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Degradation of Polycyclic Aromatic Hydrocarbons by Co-Culture of *Pleurotus ostreatus* Florida and *Azospirillum brasilense*

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Abstract: Bacterial-fungal interactions are important in the functioning of natural ecosystems. We examined possible synergistic or antagonistic effects during the degradation of polycyclic aromatic hydrocarbons (PAHs) by a fungal–bacterial co-culture. Bacteria and fungi were grown in a liquid nutrient medium supplemented with PAH substrates. The degradation of PAHs and the identification of metabolites were checked by HPLC. Enzyme activities were spectrophotometrically measured with test substrates. Compared to monocultures, the co-culture yielded higher mycelium dry weights and higher numbers of bacterial colony-forming units (CFUs). Both organisms and their co-culture transformed three- and four-ring PAHs into the corresponding quinones. The degradation of PAHs was accompanied by the production of fungal extracellular laccase and versatile peroxidase, whose activities were higher in the co-culture than they were in the monocultures. The presence of exogenous indole-3-acetic acid (IAA) boosted PAH degradation and enzyme production. The xylotrophic basidiomycete *Pleurotus ostreatus* Florida and the plant-growth-promoting rhizobacterium *Azospirillum brasilense* exerted a positive mutual effect, including increases in mycelium dry weight, number of CFUs, degradation of PAHs, and production of fungal extracellular enzymes. IAA may be a factor in the interactions of *P. ostreatus* Florida with *A. brasilense*.

Keywords: *Pleurotus ostreatus* Florida; *Azospirillum brasilense*; bacterial-fungal interactions; polycyclic aromatic hydrocarbons; degradation; laccase; versatile peroxidase; indole-3-acetic acid

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1. Introduction

Fungi and bacteria coexist in a variety of environments, from soil and food to plants and animals. Bacterial-fungal interactions are important for habitat colonization and for survival and pathogenesis [1], and they are an essential component of microbial communities in terrestrial ecosystems. In the past decade, research attention to these interactions has increased tremendously. Information is available on the mechanisms and functional responses of partners in a wide range of such interactions [2].

Bacteria, which move by flagella along the continuous liquid film on the surface of fungal hyphae [3], can use hyphae as pathways to reach inaccessible habitats [4]. Unlike bacteria, fungi can easily spread in water-deficient environments, facilitating the access of pollutant-degrading bacteria to pollutants [5]. In addition, fungal hyphae may promote the attachment of bacteria to plant roots, facilitating their entry into the rhizosphere or tissues [6]. Bacteria and fungi can form a mixed biofilm on the surface of plants, which protects them from adverse environmental conditions [7].

Most research has focused on bacterial–fungal interactions under controlled conditions [2]. How relevant this research is to the importance of bacterial–fungal interactions for the functioning of natural microbial communities in actual ecosystems is still an open question. Most often, in vitro models have explored bacterial–fungal interactions related to

food microbiology, soil ecology, bacterial control of pathogenic fungi, and mycorrhiza formation [1,8].

Studies on bacterial–fungal interactions during the degradation of natural substrates and xenobiotics are few. There exists a niche differentiation between saprotrophic fungi and bacteria. Fungi mainly degrade hard lignocellulosic materials (such as wood debris), whereas bacteria mainly utilize easy-to-degrade soluble organic compounds (such as sugars). This is evidence for a relationship between the degrading activities of fungi and bacteria [2].

Some authors believe that indole-3-acetic acid (IAA), which is a phytohormone of the auxin class, can also be a universal physiological code not only for the plants but also for bacteria and fungi. Increasing evidence shows IAA as a diffusible signal that is used for interspecies communications. This phenomenon suggests the existence of a framework, widely evolved in both eukaryotes and prokaryotes, allowing the production, transfer, and perception of IAA signals between distantly related organisms across the branches of phylogenetically diverse groups of the tree of life [9]. The production of IAA by bacteria is well-studied. Although, IAA-producing fungi are known [10], their biosynthetic pathways and role in fungal ecology have not been widely studied.

The interaction of soil bacteria with fungi is very important for the degradation of various man-made pollutants, as well as for the metabolism of natural substrates, such as cellulose, hemicellulose, and lignin. Cooperation and antagonism have been described between bacterial microflora and fungi during the degradation and mineralization of pollutants. The antagonistic interactions between soil bacteria and fungi may be caused by competition for an available carbon source [11]. In the degradation of some pollutants, fungi act synergistically with bacteria [7,12,13]. In such a case, the fungal and bacterial degradation pathways may complement each other. The cometabolic degradation leads to complete pollutant utilization, because metabolites formed by one organism are metabolized by the other. Co-cultures of bacteria and fungi have been used in the bioremediation of soils polluted by polycyclic aromatic hydrocarbons (PAHs) [14–17]. Using a bacterial-fungal culture, Boonchan et al. [18] observed a decrease in the mutagenicity of soil organic extracts, as well as an increase in the soil degradation of PAHs, as compared to the data for the native microflora and for soil inoculated with separate cultures. Similarly, soil remediation with co-cultures of *Rhodococcus* sp. and *Aspergillus terreus* or *Penicillium* sp. resulted in decreased soil toxicity and complete degradation of anthracene, phenanthrene, and pyrene [19]. This synergism may be related to the ability of fungi to transform PAHs into polar compounds that are utilized by the soil microflora. This polarity reversal leads to pollutant redistribution between the soil and water phases, which also makes polar products more accessible to bacteria [20,21].

