

Original Research

Degradation of a Model Mixture of PAHs by Bacterial–Fungal Co-Cultures

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Abstract

Background: Bacteria and fungi are the most important soil organisms owing to their abundance and the key roles they play in the functioning of ecosystems. We examined possible synergistic and antagonistic effects during the degradation of polycyclic aromatic hydrocarbons (PAHs) by co-cultures of ascomycetes and a plant-growth-promoting bacterium. **Methods:** Bacteria and fungi were grown in a liquid nutrient medium supplemented with PAHs. The PAH degradations and the identification of metabolites were checked by high-performance liquid chromatography (HPLC). Enzymatic activities were measured spectrophotometrically using test substrates. All experimental treatments were analyzed using Excel 2019 (Microsoft Office 2019, USA). **Results:** The model system included the plant-growth-promoting rhizobacterium (PGPR) *Azospirillum brasilense* and one of the following ascomycetes: *Fusarium oxysporum* (plant pathogen), *Talaromyces sayulitensis* (rhizospheric fungus), *Trichoderma viride* (plant-growth-promoting fungus, PGPF), and *Trichoderma harzianum* (PGPF). The notable results are: (1) synergistic effects consisted of more active utilization of the PAH mixture compared to individual compounds, while the PAH mixture was more actively degraded by co-cultures than monocultures; (2) three effects of mutual influence by the studied organisms were also revealed: depressing (*F. oxysporum* and *A. brasilense*), partially depressing (*T. sayulitensis* suppressed the growth of *A. brasilense* but increased the degradation of anthracene, pyrene, and fluoranthene), and positive effects (*A. brasilense* and *T. viride* or *T. harzianum*); (3) for the first time quinone metabolites of PAH degradation and extracellular oxidase and peroxidase were produced during PAH degradation by *T. sayulitensis*. **Conclusions:** The results of the study contribute to the understanding of bacterial–fungal interactions in polluted settings.

Keywords: ascomycetes; bacterial–fungal interactions; polycyclic aromatic hydrocarbons; degradation; enzymes

1. Introduction

Soil microbial communities are complex, and their phylogenetic and physiological diversity is enormous. Soil is a natural habitat for a variety of communities of organisms, which are important in the decomposition of organic matter, the cycle of carbon, and the utilization of various types of pollutants. Bacteria and fungi are the most important soil microorganisms owing to their abundance and their key roles in the functioning of ecosystems. Many fungi, together with bacteria, live in the rhizosphere of plants and contribute to their growth [1–4].

The type of soil, the amount and composition of nutrients, pH, humidity, temperature, and other factors determine the activity, diversity, and conformation of soil organism communities [5–7]. In polluted settings, these factors are supplemented by the “nature” and concentration of the pollutant [8–11].

The ecological effects exerted by fungi on soil-dwelling bacteria can be positive, neutral, or negative [3, 12]. In the hyphosphere (the area surrounding the fungal hyphae), bacterial growth is promoted by nutrient sources, which increase in availability to the bacteria through the network of hyphae. Colonization and adhesion of bacterial

cells on the surface of hyphae are important for the development of the hyphaspheric community [9,13,14]. Some bacteria, such as free-living nitrogen fixers, can penetrate fungal chlamydospores and survive in these protective conditions [15]. Fungi have strong potential to change the microenvironment through the production of extracellular enzymes and organic compounds. Bacteria can utilize fungal trehalose, mannitol, glycerol, and acetic and formic acids as their carbon sources [6]. Sometimes, coexistence with fungal mycelium is necessary for the degradation of hydrocarbons by bacteria [14]. In addition, there is a certain “specialization” between soil-inhabiting fungi and bacteria. Lignocellulosic materials such as wood residues are available for fungal degradation, whereas bacteria preferentially utilize soluble organic compounds, such as sugars [7]. Therefore, an increase in the number of fungi can lead to serious changes in the microbial community. However, the natural microflora can counteract or compete with fungi for resources that limit the growth of fungal mycelium [3].

Interactions between various microorganisms in polluted areas may change both the soil microbial communities and their functions [10], including the mineralization or degradation of pollutants. Potentially, the main reason



for the fungistatic effect of microorganisms could be competition with soil microorganisms for available carbon [16]. Yet, fuel-degrading micromycetes can exist in the form of associations with bacteria and fuel can be degraded synergistically by the partner organisms [17]. The large number of extracellular enzymes secreted by micromycetes increases the bioavailability of hydrophobic organic pollutants in the environment and promotes their efficient degradation by bacteria. The degradation pathways in fungi and bacteria may complement each other [18,19]. Such cometabolic degradation leads to complete utilization of the pollutant since metabolites formed by one organism are metabolized by others [7,10,11,20].

Bacterial and fungal cells secrete signaling molecules into the environment. These can be quorum-sensing molecules, antibiotic-like substances, metabolites, and volatile organic compounds [21]. Some authors believe that indole-3-acetic acid (IAA) is potentially such a signaling molecule, which is used in interspecies communications between bacteria and fungi [22]. IAA production by bacteria has been well studied. Although IAA-producing fungi are known [23], their biosynthetic pathways and their role in fungal ecology have not been widely studied.

Some of the most hazardous environmental pollutants are polycyclic aromatic hydrocarbons (PAHs). These aromatic hydrocarbons contain two or more fused benzene rings and enter the environment from natural and anthropogenic sources. Interest in the mechanisms of biodegradation and in the fate of PAHs in the environment is associated with their ubiquitous distribution, resistance to degradation, accumulation by soil and sediments, and toxic, mutagenic, and carcinogenic properties. PAHs are thermodynamically stable and resistant to microbial degradation owing to their hydrophobic nature, the presence of multiple benzene rings, and their low solubility in water [24–28].

Now various physical and chemical methods of PAH cleaning are available, although these technologies have several downsides, such as a regulatory burden, cost, complexity, and sometimes inefficiency in completely removing the compounds. Bioremediation is a natural degradation process, where the pollutant is either removed or converted into less harmful compounds by living organisms [29]. Despite the chemical and physical properties of PAHs, different bacteria, fungi, and algae can degrade or transform these compounds [30]. Many informative reviews have now been published on this topic [24–31]. However, most studies on microbial degradation of PAHs have been based on enzymes involved in PAH metabolism and mineralization [24,25,30,31].

Members of the genera *Sphingomonas*, *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Burkholderia*, *Mycobacterium*, *Rhodococcus*, *Nocardioideis*, and *Novosphingobium* are PAH-degrading bacteria. The biochemical pathways and the genes responsible for the bacterial degradation of PAHs have been described [24,25,27,28,30,31]. The first step

in the bacterial degradation of PAHs under aerobic conditions includes the dioxygenase-catalyzed hydroxylation of the aromatic ring. The resulting *cis*-dihydrodiols undergo rearomatization catalyzed by dehydrogenases. The aromatic rings of the formed diol intermediates are further cleaved by intra- or extradiol dioxygenases to produce metabolites, which, through a number of stages, are included in the main metabolism [25].

