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ORIGINAL PAPER

Ligninolytic enzymes production during polycyclic aromatic hydrocarbons degradation: effect of soil pH, soil amendments and fungal co-cultivation

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Abstract Soil microorganisms play an important role in the degradation of PAHs and use various metabolic pathways for this process. The effect of soil pH, different soil amendments and the co-cultivation of fungi on the degradation of PAHs in soil and on the activity of ligninolytic enzymes was evaluated. For that purpose, three fungi were studied: *Trichoderma viride*, *Penicillium chrysogenum* and *Agrocybe aegerita*. Biodegradation assays with a mixture of 200 ppm PAHs (fluorene, pyrene, chrysene, and benzo[a]pyrene—50 ppm each) were set up at room temperature for 8 weeks. The maximum laccase activity by solid state fermentation—SSF (7.43 U/g) was obtained by *A. aegerita* on kiwi peels with 2 weeks and the highest manganese peroxidase activity (7.21 U/g) was reached in 4 weeks, both at pH 7. Fluorene, pyrene, and benzo[a]pyrene achieved higher degradation rates in soil at pH 5, while chrysene was more degradable at pH 7. About 85–90% of the PAHs were degraded by fungal remediation. The highest degradation of fluorene was achieved by co-cultivation of *A. aegerita* and *P. chrysogenum*, remaining 14% undegradable. Around 13% of pyrene stay undegradable by *A. aegerita* and *T. viride* and by *A. aegerita* and *P. chrysogenum,* both systems supported in kiwi peels, while 11% of chrysene remained in soil by the co-cultivation of these fungi, supported by peanut shells. Regarding benzo[a]pyrene, 13% remained in soil after treatment with *A. aegerita.* Despite the increase in degradation of some PAHs with co-cultivation, higher enzyme production during degradation was observed when fungi were cultivated alone.

Keywords PAHs · Soil remediation · Laccase · Manganese peroxidase · Lignin peroxidase · Fungi

Introduction

Polyaromatic hydrocarbons (PAHs) are basically non-polar organic compounds with two or more aromatic rings, composed of carbon and hydrogen atoms (Ghosal et al. 2016), which are easily absorbed by soil due to their high hydrophobicity, stability and strong recalcitrant nature (Cao et al. 2020). PAHs in environment are present as mixtures, usually with other toxic compounds, and therefore co-metabolism plays a very crucial role in bioremediation of PAHs contaminated sites. Bioremediation is a low-cost but slow process, in which microorganisms degrade or transform contaminants into less hazardous or non-hazardous substances. Many strains are known to be effective as bioremediators, but only under controlled and/or optimal laboratory conditions. Although

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microorganisms exist in extreme environments, most of them still prefer optimal conditions as those created in laboratory (Leung 2004; Mary Kensa 2011; Deshmukh et al. 2016). Co-metabolism is, in this context, a phenomenon by which a recalcitrant compound is degraded in the presence of an analogous degradable compound. Several microorganisms can co-metabolize PAHs, as they are present in the environment as mixtures. Co-metabolism of one PAH could have a synergetic effect on the degradation of another PAH, specifically on the degradation of high molecular weight PAHs (Van Herwijnen et al. 2003). However, when two or more degradable PAHs are present in the medium, metabolic competition may occur (Dean-Ross et al. 2002). Wang et al. (2009) reported some changes in the enzyme production in different type of soils. A significant spectrum of PAHs is biodegradable in aqueous medium, but they are not biodegradable in soil. This fraction is classified as persistent residue with highly reduced bioavailability (Kadri et al. 2017).

Ascomycetes, basidiomycetes and deuteromycetes fungi can produce laccases and among them white-rot basidiomycetes are the most efficient lignin degraders and laccase producers (Yang et al. 2017). Laccases are a family of copper-containing oxidases found in a variety of bacteria, fungi, insects and plants (Forootanfar and Faramarzi 2015). The non-specific nature of their activity on a variety of substrates makes them the ideal catalyst for different industrial applications that are efficient, sustainable and environment friendly. Nonetheless, large-scale applications of laccases are limited by the economy and efficiency of the enzymes (Yang et al. 2017). Depending on the source of activity, peroxidases are classified as lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP). These enzymes usually catalyse the first attack on the PAH molecules (Steffen et al. 2003). MnP and LiP are heme peroxidases, mostly reported for degradation of toxic compounds by basidiomycetes and white rot fungi. Peroxidases also require the presence of hydrogen peroxide and manganese for activity (Deshmukh et al. 2016; Kadri et al. 2017).

Species belonging to the genera *Trichoderma*, *Fusarium*, *Penicillium*, *Stachybotrys*, *Aspergillus*, *Cladosporium*, *Mortierella*, *Beauveri*a, *Engyodontium* are some examples of fungi that are common in polluted soil (Anastasi et al. 2013). These fungi tend to defend the resource against potential invaders, either by sequestering critically limiting nutrients or by producing inhibitory metabolites (Anastasi et al. 2013). Moreover, they are generally characterized by high tolerance to environmental stresses such as the presence of pollutants, and are indicated as potential bioremediation agents in soil (Tigini et al. 2009). Independent of their degradation capabilities, these fungi are capable of decreasing the concentration of organic pollutants such as PAHs, polychlorinated biphenyls (PCB), chlorobenzoic acids (CBA) and endosulfan by accumulating them in intracellular lipid vesicles. Moreover, these vesicles could have a role in biodegradation (Verdin et al. 2004; Anastasi et al. 2013).