As a promising option, fungal enzymes are regarded as a powerful choice for the degradation of PAHs. Ligninolytic fungi have been extensively studied because they produce extracellular enzymes with extremely reduced substrate specificity. The ligninolytic system contains three peroxidases (lignin peroxidase, Mn-peroxidase, and versatile peroxidase) and laccase. The involvement of these enzymes in the degradation of PAHs was shown many studies. The first attack of the molecule of PAH resulted in the formation of corresponding quinones. In addition to the mentioned other fungal enzymes, such as cytochrome P450 monooxygenase and epoxide hydrolase, some dioxygenases participate in PAH degradation. Finally, the fungal degradation of PAHs resulted in the mineralization of parent compounds [22]. More often in bacterial degradation pathways, the PAHs are activated by directly incorporating molecular oxygen with the help of mono- or dioxygenases [23]. Usually, the first step in the aerobic bacterial degradation of PAHs is the hydroxylation of an aromatic ring via a dioxygenase, with the formation of a *cis*-dihydrodiol, which gets rearomatized to a diol intermediate by the action of a dehydrogenase. These diol intermediates may then be cleaved by intradiol or extradiol ring-cleavage dioxygenases, leading to intermediates that can be involved in the TCA cycle [24].

Wood-inhabiting basidiomycetes of the genus *Pleurotus* belong to ligninolytic fungi, which are some of the most active degraders of lignin in nature. They can degrade a wide range of persistent aromatics and produce a complex of extracellular nonspecific enzymes including Mn-peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16), and laccases (EC 1.10.3.2). The ability of *Pleurotus* fungi to survive in soil, compete with the indigenous microflora, and degrade soil pollutants makes them promising candidates for mycoremediation [25].

Bacteria belonging to the genus *Azospirillum* are free-living microbes that promote plant growth (PGPR). The most accepted theory regarding the mechanism of action of *Azospirillum* is its growth promotion, which includes nitrogen fixation and phytohormone (including IAA), polyamine, and trehalose production. There are reports on using *Azospirillum* strains in the pollutant (phenol, benzoate, protocatechuate, catechol, phenanthrene, and crude oil) degradation process [26,27]. The production of laccase and ligninolytic peroxidases, such as fungi, by *Azospirillum* are also known [28,29].

The study of the interaction mechanisms operating in bacterial–fungal systems is an integral part of research into the functioning of natural ecosystems in polluted settings and is necessary for the design of bioremediation technologies. Here, we sought to identify possible synergistic or antagonistic effects during PAH degradation by a co-culture of the basidiomycete *Pleurotus ostreatus* and the rhizobacterium *Azospirillum brasilense*.

2. Materials and Methods

P. ostreatus f. Florida (IBPPM 540) and *A. brasilense* SR80 (IBPPM 24) were obtained from the IBPPM RAS Collection of Rhizosphere Microorganisms (http://collection.ibppm.ru, accessed on 23 July 2022).

The fungus was grown on a modified basidiomycetes-rich medium [30] composed as follows (g/L): NH_4NO_3, 0.724; KH_2PO_4, 1.0; $MgSO_4 \times 7H_2O$, 1.0; KCl, 0.5; yeast extract, 0.5; $FeSO_4 \times 7H_2O$, 0.01; $ZnSO_4 \times 7H_2O$, 0.0028; $CaCl_2 \times 2H_2O$, 0.033; glucose, 10.0; peptone, 10.0; agar, 15.0; (pH 6.0). The growth of *P. ostreatus* mycelium was controlled by the weight method [31].

Bacteria were grown on R2A medium [32] composed as follows (g/L): yeast extract, 0.5; peptone, 0.5; casein hydrolysate, 0.5; glucose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; K_2HPO_4, 0.3; $MgSO_4 \times 7H_2O$, 0.05; agar, 15.0. Growth was controlled by direct plating of multiple dilutions of the culture medium on R2A agar and by counting the CFUs.

Phenanthrene (∼90%), pyrene (∼90%), anthracene (≥95%), fluoranthene (≥97%), fluorene (≥99%), and indole-3-acetic acid (IAA; Pestanal®, analytical standard) were all purchased from Fluka (Buchs, Switzerland).

For degradation studies, we used a medium of the following composition (g/L): glucose, 4.5; fructose, 4.5; L-asparagine, 1.5 [33]. A mixture of PAHs (anthracene, phenanthrene, fluorene, pyrene, and fluoranthene; 1 mg of each) was added to a final total PAH concentration of 5 mg per 100 mL. *P. ostreatus* (20 mg of dry weight/about 200 mg of wet weight) and *A. brasilense* (3×10^{-5} CFUs), both singly and in combination, were grown at 24, 30, or 37 °C in 0.3-L Erlenmeyer flasks on a shaker New Brunswick™ Excella®E24/E24R Shaker (Eppendorf, Nürtingen, Germany) at 130 rpm. At certain time intervals, an aliquot (2 mL) was taken from the flasks and the activity of extracellular enzymes and the concentration of IAA were determined as described below.

After 14 days, the contents of the flasks were extracted three times with 5 mL of chloroform, and the extracts were evaporated. The sorption of PAHs by fungal mycelium did not exceed 5–7%. PAH content was analyzed by HPLC on an Agilent Technologies 1220 Infinity II LC chromatograph (Agilent Technology, Waldbronn, Germany) equipped with a 4.6 × 150-mm ZORBAX Eclipse PAH 5-Micron column and a 254-nm UV detector. The solvent system was H_2O:acetonitrile, the linear gradient was 40–100% acetonitrile, and the time was 15 min. The PAHs and their metabolites were analyzed by comparing the retention times with those of standard compounds.

IAA content was analyzed by HPLC on an Agilent Technologies 1220 Infinity II LC chromatograph (Agilent Technology, Waldbronn, Germany) equipped with a 4.6 × 50-mm ZORBAX Eclipse Plus C18 5-Micron column and a 254-nm UV detector. The solvent system was H_2O (acidified with H_3PO_4 to pH 2.5):acetonitrile, the linear gradient was 10–90% acetonitrile, and the time was 15 min.