Fungi can play a major role in the detoxification of toxic PAHs either by using them as their sole carbon source or through cometabolism while utilizing other substrates [4]. The main fungal “players” in polluted soils are members of the phyla Ascomycota and, to a lesser extent, Basidiomycota. Ascomycetes are the dominant fungal phylum in polluted environments, where they can transform or degrade PAHs. According to several authors, PAH-contaminated soils are mainly colonized by members of the genera *Penicillium*, *Fusarium*, *Trichoderma*, *Paecilomyces*, and *Debaryomyces* [32]. PAH-metabolizing ascomycetes can be conditionally divided into two groups. Members of one group, such as basidiomycetes, can degrade and mineralize PAHs through their extracellular ligninolytic (lignin peroxidase, Mn-peroxidase, laccase) and intracellular (cytochrome P450, epoxide hydrolase) enzymes. The products of the first attack on the PAH molecule are the corresponding *trans*-dihydrodiols and quinones, which, through a series of steps, are included in the basal metabolism [4,30]. This group includes members of the genera *Fusarium* [10], *Cladosporium* [33], *Trematophoma* [34], and *Trichoderma* [35]. However, since the expression of extracellular lignin peroxidase, Mn-peroxidase, and laccase in ascomycetes requires the presence of lignocellulosic substrates, some authors believe that they only have a limited contribution to the degradation of PAHs in natural ecosystems [32]. The other largest group of ascomycetes, which do not produce extracellular ligninolytic enzymes (*Aspergillus niger*, *Cunninghamella elegans*, *Cunninghamella japonica*, *Neurospora crassa*, *Penicillium chrysogenum*, *Mucor circinelloides*, and others), transforms and detoxifies PAHs; similar to in bacteria, the enzyme systems that catalyze these processes are intracellular. The end products are glucuronide, glucoside, xylose, and sulfate conjugates [30,32,35].

Although bacterial and fungal degradation of PAHs is well known, the collaboration among soil microorganisms during PAH degradation remains poorly understood [36]. Few studies have explored the bacterial–fungal interactions during PAH degradation, and most of them have used bacterial–fungal consortia to remediate contaminated soils, including the effects of bioaugmented cultures/cocultures on degradation by indigenous microbiota. The few studies on this topic are summarized in the review of Bokade *et al.* [4]. These studies revealed interactions between fungi and bacteria, suggesting they played synergistic roles in degradation [36–40]. Fungi have shown bet-

ter transformation of PAHs to soluble hydroxylated or oxidized metabolites, which can be readily available for uptake by other organisms in the microbial community, which leads to complete and improved mineralization of PAHs [4]. Fungi and bacteria may act synergistically while co-existing in PAH-polluted soil in a way that fungi facilitate the transformation of insoluble substrates by utilizing plant and bacterial exudates available in the soil matrix [36]. For example, the degradation of benzo[a]pyrene by a *Fusarium solani* and *Rhodococcus erythropolis* co-culture resulted in a higher CO₂ production rate and CO₂ percent yield, compared to when using both organisms alone [41]. A mixed culture of *Phlebia brevispora* and bacteria (*Enterobacter* sp. and *Pseudomonas* sp.) improved pyrene and benzo[a]pyrene degradation to 92% and 72%, respectively [42]. Our previous studies have shown that the xylophilic 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* [43]. Thus, the application of such synergistically acting mixed fungal–bacterial cultures could be a viable strategy for the remediation of contaminated sites [4].

Here we first study the possible synergistic/antagonistic effects during the degradation of a mixture of PAHs by model bacterial–fungal systems, which include soil-inhabiting ascomycetes belonging to different ecological–physiological groups: *Fusarium oxysporum* (plant pathogen), *Talaromyces sayulitensis* (rhizospheric fungus), *Trichoderma viride* (PGPF), and *Trichoderma harzianum* (PGPF) alongside the plant-growth-promoting rhizobacterium *Azospirillum brasilense* (PGPR).

2. Materials and Methods

2.1 Microorganism, Growth Assay, and Storage

Fusarium oxysporum Schltdl. (IBPPM 543) MG593980, *Talaromyces sayulitensis* Visagie, N. Yilmaz, Seifert and Samson (IBPPM 664) KJ775206, and *Azospirillum brasilense* SR80 (IBPPM 24) QXHE00000000 were obtained from the IBPPM RAS Collection of Rhizosphere Microorganisms (<http://collection.ibppm.ru>, accessed on 23.07.2022). *Trichoderma harzianum* and *Trichoderma viride* were obtained from the collection of the Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences.

The fungi were grown on a modified basidiomycetes-rich medium [44] (g/L): NH₄NO₃, 0.724; KH₂PO₄, 1.0; MgSO₄ × 7H₂O, 1.0; KCl, 0.5; yeast extract, 0.5; FeSO₄ × 7H₂O, 0.01; ZnSO₄ × 7H₂O, 0.0028; CaCl₂ × 2H₂O, 0.033; glucose, 10.0; peptone, 10.0; agar, 15.0; (pH 6.0). The growth of fungal mycelium was controlled by the weight method [45].

Bacteria were grown on R2A medium [46] (g/L): yeast extract, 0.5; peptone, 0.5; casein hydrolysate, 0.5; glucose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; K₂HPO₄, 0.3; MgSO₄ × 7 H₂O, 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.

The organisms were checked for antagonistic activity by the double-culture method [47].

2.2 Chemicals

Phenanthrene (~90%), pyrene (~90%), anthracene (≥95%), fluoranthene (≥97%), fluorene (≥99%), and IAA (Pestanal®, analytical standard) were all purchased from Fluka (Buchs, Switzerland).

2.3 PAHs Degradation Assay

For degradation studies, we used a medium previously composed by Tsivileva *et al.* [48] (g/L): glucose, 4.5; fructose, 4.5; L-asparagine, 1.5; KH₂PO₄, 1.0; K₂HPO₄, 1.0; Na₂MoO₄ × 2H₂O, 0.002; CaCl₂, 0.02; MgSO₄ × 7H₂O, 0.2; NaCl, 0.1; NH₄Cl, 1.0. A mixture of PAHs (anthracene, phenanthrene, fluorene, pyrene, and fluoranthene; 1 mg of each) was added to a final PAH concentration of 5 mg per 100 mL. Fungi (20 mg of dry weight or about 10⁴ CFU) and *A. brasilense* (3 × 10⁵ CFU), both singly and in combination, were grown at 24, 30, or 37 °C in 0.3 L Erlenmeyer flasks on an Environmental Shaker–Incubator ES20/60 (BioSan, Latvia) at 130 rpm. At certain time intervals, an aliquot (2 mL) was removed from the flasks and the extracellular enzyme activities and the IAA concentration 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 [43,45,49]. The sorption of the PAHs by fungal mycelium did not exceed 5–7%.

