted around 45% decrease in the content of 15 PAH sum in soil naturally polluted with crude oil derivatives. Liste and Felgentreu [17] 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.

Significant for the bioremediation of soils polluted with PAHs are sorption processes [29]. Plants are capable – through the root system – of absorbing various organic compounds, depending on their relative lipophilicity [9, 15]. Compounds uptaken by the plant may accumulate in the roots or become permanently built into its structure, for example lignin, which is an example of pollution phytostabilization [6, 7, 22]. 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 is not advantageous to the environment because it causes atmospheric pollution. Moreover, the presence of plants in the soil intensifies humification [2, 30], as the organic compounds of the pollutant are built into humus components. When they are immobilized in such a way they 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 [1, 20, 31-33].

In the root area of plants, an increased bioremediation rate of organic pollutants is observed in comparison with non-rhizospheric soil [34-37]. This is related first of all to the metabolic activity of microflora, which populates the rhizosphere in great numbers. It turns out that of significant importance are also microorganisms directly connected with the plants that live inside roots, stems, and leaf tissues [38, 39]. Examples of such microorganisms are the strains *Azospirillum* spp. and *Pseudomonas stutzeri*. *Rhizobacteria* from genus *Azospirillum*, which fix free nitrogen and which are classified as optional endophytes capable of colonizing both the external surface of the root and the inside intracellular space, favorably affecting plant growth and development [11, 24-26].

The conducted studies make it possible to preliminarily positively evaluate the effect of plant inoculation with *Azospirillum* spp. and *Pseudomonas stutzeri* against PAH degradation in soils naturally and artificially polluted with these compounds. The effect was observed in all the studied soils newly polluted with PAHs, particularly in calcareous rendzina and chernozem, but also in lessives, slightly acid, with poor physical properties, as well as in brown aged soil polluted with crude oil.

Conclusions

1. The application of meadow feascue inoculation with *Azospirillum* spp. and *Pseudomonas stutzeri* had a positive effect on the degradation processes of polycyclic aromatic hydrocarbons in soils freshly polluted with a mixture of PAHs and diesel fuel.
2. A statistically significant PAH degradation degree was found in calcareous rendzina and chernozem freshly polluted with a mixture of PAHs and diesel fuel.
3. The ability of strains *Azospirillum* spp. and *Pseudomonas stutzeri*, which populate the root area and the interior of grass roots, to fix nitrogen and to use aromatic hydrocarbons as the only source of carbon and energy suggest a potential possibility to use these strains for the bioremediation of soils polluted with PAHs at limited habitat supplementation with nitrogen fertilizers.

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