Laccase activity was measured by the oxidation rate for 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) at 436 nm (ε = 29,300 $M^{-1}cm^{-1}$), according to Niku-Paavola et al. [34]. Versatile peroxidase was measured by the oxidation rate for 2,6-dimethoxyphenol (DMP) with H_2O_2 and Mn^{2+} at 468 nm (ε = 14,800 $M^{-1}cm^{-1}$), according to Martinez et al. [35]. One unit of enzyme activity was defined as the amount required to catalyze the formation of 1 µmol of product per minute and is expressed in arbitrary units (µmol/min/mL of enzyme preparation (U/mL)).

In separate experiments the effect of IAA on PAH degradation and extracellular enzyme production was investigated by supplementing the culture medium with IAA to a final concentration of 1 mg/100 mL. At certain time intervals, an aliquot (2 mL) was taken from the flasks and the activity of extracellular enzymes and the concentration of IAA were determined too.

All experimental treatments and all analyses were repeated at least three times. Each experiment was repeated at least three times. Results were analyzed with Excel 2003 (Microsoft Office 2003, USA) and Origin 7 software.

3. Results

3.1. Degradation of the PAH Mixture

Before *A. brasilense* SR80 and *P. ostreatus* Florida were co-cultured in the liquid medium, their antagonistic activity on R2A agar was investigated by the double-culture method [36,37]. The results showed an absence of mutual antagonism (data not shown). The optimal temperatures to grow the organisms were 37 °C (*A. brasilense* SR80), 24 °C (*P. ostreatus* Florida), and 30 °C (co-culture).

At 24 and 30 °C, co-culturing of *P. ostreatus* Florida and *A. brasilense* SR80 in liquid medium increased the dry weight of the fungal mycelium and the bacterial CFU number (Table 1). Increasing the temperature to 37 °C inhibited mycelium growth.

Table 1. Effect of PAHs and co-culture on growth variables of *P. ostreatus* and *A. brasilense*.

Culture	Bacterial Count (CFU × 10^7/mL)			Mycelium Dry Weight (mg)		
	24 °C	30 °C	37 °C	24 °C	30 °C	37 °C
A. brasilense	1.4 ± 0.4	2.0 ± 0.4	14.6 ± 1.9	-	-	-
A. brasilense + PAHs	1.0 ± 0.4	0.2 ± 0.1	10.8 ± 0.9	-	-	-
P. ostreatus	-	-	-	212.4 ± 12.9	353.5 ± 18.3	9.2 ± 2.7
P. ostreatus + PAHs	-	-	-	154.5 ± 8.8	295.0 ± 30.1	22.7 ± 4.3
P. ostreatus + *A. brasilense*	0.5 ± 0.0	3.2 ± 0.2	109.0 ± 8.8	270.5 ± 12.1	641.4 ± 27.0	40.3 ± 7.5
P. ostreatus + *A. brasilense* + PAHs	6.1 ± 0.9	40.5 ± 7.4	83.0 ± 2.8	153.8 ± 15.0	73.4 ± 10.1	13.4 ± 2.7

The presence of the PAH mixture significantly reduced mycelium dry weight, as compared to the control, possibly as the result of inhibition of fungal growth by the PAHs and/or their metabolites. The CFU number in *A. brasilense* SR80 also decreased in the presence of the PAHs but increased in the dual culture (Table 1). It may be that, in this case, fungal metabolites of PAHs may be an additional source of carbon for the bacteria. This hypothesis will be the subject of further research.

During the disappearance of PAHs, we evaluated the activity of extracellular oxidative enzymes. *A. brasilense* SR80 did not produce extracellular laccase or peroxidase; by contrast, *P. ostreatus* Florida did produce laccase and versatile peroxidase in all treatments. At 30 and 37 °C, the activities of both enzymes were significantly lower than they were at 24 °C. The PAHs and/or their metabolites induced the production of versatile peroxidase. Laccase and versatile peroxidase activities were the highest in the co-culture (Table 2).

Table 2. Effect of growth conditions on activities of *P. ostreatus* extracellular enzymes.

Culture	Laccase Activity (U/mL)			Versatile Peroxidase Activity (U/mL)		
	24 °C	30 °C	37 °C	24 °C	30 °C	37 °C
P. ostreatus	2.4 ± 0.6	0.7 ± 0.1	0.2 ± 0.03	1.8 ± 0.4	0.1 ± 0.02	0.1 ± 0.01
P. ostreatus + PAHs	3.3 ± 0.8	0.2 ± 0.05	0.06 ± 0.01	4.1 ± 0.5	3.7 ± 0.8	0.6 ± 0.02
P. ostreatus + *A. brasilense*	3.1 ± 0.7	1.8 ± 0.3	0.12 ± 0.03	3.0 ± 0.8	0.1 ± 0.01	0.06 ± 0.01
P. ostreatus + *A. brasilense* + PAHs	8.9 ± 1.0	2.2 ± 0.9	0.36 ± 0.08	7.1 ± 1.2	2.5 ± 0.5	0.3 ± 0.04

Both organisms, alone and in combination, utilized the PAHs. Fluorene was the most accessible of all PAHs used, because it is relatively well-soluble (0.39–1.98 mg/L) and has a low ionization potential (7.88–8.2 eV) [38]. Phenanthrene (at 30 °C), fluoranthene, and pyrene were hard to use by bacteria (Figure 1); by contrast, all PAHs were available for fungal utilization. At an elevated temperature, however, only phenanthrene was subject to fungal utilization, and fluorene and phenanthrene were subject to bacterial utilization. This may have been the result of poor fungal growth at high temperatures. Although *A. brasilense* SR80 grew well, it degraded the PAHs to a worse degree at 37 °C than it did at 24 and 30 °C.