2.4 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1220 Infinity II LC chromatograph (Agilent Technology, Waldbronn, Germany) equipped with a 254 nm UV detector.

The PAH content was analyzed on a 4.6 × 150 mm ZORBAX Eclipse PAH 5-Micron column. The solvent system was H₂O:acetonitrile, the linear gradient was 40–100% acetonitrile, and the duration was 17 min. The PAHs and their metabolites were analyzed by comparing the retention times (RT) with those of standard compounds (RT, min): carboxybenzaldehyde (1.938 min), 9,10-phenanthrenequinone (5.145 min), 1-hydroxy-2-naphthoic acid (5.823 min), 9-fluorenone (6.856 min), 9,10-anthraquinone (7.320 min), fluorene (8.844 min), phenanthrene (9.353 min), anthracene (9.888 min), pyrene (10.382 min), and fluoranthene (10.837 min).

IAA content was analyzed on a 4.6 × 50 mm ZORBAX Eclipse Plus C₁₈ 5-Micron column. The solvent sys-

tem was H₂O (acidified with H₃PO₄ to pH 2.5): acetonitrile, the linear gradient was 10–90% acetonitrile, and the duration was 15 min.

2.5 Enzymatic Assays

Enzyme activity was determined by an Evolution 60 (840-208700, Thermo Scientific™, Madison, WI, USA). Laccase activity was measured by the oxidation rate of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) at 436 nm ($\epsilon = 29,300 \text{ M}^{-1}\text{cm}^{-1}$), according to Niku-Paavola *et al.* [50]. Peroxidase was measured by the oxidation rate of 2,6-dimethoxyphenol (DMP) with H₂O₂ at 468 nm ($\epsilon = 14800 \text{ M}^{-1}\text{cm}^{-1}$), according to Martínez *et al.* [51] and by ABTS oxidation, as described above, but in the presence of 0.1 mM H₂O₂. The peroxidase activity was calculated as the difference between the values obtained in the presence and absence of H₂O₂. One unit of enzyme activity was defined as the amount required to catalyze the formation of 1 μmol of product per min and is expressed in relative units ($\mu\text{mol}/\text{min}/\text{mL}$ of enzyme preparation (U/mL)).

2.6 Statistical Analysis

All experimental treatments and all analyses were repeated at least three times. The obtained experimental data were statistically processed, average values were calculated, and the standard deviation (SD) was used for comparisons. Calculations were carried out using Microsoft Excel 2019 (Microsoft, WA, USA).

3. Results and Discussion

We used four soil-inhabiting ascomycetes that can degrade a number of pollutants and belong to different ecological–physiological groups (*Fusarium oxysporum*, *Talaromyces sayulitensis*, *Trichoderma harzianum*, and *Trichoderma viride*) [49,52]. We also used the PGPR *Azospirillum brasilense*, which can degrade crude oil [53,54].

Fusariums are ubiquitous in soils and plant rhizospheres [10]. Most *Fusarium* species lead a saprotrophic lifestyle in soil and use lignin and complex carbohydrates as food sources [55]. An increase in the number of species in this genus in the rhizosphere of plants growing on oil-contaminated soil was also described by Mohsenzaden *et al.* [56]. *Fusariums* are involved in the degradation of hazardous pollutants, including oil, synthetic dyes, PAHs, herbicides, and many others [57,58]. The ascomycete *F. oxysporum* IBPPM 543 was isolated from old sleepers contaminated by creosote and was highly degradative towards a wide range of pollutants [49,52].

The ubiquitous members of the genus *Trichoderma* are well known because of their plant-growth-promoting properties (PGP-fungi, PGPF) [22,59,60], their ability to utilize lignocellulosic materials [61,62], and also because of their degradative activity towards synthetic dyes [62–64], nitrocellulose [65], bisphenol A [66], carbamazepine and clar-

ithromycin [67], polyethylene [68], polyethylene terephthalate [52], oil [69], diesel fuel [60], and PAHs [35]. *Tr. longibrachianum* [70,71], *Tr. hamatum*, *Tr. reesei*, *Tr. koningii* [35], *Tr. viride* [72], *Tr. virens*, *Tr. harzianum* [58,72], and *Tr. asperellum* [73] are all known PAH degraders.

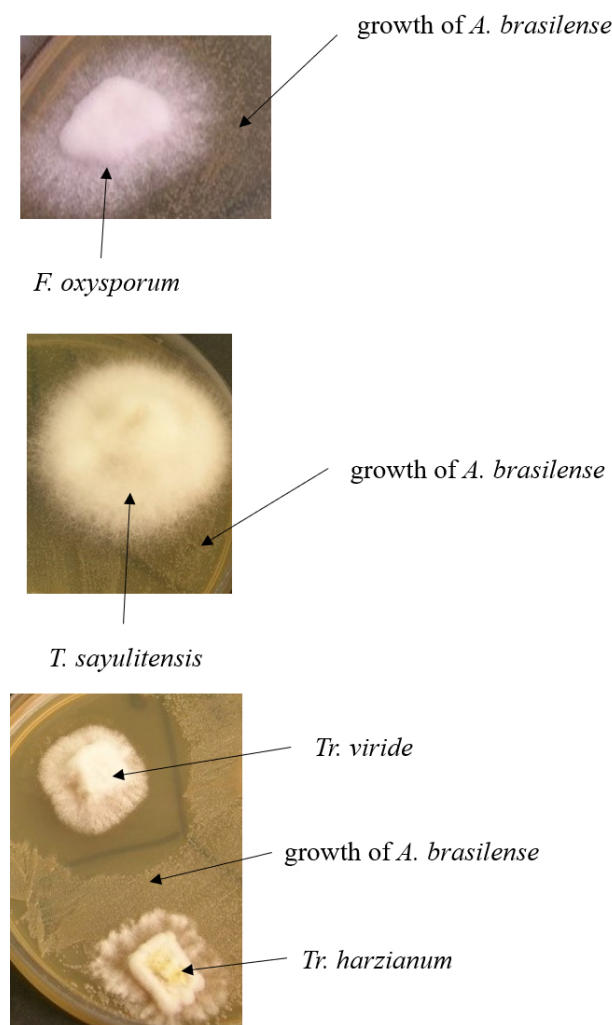


Fig. 1. Growth of bacterial–fungal co-cultures on R2A agar medium.