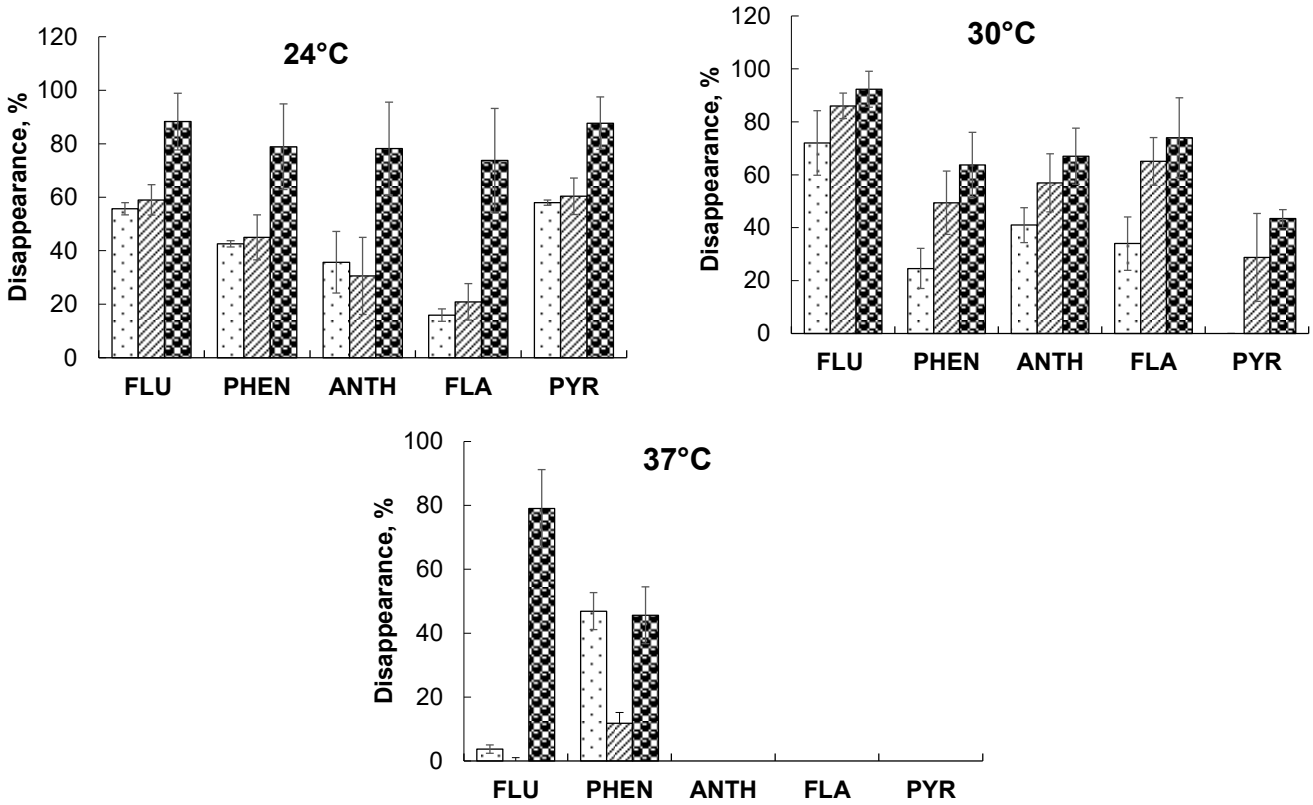

Figure 1. PAH degradation by *A. brasilense* SR80 (⁘), *P. ostreatus* Florida (▨), and *A. brasilense* + *P. ostreatus* (▩) at different temperatures: FLU—fluorene, PHEN—phenanthrene, ANTH—anthracene, FLA—fluoranthene, PYR—pyrene.

When the co-culture was used, synergism was noted in the utilization of anthracene, fluoranthene (at 24 °C), pyrene (at 30 °C), and fluorene (at 37 °C). Pyrene, inaccessible to *A. brasilense* SR80 at 30 °C, was metabolized by 43%, and fluorene, inaccessible to *P. ostreatus* at 37 °C, was metabolized by 78% (Figure 1).

The fungal degradation products of fluorene (9-fluorenone), phenanthrene (9,10-phenanthrenquinone), and anthracene (9,10-anthraquinone) were identified by HPLC. The product of the bacterial degradation of fluorene was 9-fluorenone. The same quinone metabolites were detected during PAH degradation by the co-culture.

3.2. Degradation of Fluorene and Its Main Metabolite, 9-Fluorenone

According to the obtained data, fluorene and its degradation product, 9-fluorenone, were chosen for further study of the metabolism by co-cultures. The chosen temperature of 30 °C was optimal for the growth of both cultures. PAH degradation by the fungus and the co-culture was accompanied by yellow coloration of the growth medium, which may indicate the formation of quinone metabolites [39].

The presence of fluorene or 9-fluorenone in the growth medium decreased the mycelium dry weight, as compared to the PAH-free control (Table 1). The co-culturing of *P. ostreatus* Florida and *A. brasilense* SR80 increased the mycelium dry weight (Table 3). The CFU value of *A. brasilense* SR80 alone also decreased in the presence of 9-fluorenone, but it increased by many times in the co-culture.

Table 3. Growth and enzymatic activity of *P. ostreatus* Florida and *A. brasilense* SR80 monocultures and the co-culture during biodegradation of fluorene and 9-fluorenone.