However, there is limited information on the degradative activity of *Talaromyces* fungi. For example, their ability to metabolize lignocellulosic substrates was described [74]. *Talaromyces* sp. can utilize oil, *n*-alkanes [18], and kerosene [17]; *T. spectabilis*, a mixture of pyrene and phenanthrene [75]; *T. astroroseus*, dichlorvos [1]. The rhizospheric fungus *T. purpurogenus* has been used in combination with plants to restore soil contaminated by crude oil [76]. *Talaromyces sayulitensis* strain IBPPM 664 was isolated in our studies from the rhizosphere of miscanthus grown in a zinc-polluted area. Preliminary studies have shown its ability to utilize oil and polyethylene terephthalate as the sole sources of carbon [52].

The associative bacteria *Azospirillum* are well known for their plant-growth-promoting properties, while the positive effect of PGPR on mycorrhizosphere communities is also known [77]. However, the co-cultivation of *Azospirillum* with xylotrophic basidiomycetes that do not form mycorrhiza may promote the growth of the latter. For example, the high potential of bacterial–fungal cultures (based on *Azospirillum* and cultivated fungi) has been shown in the effective production of mycelial biomass and fruiting of basidiomycetes [48].

3.1 Interaction between Fungi and *A. brasilense*: The Double-Culture Method

We first evaluated the mutual influence of the fungi and bacteria by the double-culture method. This method is often used to identify the antagonistic effect of an organism on a particular pathogen. Antagonists grow faster than pathogens and form zones of inhibition, thereby limiting pathogen growth. For example, a similar test was used to examine the ability of a number of *Trichoderma* species to suppress the growth of *Sarocladium oryzae* [78]. In our experiments, no antagonistic activity was found in the following pairs: *A. brasilense*–*F. oxysporum*, *A. brasilense*–*T. sayulitensis*, and *A. brasilense*–*Tr. harzianum*. From Fig. 1 we can see the active growth of the mycelium of the studied fungi and *A. brasilense*. By contrast, *Tr. viride* suppressed the growth of *A. brasilense* on agar, as evidenced by the absence of bacterial growth on the agar with active growth of fungal mycelium (Fig. 1). In earlier studies by Kalaiselvi *et al.* [78], *Tr. harzianum* was more active than *Tr. viride* and some other *Trichoderma* species at inhibiting the growth of *Sarocladium oryzae*. Apparently, the ability to inhibit the growth of bacteria is determined not only by the *Trichoderma* species but also by the bacterial species.

3.2 Degradation of Phenanthrene, Anthracene, Fluorene, Pyrene, and Fluoranthene and of their Mixtures by the Ascomycetes

Anthracene and fluoranthene were the least available of all the fungi (Fig. 2A). This may have been due to the low solubilities and high ionization potentials of these compounds [24–28]. Among the ascomycetes used, *F. oxysporum* was the weakest degrader, whereas *T. sayulitensis* and *Tr. harzianum* showed the highest degradative activity towards the PAHs. The other *Trichoderma* member, *Tr. viride*, intensely metabolized only low-molecular-weight PAHs (fluorene and phenanthrene, $67 \pm 3\%$ and $54 \pm 5\%$, respectively) (Fig. 2A). Previously, Zafra *et al.* [35] showed that low-molecular-weight PAHs (naphthalene, phenanthrene, and anthracene) are more accessible for degradation by *Trichoderma*. These data were confirmed by other authors. For example, *Tr. longibrachiatum* metabolized between 70 and 90% of the phenanthrene depending on the cultivation conditions [71]. Another study showed that the same fungus metabolized up to 70% of phenan-

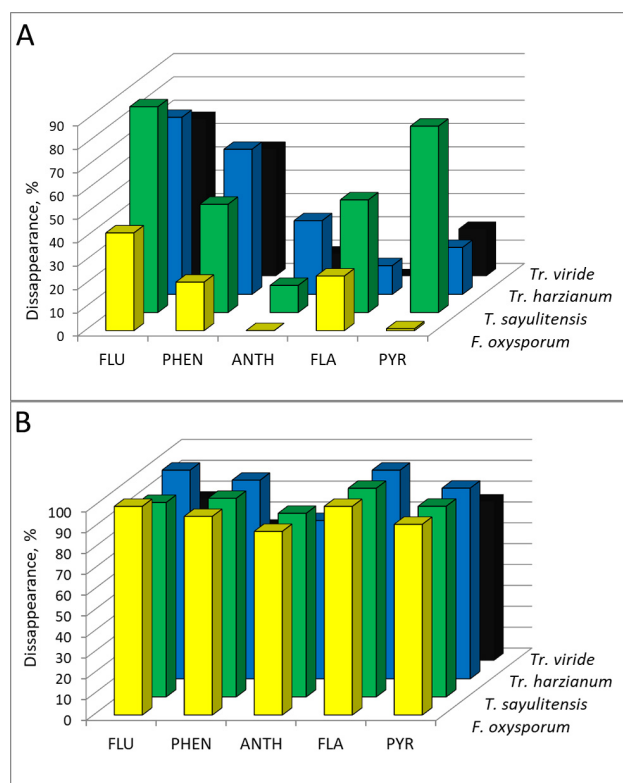


Fig. 2. Degradation of individual polycyclic aromatic hydrocarbons (PAHs) (A) and their model mixture (B) by the ascomycetes. FLU, fluorene; PHEN, phenanthrene; ANTH, anthracene; FLA, fluoranthene; PYR, pyrene. ■ *F. oxysporum*; ■ *T. sayulitensis*; ■ *Tr. harzianum*; ■ *Tr. viride*. The standard deviation (SD) did not exceed 10% of the basic values.

threne, anthracene, fluoranthene, and pyrene at a starting concentration of 25 mg/L [60]. *Tr. harzianum* metabolized 27–28% of the initially added 46.8 mg/L of anthracene [79]. *Tr. harzianum*, *Tr. pseudokoningii*, and *Tr. viride* metabolized pyrene [72]. *Tr. reesei* cometabolized 54% of the benzo[a]pyrene, at a starting concentration of 20 mg/L [80].

Each of 9-fluorenone, 9,10-phenanthrenquinone, and 9,10-anthraquinone were identified as the main metabolites in the degradation of fluorene, phenanthrene, and anthracene, respectively, in *F. oxysporum* and *T. sayulitensis*. This was confirmed by the retention times (RTs) of the identified metabolites against those of the commercial compounds. The formation of quinones as the first metabolites of the PAH degradation by *Fusarium* is well known. For example, *F. solani* degrades anthracene and benzo[a]anthracene via the corresponding quinones, which is followed by the formation of phthalic acid [81]. Although *Talaromyces* members can degrade PAHs, no information is available on the key metabolites [82]. In our study, quinone metabolites of PAH degradation were found in *T. sayulitensis* for the first time. Moreover, 1-hydroxy-2-naphthoic acid was identified as a metabolite of phenanthrene degra-

Fig. 3A

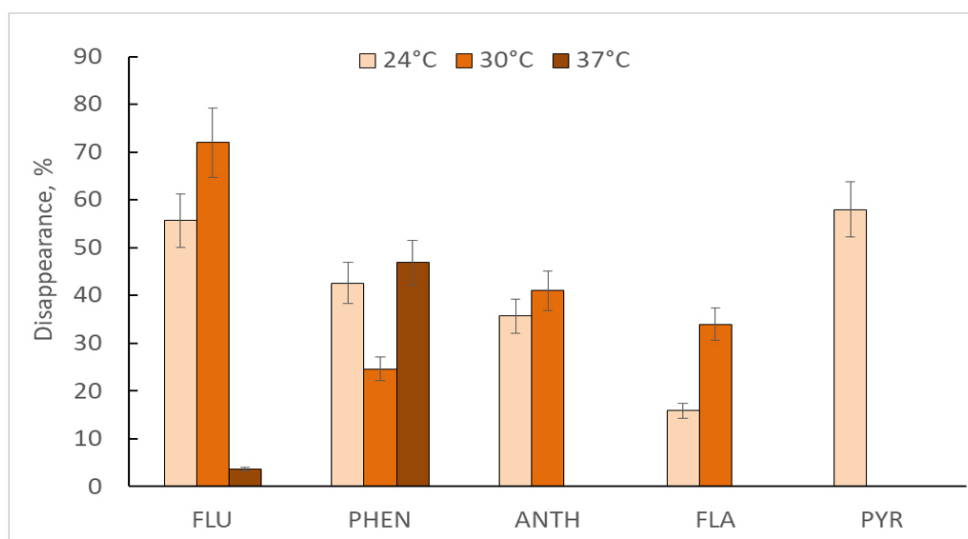


Fig. 3B

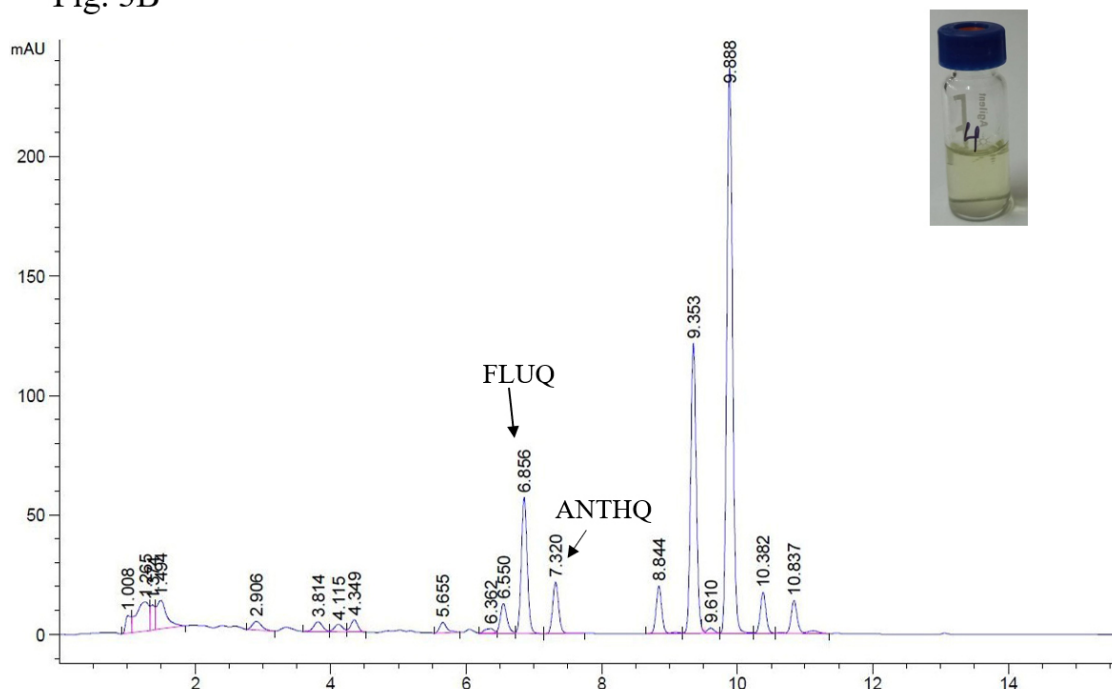


Fig. 3. Degradation of the PAH model mixture (A) and chromatogram of the culture medium after growth of *A. brasilense* SR-80 (B). FLU, fluorene; PHEN, phenanthrene; ANTH, anthracene; FLA, fluoranthene; PYR, pyrene; FLUQ, 9-fluorenone; ANTHQ, 9,10-anthraquinone.

gradation by *Tr. harzianum*, whereas no quinone metabolites were found. This is consistent with data from Hadibarata *et al.* [83], who showed that *Trichoderma* sp. S019 metabolized up to 72% of the phenanthrene to produce 1-hydroxy-2-naphthoic acid, salicylaldehyde, salicylic acid, and catechol through the pathway described in the bacterium. We did not detect PAH degradation metabolites in *Tr. viride*, which may have been due to the low degradative activity of this fungus.

All ascomycetes utilized the model mixture of PAHs (equal amounts of fluorene, phenanthrene, anthracene, pyrene, and fluoranthene) by almost 100% (Fig. 2B). A similar effect was described by Cobas *et al.* [71], who showed that *Tr. longibrachiatum* immobilized on nylon in a bioreactor utilized a mixture of three PAHs (phenanthrene, benz[a]anthracene, and pyrene) faster than it utilized individual PAHs.

3.3 Degradation of Phenanthrene, Anthracene, Fluorene, Pyrene, and Fluoranthene and of their Mixtures by *A. brasilense*

Bacteria in general prefer aerobic PAH degradation to be catalyzed by dioxygenase enzymes. There is no information on the ability of *Azospirillum* to degrade PAHs in the reviews we described [26,28]. Previously, we had shown that the PGPR *A. brasilense* SR-80 can utilize oil as its carbon source [53,54]. Without denying the presence of a dioxygenase pathway in *Azospirillum*, we further assumed the presence of an alternative pathway since the formation of PAH quinones at the first stage of degradation testifies in favor of an alternative pathway [43]. Subsequently, we found that this strain could cometabolize only fluorene and anthracene in the presence of a carbon source (glucose, fructose), with the removal of the pollutant identified as $20 \pm 6\%$ and $23 \pm 4\%$, respectively. During cultivation with a mixture of PAHs, *A. brasilense* SR-80 could cometabolize all the compounds used. However, the availability and degree of utilization of individual PAHs depended on the cultivation temperature. Utilization was the most intense at 30 °C (Fig. 3A). PAHs are known to be more greatly degraded in a mixture compared to individual PAHs. Thus, the co-metabolism of the PAHs shows synergistic effects on the biodegradation of other PAHs, especially high molecular weight PAHs [29].