Culture	Growth				Enzymatic Activity (U/mL)			
	Bacterial Count (CFU × 10^7/mL)		Mycelium Dry Weight (mg)		Laccase		Versatile Peroxidase	
	FLU [1]	FLUQ [2]	FLU	FLUQ	FLU	FLUQ	FLU	FLUQ
A. brasilense	0.06 ± 0.01	0.02 ± 0.004	-	-	0	0	0	0
P. ostreatus	-	-	309.9 ± 12.1	303.8 ± 17.0	1.90 ± 0.4	1.12 ± 0.3	0.83 ± 0.01	0.67 ± 0.07
P. ostreatus + *A. brasilense*	1.8 ± 0.03	1.3 ± 0.04	318.0 ± 26.0	350.6 ± 15.8	2.61 ± 0.7	1.35 ± 0.3	1.13 ± 0.3	0.92 ± 0.09

[1] FLU—fluorene, [2] FLUQ—9-fluorenone.

We also evaluated the activity of extracellular oxidative enzymes during the degradation of fluorene and 9-fluorenone (Table 3). *A. brasilense* SR80 was not found to produce extracellular laccase or peroxidase. The production of laccase and versatile peroxidase by *P. ostreatus* Florida was lower in the presence of 9-fluorenone than in the presence of fluorene. This was expected, because the inhibition of these enzymes by PAH quinones is well-known. In the co-culture, the activities of both enzymes were higher than those in the fungus alone.

HPLC showed that fluorene and its fungal degradation intermediate 9-fluorenone were metabolized by both organisms alone and in combination (Figure 2). The degradation of fluorene by *A. brasilense* SR80 was as high as 74%, whereas that of 9-fluorenone was 40%.

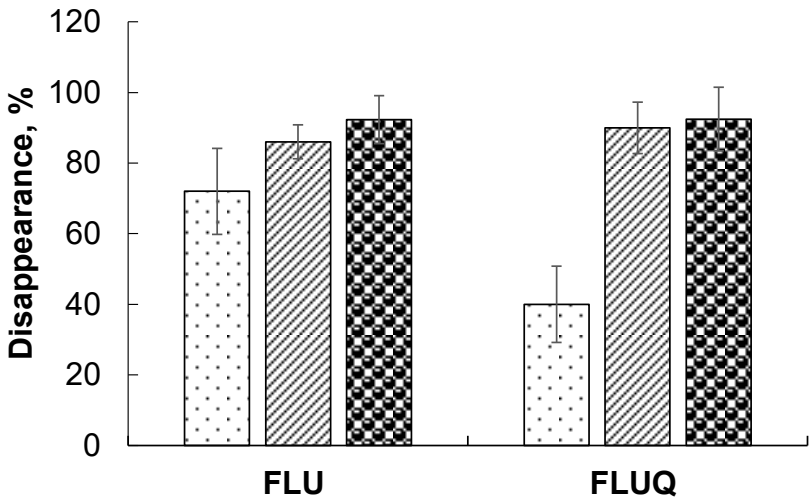

Figure 2. Degradation of fluorene and 9-fluorenone by *A. brasilense* SR80 (⬚), *P. ostreatus* Florida (▨), and *A. brasilense* + *P. ostreatus* (▩).

Comparison of the retention times (RTs) of the identified metabolites with those of standard compounds showed that *P. ostreatus* Florida metabolized fluorene to 9-fluorenone

and two unidentified metabolites with RTs of 0.817 and 2.668 min. In addition, some compounds with RTs of 1.304 and 12.375 min were detected in the co-culture (Figure S1).

The degradation 9-fluorenone by *P. ostreatus* Florida yielded two unidentified metabolites with RTs of 0.817 and 2.668 min. The same metabolites were detected after 9-fluorenone was degraded by the co-culture (Figure S2).

Thus, *P. ostreatus* Florida, separately and in combination with *A. brasilense* SR80, metabolized fluorene to provide 9-fluorenone and several unidentified metabolites, two of which, with RTs of 0.817 and 2.668 min, were detected after 9-fluorenone utilization and, apparently, are products of deeper degradation of the starting compounds.

3.3. Effect of IAA on Fungal Growth, Enzyme Activity, and PAH Degradation

The accelerated mycelium growth and the consequent increase in fungal biomass may have been the result of bacterial production of IAA.

We examined the effect of exogenous IAA on extracellular enzyme production and PAH degradation by the *P. ostreatus* component of the co-culture. The addition of exogenous IAA into the growth medium did not significantly affect mycelium production by the fungus, regardless of the presence of PAHs. Two weeks into the experiment, the amounts of IAA were 0.23 µg/mL in the absence of PAHs and 0.13 µg/mL in the presence of PAHs.

The presence of IAA in the culture medium increased the degradation of all PAHs but fluorene by 24–41% (Figure 3).

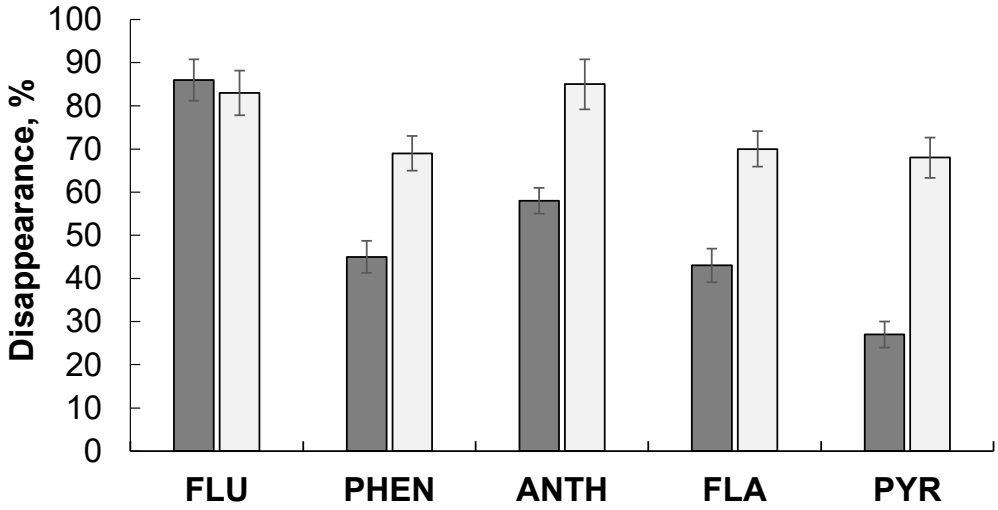

Figure 3. Effect of IAA on PAH degradation by *P. ostreatus* Florida: ▬—control (without IAA); □—with IAA.

A similar picture emerged for extracellular enzyme activity (Figure 4). IAA caused laccase activity to increase by about 1.5 times, regardless of the presence of PAHs in the culture medium.

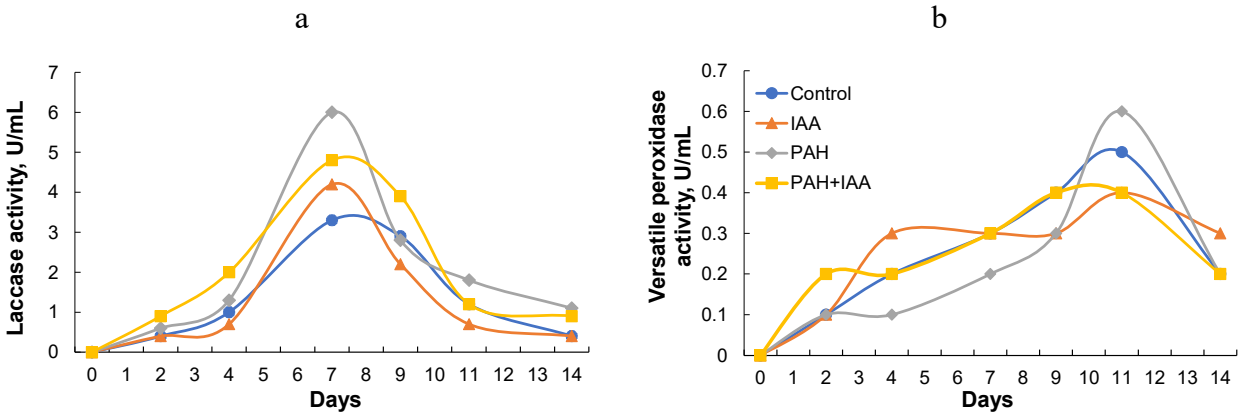

Figure 4. Effect of IAA on production of ligninolytic enzymes by *P. ostreatus* Florida: (**a**) laccase; (**b**) versatile peroxidase; ●—control (without PAHs or IAA); ▲—IAA; ♦—PAHs; ■—PAHs + IAA.

We also evaluated IAA production by *A. brasilense*, both as a monoculture and as a component of the co-culture (Figure 5).

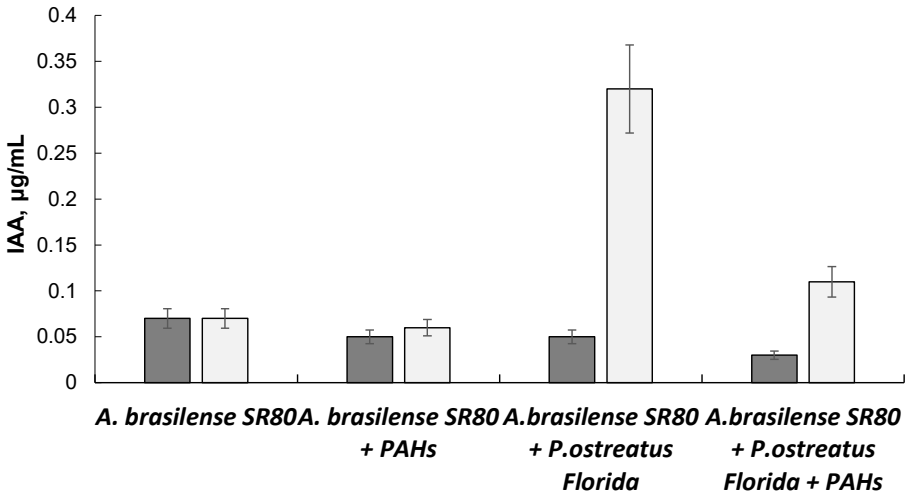

Figure 5. Effect of PAHs and *P. ostreatus* Florida on IAA production by *A. brasilense* SR80: ▬—7 days; ▭—14 days.

The concentration of IAA produced by *A. brasilense* alone was as high as 0.05–0.07 µg/mL and was barely affected by PAHs. A significant increase in IAA production (up to 0.32 µg/mL) was noted in the co-culture at the end of Week 2 of growth.

4. Discussion

In nature, fungi interact with other organisms (in particular, with rhizosphere bacteria), which may affect their degradative activity. *P. ostreatus* Florida was chosen as a model object for study because it is a potent degrader of a wide range of pollutants, including oil and PAHs [40], and because it can be used in mycoremediation [41]. Bacteria associated with *P. ostreatus* mycelium are mainly in the genera *Curvibacter*, *Pseudomonas*, *Bacillus*, *Cupriavidus*, *Pelomonas*, and *Propionbacterium* [42].