Fig. 3 shows a yellow-colored extract of the culture liquid (vial) and a chromatogram of metabolites. HPLC allowed the identification of the degradation products of fluorene (9-fluorenone, RT 6.856 min) and anthracene (9,10-anthraquinone RT 7.320 min) (Fig. 3B).

3.4 Degradation of a Mixture of PAHs by Bacterial–Fungal Co-Cultures

The PAHs are presented in the environment as the mixture; thus, the bioremediation of contaminated sites could be crucially performed by co-metabolism. In our experiments, the PAH mixture was degraded by co-cultures, which included *F. oxysporum*, *T. sayulitensis*, *Tr. viride*, *Tr. harzianum*, and *A. brasilense* at temperatures of 24, 30, and 37 °C, which are optimal for the growth of the fungi, co-cultures, and bacterium, respectively. The growth of *F. oxysporum* and *T. sayulitensis* was inhibited in the co-cultures, regardless of the cultivation temperature. An increase in the amount of mycelium in the co-cultures was noted only for the *Trichoderma* fungi (Table 1). Yet, it was impossible to calculate the CFUs after plating the co-cultures on the agar medium owing to the complete overgrowth of agar with mycelium.

Apparently, during submerged cultivation of *F. oxysporum* and *T. sayulitensis*, the bacterial and fungal cultures inhibited each other's growth. It is known that many bacterial–fungal interactions are mediated by the production of various metabolites. For example, fusaric acid produced by *F. oxysporum* regulates the transcription of a num-

ber of genes in *Pseudomonas chlororaphis* [5]. Competition between *F. oxysporum* and *Arthrobacter oxydans* was shown in the presence of PAHs during submerged cultivation [10]. Perhaps similar effects were observed in our experiments. The lack of growth inhibition during solid-state cultivation of the *F. oxysporum*–*A. brasilense* and *T. sayulitensis*–*A. brasilense* pairs may have resulted from a lack of production of such metabolites. Thus, the absence of antagonistic mutual influence by the bacteria and fungi, found under solid-state cultivation, will not necessarily indicate a similar effect under submerged cultivation.

Studies on the degradation of the PAH mixture showed that all compounds included in the mixture were available for fungal degradation. There were no synergistic effects on PAH degradation in the co-cultures with *F. oxysporum* (Table 2). A significant decrease in the content of individual PAHs was the result of their fungal degradation. Similar data were obtained by Thion *et al.* [10], who showed the co-existence of *F. oxysporum* and *Arthrobacter oxydans* in sterile sand in the presence of PAHs. However, co-inoculation did not noticeably affect the degradation of the mixture of PAHs (phenanthrene, pyrene, and dibenz[a,h]anthracene) under these conditions [10]. However, synergistic effects of co-cultivation of *Fusarium* and bacterial strains have also been described. For example, a co-culture of *Fusarium* sp. and *Ochrobactrum anthropi* metabolized fluoranthene [84], and a co-culture of *F. solani* and *Rhodococcus erythropolis* metabolized benzo[a]pyrene [41] more efficiently than its individual cultures.

T. sayulitensis intensely metabolized the PAHs in the mixture at 30 °C, both alone and in combination with *A. brasilense* (Table 2). Synergistic effects were found at 24 and 37 °C. At 24 °C, *T. sayulitensis* did not metabolize the PAHs, despite the vigorous growth of the fungus; in the co-cultures, the degradation increased, compared to *A. brasilense* alone. At 37 °C, the *A. brasilense* monoculture did not utilize anthracene, pyrene, or fluoranthene, whereas all PAHs were available in the *T. sayulitensis* monoculture. In the co-cultures, increased degradation of anthracene, pyrene, and fluoranthene was noted. The synergistic effects we found confirmed the previous data by Shapiro *et al.* [17], who showed that *Talaromyces* micromycetes associated with bacteria can grow on kerosene, which suggests a degradative mechanism based on the synergism of bacteria and fungi [17].

Positive effects, resulting in increased PAH degradation, were found when the plant-growth-promoters *A. brasilense* and *Tr. viride* or *Tr. harzianum* were co-cultivated (Table 2). Ascomycetes of the genus *Trichoderma* are characterized by catabolic degradation of PAHs: *Tr. reesei* [80], *Tr. asperellum* [35], *Tr. longibrachiatum* [60,71], and *Tr. harzianum* [72,79]. The utilization of PAHs (benz[a]anthracene) as the sole carbon source was shown only for *T. longibrachiatum* [70]. In our experiments, for all ascomycetes, PAH degradation was possible

Table 1. Dry weight of mycelium in mono- and co-cultures.

Treatments	Dry weight of mycelium (mg)					
	Without PAHs			With PAHs		
	24 °C	30 °C	37 °C	24 °C	30 °C	37 °C
monocultures						
<i>F. oxysporum</i>	196.6 ± 10.1	306.1 ± 11.3	39.1 ± 10.1	163.1 ± 16.6	203.2 ± 15.7	21.8 ± 9.9
<i>T. sayulitensis</i>	374.1 ± 25.9	349.6 ± 18.7	377.4 ± 33.4	282.0 ± 19.0	344.3 ± 30.3	325.4 ± 13.6
<i>Tr. harzianum</i>	278.3 ± 18.6	222.8 ± 23.9	220.1 ± 18.3	178.7 ± 17.8	229.1 ± 29.5	207.4 ± 14.4
<i>Tr. viride</i>	286.3 ± 20.1	252.9 ± 30.3	250.0 ± 18.8	200.3 ± 15.8	228.1 ± 18.3	218.9 ± 21.0
co-cultures with SR80						
<i>F. oxysporum</i>	150 ± 8.8	154.1 ± 20.0	36.2 ± 6.9	144.1 ± 10.9	176.4 ± 17.0	25.5 ± 8.6
<i>T. sayulitensis</i>	336.3 ± 23.9	322.8 ± 17.0	259.2 ± 17.6	278.6 ± 14.8	329.1 ± 29.1	223.7 ± 12.9
<i>Tr. harzianum</i>	290.2 ± 32.5	249.6 ± 24.5	253.4 ± 16.9	262.2 ± 20.1	244.3 ± 15.6	250.1 ± 12.8
<i>Tr. viride</i>	300.0 ± 16.6	292.1 ± 24.6	298.1 ± 13.9	272.5 ± 23.5	277.0 ± 15.0	277.0 ± 15.1

PAHs, polycyclic aromatic hydrocarbons.

only under the conditions of cometabolism. We identified the degradation products by the co-cultures (fluorene as 9-fluorenone and anthracene as 9,10-anthraquinone), which corresponds to the data presented above.

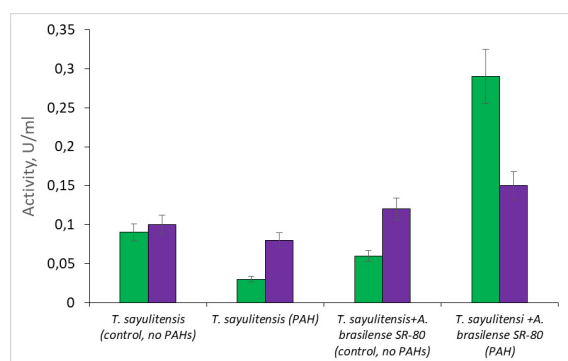


Fig. 4. Production of extracellular enzymes during PAH degradation. ■ – oxidase; ■ – peroxidase.

3.5 Extracellular Enzymes of the Ascomycetes

The initial steps in PAH degradation by many micromycetes are catalyzed by cytochrome P450 monooxygenase and epoxide hydrolase. The reaction products catalyzed by these enzymes can be *trans*-dihydrodiols, phenols, quinones, and dihydrodiol epoxides, which can further form glucuronide, glucoside, xylose, and sulfate conjugates. However, some species of ascomycete can produce laccases, peroxidases, and dioxygenases that break the aromatic ring [35,73].

The production of laccases and peroxidases by various *Fusarium* species is well known [81,85]. The involvement of these enzymes in the degradation of PAHs by *Fusarium* has also been discussed [86,87]. We have previously shown that, unlike other known strains of *F. oxysporum*, the IBPPM 543 strain of *F. oxysporum* produces peroxidase

(without laccase) during the degradation of pollutants [49]. The activity of this enzyme in the presence of PAHs was induced only at 24 °C, which is consistent with our previous results [49]. The enzyme activity was the highest (2.2 U/mL) during cultivation at 30 °C. However, following an increase in temperature to 37 °C, the extracellular activity of peroxidase decreased, which may be due to both the fungal growth being inhibited at this temperature and a decrease in the enzymatic stability as the temperature increased. In co-cultures, peroxidase activity decreased at 24 and 37 °C and slightly exceeded the control value only at 30 °C (3.3 U/mL).

We found the production of two extracellular enzymes: oxidase (according to the oxidation of ABTS) and peroxidase (according to the oxidation of 2,6-dimethoxyphenol in the presence of H₂O₂) by the fungus *T. sayulitensis* only at 30 °C (Fig. 4). The activity of both enzymes was low and increased when the PAHs were added to the co-cultures.

Although the ability of *Talaromyces* to metabolize lignocellulose and produce hemicellulases is well known [74], there is no information on the production of enzymes that catalyze the degradation of the lignin component. Enzymes have been described in some *Talaromyces* members that, hypothetically, can be related to the degradation of aromatic compounds and pollutants, such as PAHs. For example, cytochrome C peroxidase, glutathione peroxidase, catalase peroxidase, DyP peroxidase, and a hybrid of ascorbate peroxidase and cytochrome C were found in *T. stipitatus*. Catalase peroxidase was found in *T. termophilus* [88], while in *T. asrtoroseus*, which degrades dichlorvos, genes encoding catechol 1,2-dioxygenase were found to be expressed [1].

As mentioned above, *Trichoderma* members are characterized by the presence of both intra- and extracellular enzymes of the initial attack on the PAH molecules. *Trichoderma* dioxygenases can catalyze the initial attack on the aromatic ring to form unstable *cis*-dihydrodiols, which can

Table 2. Degradation of PAHs by mono- and co-cultures of ascomycetes.

PAHs	PAH disappearance, %							
	monocultures				co-cultures with <i>A. brasilense</i>			
	<i>F.oxysporum</i>	<i>T.sayulitensis</i>	<i>Tr.harzianum</i>	<i>Tr.viride</i>	<i>F.oxysporum</i>	<i>T.sayulitensis</i>	<i>Tr.harzianum</i>	<i>Tr.viride</i>
24 °C								
FLU	54 ± 7.8	0 ± 0.1	86.7 ± 7.7	60.8 ± 1	50.9 ± 6.3	90.0 ± 7.3	100 ± 1.0	77.7 ± 1
PHEN	84 ± 10.1	0 ± 0	72.2 ± 8.3	49.6 ± 1	81.1 ± 7.6	83.0 ± 3.9	95.2 ± 10.9	64.3 ± 5
ANTH	64.9 ± 3.8	0 ± 1.0	60.1 ± 5.9	49.5 ± 6	69.5 ± 6.3	74.8 ± 12.0	75.9 ± 7.2	66.9 ± 2
FLA	70.5 ± 6.9	0 ± 0.2	90.1 ± 11.0	38.8 ± 8	65.7 ± 8.9	78.0 ± 9.1	100 ± 0	59.0 ± 1
PYR	67.9 ± 9.9	0 ± 0.2	83.3 ± 3.7	56.6 ± 9	63.7 ± 8.0	78.4 ± 5.4	91.4 ± 9.4	76.3 ± 1
30 °C								
FLU	45.7 ± 9.6	85.0 ± 7.8	84.8 ± 12.9	59.9 ± 3	40.5 ± 10.1	78.8 ± 8.8	100 ± 1.0	75.9 ± 1
PHEN	77.3 ± 3.8	62.4 ± 7.6	70.0 ± 7.1	42.4 ± 5	75.7 ± 4.9	61.2 ± 2.3	93.4 ± 3.0	65.1 ± 3
ANTH	58.1 ± 3.2	45.7 ± 5.9	60.2 ± 6.6	45.3 ± 5	58.4 ± 8.3	7.8 ± 2.2	78.8 ± 7.7	65.7 ± 3
FLA	63.8 ± 10.1	43.5 ± 5.0	88.5 ± 3.0	28.1 ± 8	60.0 ± 9.0	8.6 ± 2.9	100 ± 0	59.5 ± 1
PYR	62.1 ± 2.8	43.5 ± 6.0	78.4 ± 2.8	49.9 ± 7	57.6 ± 3.3	49.2 ± 5.9	100 ± 0	77.0 ± 5
37 °C								
FLU	28.4 ± 7.6	99.0 ± 1.0	69.5 ± 13.2	53.8 ± 1	44.6 ± 8.6	100 ± 0	100 ± 0	73.2 ± 5
PHEN	57.0 ± 12.0	72.0 ± 5.9	50.7 ± 6.5	40.1 ± 7	51.6 ± 8.8	98.0 ± 2.2	92.9 ± 0	60.9 ± 5
ANTH	0 ± 0.3	34.5 ± 9.7	50.5 ± 5.5	40.0 ± 1	0 ± 0	96.5 ± 3.8	72.9 ± 1.8	60.3 ± 9
FLA	0 ± 0	59.5 ± 4.6	73.9 ± 6.7	18.9 ± 5	0 ± 0	93.6 ± 2.0	100 ± 0	54.6 ± 7
PYR	0 ± 0	49.2 ± 4.0	68.8 ± 4.9	35.5 ± 9	0 ± 0	93.1 ± 2.0	100 ± 0	78.1 ± 5