Azospirillum bacteria are well-known for their associative plant-growth-promoting characteristics [43,44] and have recently been proposed for use in environmental biotechnology [45,46]. The oil-oxidizing activity of *Azospirillum* was previously shown, with *A. brasilense* SR80 [26,47] as an example. In addition, strain SR80 was found to have ligninolytic activity, which manifested as the production of laccase and a number of peroxidases [29].

Plant-growth-promoting rhizobacteria (PGPR) are beneficial to mycorrhizal communities [48]. However, the co-culturing of *Azospirillum* with xylotrophic basidiomycetes that do not

form mycorrhizae was not reported before the pioneering research at the Microbiology Laboratory of our institute [33,49]. That research made a strong case for the use of bacterial-fungal cultures for the effective production of mycelium biomass and fruiting of basidiomycetes [33].

An interesting aspect of such co-cultures is their degradative activity toward various kinds of pollutants. There are few reports in this area and only one that deals with the effect of bacteria on the degradative activity of *P. ostreatus* [50]. The objective of the work [50], however, was to explore bacterial effects on the fungal degradation of the anthraquinone dye Remazol Brilliant Blue R. *Pseudomonas fluorescens* and *Bacillus licheniformis*, known for their antagonism to fungi, did not strongly affect the growth of mycelium or the degradative activity of *P. ostreatus* [50]. If, however, a PGPR and a degradative fungus are used simultaneously, the interaction between them is inevitable, which is what we have examined in our model system.

The co-culturing of *P. ostreatus* Florida with *A. brasilense* SR80 at 24 and 30 °C increased the dry weight of mycelium and the number of CFUs. A similar picture was observed by Tsivileva et al. [33], who showed a significant increase in fungal dry biomass in a co-culture of *P. ostreatus* and *A. brasilense* SR80. In our experiments, increasing the temperature further to 37 °C inhibited fungal growth.

The presence of PAHs and/or their metabolites in the culture medium inhibited the growth of *P. ostreatus* and *A. brasilense* SR80 as monocultures. However, the CFU value of *A. brasilense* SR80 increased in the co-culture, which may have resulted from the bacterial utilization of the fungal metabolites of the PAHs as an additional carbon source.

Both organisms degraded the PAHs under cometabolism conditions. It is well-known that ligninolytic fungi are degradative to PAHs [22]. By contrast, this study is the first to have found that *A. brasilense* can transform or degrade PAHs. Fluorene, which is relatively well-soluble and has a low ionization potential, was intensely utilized by both *P. ostreatus* and *A. brasilense* SR80. The co-culture acted synergistically, increasing the degradation of anthracene, fluoranthene, pyrene, and fluorene. At 30 °C, pyrene was unavailable to *A. brasilense* SR80 alone but was metabolized by 43% in the co-culture. In turn, fluorene was unavailable for fungal degradation at 37 °C but was metabolized by 78% in the co-culture. These data also indicate a positive interaction between the xylotrophic basidiomycete and the PGPR in a PAH-polluted setting.

PAH quinones (9-fluorenone, 9,10-phenanthrenquinone, and 9,10-anthraquinone), detected by HPLC as resulting from PAH degradation, were identical for both the fungal monoculture and co-culture. The formation of the corresponding quinones at the first stage of PAH degradation by ligninolytic fungi is well-known. The reactions of ligninolytic peroxidases and laccases lead to the formation of these products [22,24]. In our experiments, the formation of quinone metabolites in the fungal monoculture and co-culture with *A. brasilense* SR80 can also be the result of the catalytic action of laccase and versatile peroxidase produced by this fungus. 9-fluorenone revealed as a product of the bacterial degradation of fluorene can be an indirect confirmation of the participation of bacterial laccase in the degradation of this PAH. The study of metabolism of PAHs in *A. brasilense* SR80 and the involvement of laccases will be the subject of further research.

P. ostreatus Florida, singly and as combined with *A. brasilense* SR80, metabolized not only fluorene but also its metabolite, 9-fluorenone, yielding a number of unidentified metabolites, two of which (RTs 0.817 and 2.668 min) were apparently products of deeper degradation of the starting compounds. They appeared on a later day of cultivation and disappeared with an increase in cultivation time. In the presence of 9-fluorenone, the CFU number of *A. brasilense* SR80 in the co-culture increased many times, indicating utilization of the formed metabolites. One from the key metabolites of PAH degradation by fungi is phthalic acid, which, through a number of stages, is included in the main metabolism [22,24]. However, the retention times of the metabolites, revealed in our experiments, do not correspond to phthalic acid. At this stage of the study, we were unable to identify these compounds. Their identification will be the subject of further research.

The formation of PAH quinones is associated with the fungal production of ligninolytic enzymes (laccase, Mn peroxidase, versatile peroxidase, and lignin peroxidase) [22]. *A. brasilense* SR80, too, is a producer of ligninolytic enzymes [29]. This may suggest that *P. ostreatus* and *A. brasilense* use similar mechanisms to initially attack PAH molecules. This hypothesis will be the subject of further research.