FLU, fluorene; PHEN, phenanthrene; ANTH, anthracene; FLA, fluoranthene; PYR, pyrene.

subsequently be metabolized via the catechol pathway [83]. The presence of PAHs resulted in a significant increase in the activity of catechol 1,2- and 2,3-dioxygenases at the initial stages of degradation [73]. The phenanthrene degradation pathway described by Hadibarata *et al.* [83], includes the action of catechol-1,2- and 2,3-dioxygenases in the initial stage of oxidation, with 1-hydroxy-2-naphthoic acid, salicylic acid and catechols as the main metabolites. Ultimately, this may explain the ability of some *Trichoderma* species to utilize PAH as their sole carbon source.

The production of extracellular enzymes, similar to the ligninolytic enzymes in basidiomycetes, has been described in *Trichoderma*. For example, *Tr. tomentosum* produced Mn-peroxidase and lignin peroxidase during the degradation of azo dyes [64]. Laccase was found in *Tr. harzianum*, which degrades bisphenol A and utilizes phenols in *Tr. viride* [66]. However, in this study, the enzyme was partially purified, and its activity was tested through the oxidation of guaiacol, which is a non-specific substrate of both laccases and tyrosinases. Sowmya *et al.* [68] described laccase and Mn-peroxidase during the degradation of polyethylene by *Tr. harzianum*. However, the activity of these enzymes was only checked through the formation of colored zones during the growth of the fungus on an agar medium with guaiacol, which also raises doubts about the nature of the identified enzymes. More correct testing of extracellular laccase (by ABTS) and Mn-peroxidase (by vanil-

lyl acetone) revealed no activities of these enzymes during the degradation of benzo[a]pyrene by *Tr. reesei* [80]. Additionally, activities of laccase (by ABTS) and peroxidases (by phenol red and veratryl alcohol) during PAH degradation were revealed in *Tr. asperellum* [74]. The ability of *Tr. longibrachiatum* to degrade PAHs was accompanied by the production of phenol oxidase, which was identified following the oxidation of gallic acids; this is a more correct name for the extracellular oxidative enzyme in this case [60]. In addition, extracellular laccase production has been described in *Tr. atroviride*, *Tr. harzianum*, and *Tr. asperellum* [74], the oxidation of PAHs by this enzyme may represent an alternative route for the initial attack of the molecules of these compounds.

In our experiments, the activities of extracellular oxidases and peroxidases in *Tr. viride* or *Tr. harzianum* under these studied conditions was not found. Apparently, under these conditions, the degradation of PAHs by *Tr. viride* or *Tr. harzianum* proceeds along the bacterial pathway described by Hadibarata *et al.* [83]. The 1-hydroxy-2-naphthoic acid that we identified as the main metabolite of phenanthrene degradation confirms this assumption.

3.6 IAA Production

Trichoderma are ubiquitous ascomycetes with a number of growth-promotion properties, including the synthesis of IAA, ACC-deaminase, siderophores, acid phosphatase,

and phosphate-dissolving agents under biotic and abiotic stress [60,89]. For example, IAA production was found in *Tr. longibrachiatum* [89]. *Tr. virens* did not produce IAA, yet ACC deaminase was detected [60]. We identified the ability of *Tr. viride* or *Tr. harzianum* to produce IAA during PAH degradation, although the IAA concentration was low and did not exceed 0.1 µg/mL. The other two ascomycetes did not produce IAA.

4. Conclusions

Bacterial–fungal interactions are important for the natural ecosystems to function since they contribute largely to the bioremediation of contaminated soils. To study the mutual influence of bacteria and fungi during the degradation of PAHs, we used a model system that included the plant-growth-promoting rhizobacterium *Azospirillum brasilense* and one of the following ascomycetes: *Fusarium oxysporum* (phytopathogen), *Talaromyces sayulitensis* (rhizospheric), *Trichoderma viride*, or *Trichoderma harzianum* (plant-growth-promoting fungi). Although *Talaromyces* members can degrade PAHs, no information is available on the key metabolites. In this study, quinone metabolites of PAH degradation were found in *T. sayulitensis* for the first time.

Two synergistic effects were found: (1) all fungi tested and *Azospirillum* were more active in the degradation of the PAH mixture than the individual compounds; (2) PAHs were utilized more efficiently in the bacterial–fungal cultures than in the monocultures of these individual microorganisms. Three effects of mutual influence by the studied organisms were also revealed. In the co-cultures, *F. oxysporum* and *A. brasilense* had a depressing effect on each other, which was expressed via a decrease in the dry weight of mycelium, the number of CFUs, and the degradation of PAHs. In the co-cultures, *T. sayulitensis* suppressed the growth of *A. brasilense*; however, the degradation of anthracene, pyrene, and fluoranthene was increased under these conditions. Positive effects were found during the joint cultivation of PGPR bacteria (*A. brasilense*) and PGPF ascomycetes (*Tr. viride* or *Tr. harzianum*), which manifested as increases in the dry weight of mycelium and in the degradation of PAHs. Moreover, 9,10-anthraquinone, 9-fluorenone, and 9,10-phenanthrenquinone were identified as the main metabolites, regardless of the experimental conditions. The production of indole-3-acetic acid and extracellular peroxidases by *Trichoderma* was found also.

Our studies revealed the presence of enzymes (peroxidase, oxidase) in two fungi (*F. oxysporum*, *T. sayulitensis*), which were similar to the ligninolytic enzymes. Perhaps they also participate in the degradation of PAHs, as evidenced by the formation of PAH-quinones, although no similar enzymes or PAH-quinones were found in *Tr. viride* or *Tr. harzianum*. Apparently, *Trichoderma* fungi degrade the PAHs through the cytochrome P450 monooxygenase pathway.

Abbreviations

IAA, indolile-3-acetic acid; PAHs, polycyclic aromatic hydrocarbons; PGPR, plant-growth-promoting rhizobacteria; HPLC, High Performance Liquid Chromatography; RT, retention time.

Availability of Data and Materials

The data and materials generated during the current study are available from the corresponding author.

Author Contributions

NP designed the research study. AB performed auxiliary experiments. NP and AB performed the research. AM and OT provided help and advice on bacterial part of studies and discussion of the data obtained. NP analyzed the data. NP and AM wrote the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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