The ability of *Azospirillum* bacteria to metabolize aromatic compounds can be regarded as the need for adaptation to the rhizosphere, a habitat saturated with various organic substrates [51]. However, information on the metabolism of aromatic compounds (especially PAHs) by *Azospirillum* has not yet been systematized. A few authors have reported that *Azospirillum* can utilize benzoate, salicylate, gentisate, naphthalene, and some other aromatic substrates as carbon sources [52–54]. In *A. brasilense* and *A. lipoferum*, Chen et al. [52] detected the activities of catechol 2,3-dioxygenase (growth with benzoate and catechol) and protocatechuate 4,5-dioxygenase (growth with 4-hydroxybenzoate and protocatechuate), and in *A. lipoferum*, they also detected the activities of catechol 1,2-dioxygenase (growth with benzoate, catechol, and naphthalene) and protocatechuate 3,4-dioxygenase (growth with 4-hydroxybenzoate, protocatechuate, and 4-toluate). Cruz-Hernandez et al. [54] showed that *Azospirillum* growth with aromatic substrates is accompanied by the production of biosurfactants. In addition, bioinformatic analysis of the genome of *A. brasilense* strain CBG-497 led the authors to record 19 sequences encoding the degradation of aromatic compounds. Of these, 11 were associated with the metabolism of central aromatic intermediates and 5 were involved in peripheral catabolic pathways, whose function is associated with the degradation pathways for quinate, benzoate, salicylate, gentisate, and toluene [54]. Our studies of PAH degradation by *A. brasilense* SR80 show that this strain can degrade phenanthrene, fluorene, and anthracene, yielding the corresponding quinones (unpublished data). Full genome analysis of strain SR80 did not reveal any genetic determinants corresponding to the dioxygenase conversion of these PAHs, but laccase genes were found. The laccase activity of *A. brasilense* SR80 was described earlier [29]. Considering that quinones are the products of the primary attack on the PAH molecules by laccases, including bacterial ones [55], we believe that PAH conversion by *A. brasilense* SR80 is mediated by laccases and yields quinones.

Examination of extracellular laccase and peroxidase activities showed that, in all experimental treatments, *P. ostreatus* Florida produced laccase and versatile peroxidase. These extracellular enzymes, however, were not found in *A. brasilense* SR80. This may be because the SR80 enzymes are localized either intracellularly or on the cell wall surface, such as laccase from *A. lipoferum* as described by Diamantidis [28]. Laccase and versatile peroxidase activities were the highest in the co-culture. A similar increase in extracellular laccase production was found by Flores et al. [56], who co-cultured *P. ostreatus* with some species of *Trichoderma*. The authors suggested that the production of lytic enzymes by *Trichoderma* leads to the destruction of *P. ostreatus* cells, which, in turn, increases the amount of extracellular laccase. The increased production of extracellular enzymes in the co-culture may be explained in several ways: (1) *P. ostreatus* responded to the stress caused by the presence of *Azospirillum*. (2) The *Azospirillum* metabolites, including PAH degradation products, could induce these enzymes. (3) The lytic enzymes of *Azospirillum* destroyed the *P. ostreatus* cell wall and promoted enzyme release. All these assumptions require further research.

The accelerated growth of mycelium and, consequently, the increase in fungal biomass may have resulted from the bacterial production of IAA, as shown for dual cultures of *Pleurotus eryngii* and *Pseudomonas* sp. P7014 [57] and of *P. ostreatus* and *Bacillus cereus*, *Bacillus aryabhattai*, or *Acinetobacter pitti* [58]. Bacteria that promote the growth of higher fungi have recently been designated mushroom-growth-promoting bacteria (MGPB) [59]. Adding *Pseudomonas* sp. P7014 and its supernatant liquid to the growth medium of *P. eryngii* increased the mycelium growth rate by 1.6 times [8]. Analysis of ethyl acetate extracts of the culture liquid of this bacterium showed the presence of compounds that

promote fungal growth, including IAA. The IAA concentration needed to accelerate fungal growth is less than 10 nM [57].

In this study, the addition of exogenous IAA to the culture medium did not noticeably affect the growth of *P. ostreatus* mycelium, regardless of the presence of PAHs. However, it increased the degradation of all PAHs but fluorene by 24–41% and promoted the production of fungal extracellular laccase. In this case, apparently, IAA and/or the products of its utilization by *P. ostreatus* induced the laccase. In turn, the increased laccase production could lead to increased PAH degradation.

To test whether IAA affected growth, PAH degradation, and enzyme production, we determined its content in the *A. brasilense* monoculture and in the co-culture with *P. ostreatus*. The IAA concentration in the *A. brasilense* SR80 monoculture was low (0.05–0.07 µg/mL), although this strain produces up to 20–30 µg/mL of IAA in the Nfb medium supplemented with tryptophan [26]. As a rule, IAA production in *Azospirillum* is tryptophan-dependent, but a minor, tryptophan-independent IAA synthetic pathway is also present in these bacteria [60]. In this study, the medium did not contain exogenous tryptophan. Apparently, the synthesis of IAA in the *A. brasilense* SR80 monoculture proceeds by way of the minor pathway, which explains the low IAA concentration. In the co-culture (Week 2 of growth), IAA production increased significantly (up to 0.32 µg/mL). In this case, fungi may contribute to IAA synthesis, as shown for another *Pleurotus* member, *P. pulmonarius* [10].

5. Conclusions

Bacterial–fungal interactions in the presence of PAHs were investigated in a model system. A positive mutual influence was found between the xylotrophic basidiomycete *P. ostreatus* Florida and the PGPR *A. brasilense* SR80. This influence manifested as an increase in the production of fungal mycelium, the number of bacterial CFUs, the degradation of PAHs, and the production of fungal extracellular enzymes. The identified PAH quinones were the same for both fungal and co-cultures. The product of the bacterial degradation of fluorene was 9-fluorenone, in whose presence the CFUs of *A. brasilense* SR80 increased in the co-culture. This increase may indicate the utilization of the resulting metabolites. One factor of mutual influence between *P. ostreatus* Florida and *A. brasilense* SR80 is their produced IAA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol2040056/s1, Figure S1: Chromatograms of culture liquid extracts after fluorene degradation; Figure S2: Chromatograms of culture liquid extracts after 9-fluorenone degradation.

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