On the other hand, amendment of contaminated soil with organic matter has been reported to increase the degradation of PAHs, as well as of other pollutants (Wan et al. 2003; Briceño et al. 2007). It can accelerate or increase biodegradation by the stimulation of microbial activity due to nutrient incorporation to the soil. Kiwi peels and peanut shells are organic residues from food processing industry. They are low cost and highly available residues in Portugal. In 2018, there were 35,410 tons of kiwi fruit produced in Portugal, representing 1.82% of the world production, and up to 30% are peels (Soquetta et al. 2016). The peanut industry is one of the main generators of agro-industrial residues, of which 25% are shells (Perea-Moreno et al. 2018). Annually, there is a world production of this residue of around 11,000,000 tons from the peanut industry that is still unexplored (Duc et al. 2019). These residues are rich in lignocellulose and a potential source of carbon and energy for microorganisms.

The purpose of this study is to elucidate the enzymatic mechanisms involved in the degradation of a mixture of four PAHs by *Trichoderma viride, Penicillium chrysogenum* and *Agrocybe aegerita*. To that, the production of ligninolytic enzymes, namely laccase, manganese peroxidase and lignin peroxidase, during the biodegradation of PAHs in soil by fungal strains was assessed. Furthermore, the effects of different pH of soil, of organic amendments (kiwi peels and peanuts shells) and of co-cultivation of fungi on PAHs degradation and enzyme activities were evaluated and discussed.

Material and methods

Chemicals

Four EPA-priority PAHs were used in this study. Fluorene and pyrene were purchased from Acros Organics, and chrysene and benzo[a]pyrene from Sigma-Aldrich. ABTS and MBTS were acquired from Alfa Aesar. DMAB was obtain from Sigma-Aldrich, and acetonitrile (HPLC grade) and polyvinylpolypyrrolidone were purchased from Fisher Scientific.

Fungal strains

Three fungal strains were studied in this work, namely *T. viride* (EXF8977), *P. chrysogenum* (EXF1818) and *A. aegerita* (EXF3253). These strains were obtained from Infrastructural Centre Mycosmo, MRIC UL, Slovenia. Stock cultures were maintained on malt extract agar plates, at 4 °C.

Substrate pretreatment

Kiwi peels were cut to 1 cm^2 and pretreated with 83 mM of KOH at room temperature (≈ 26 °C) for 20 min, to neutralise organic acid (Stredansky and Conti 1999). After that, they were washed two times with deionised water and dried at 60 °C. Peanut shells were minced to a size of 6 mm, washed, and dried at 60 °C. Prior to use, substrates were autoclaved at 121 °C for 15 min.

Enzyme production

Fungal inoculum

The liquid inoculum was prepared by cutting four agar plugs (5 mm × 5 mm) from malt extract agar plates. These were extruded through a syringe into 500 mL Erlenmeyer flasks containing 200 mL of sterile malt extract (2% w/v). Fungi were cultivated at room temperature, with continuous agitation at 120 rpm, for 3 days for *T. viride* and 5 days for *P. chrysogenum* and *A. aegerita.*

SSF in Erlenmeyer flasks

The 100 mL Erlenmeyer flasks were filled with 20 g of kiwi peels or peanuts shells. These substrates were autoclaved at 121 °C for 15 min and inoculated with 1 mL of fungal biomass per flask and left at room temperature for 6 weeks, with samples collection once a week. The mixture was suspended in 40 mL of 50 mM sodium acetate buffer (pH 5) or phosphate buffer (pH 7) and mixed continuously (100 rpm) for 1 h at room temperature. This suspension was filtered through a nylon cloth and the filtrate was centrifuged at 7500×*g* for 15 min and used for enzymatic assays. SSF was performed in duplicate.

Soil preparation

Commercially available Naturaduba Agro soil (Gintegral—Gestão Ambiental, S.A.), presenting 49.1% organic material, 0.99% N, 0.20% P_2O_5, 0.49% K_2O, 2.17% Ca, 0.26% Mg and 0.17% S was used in this work. The soil was first heated for 2 days at 60 °C. The initial soil pH was 7.2 and the pH adjustment to 5.0 was made with HCl. Four EPA-priority PAHs were used in this study, at 50 ppm each, namely fluorene, pyrene, chrysene, and benzo(a)pyrene. They exist as crystalline solids at room temperature. Stock solution of 1000 ppm for each individual PAH was prepared in acetonitrile and further diluted to the desired concentration. After acetonitrile evaporation, the soil was left at 4 °C for 60 days.

Degradation assays

The degradation assays were done in 500 mL flasks with 180 g of previously spiked soil, pH adjustment, 20 g of soil amendment and a total of 10 mL of fungal inoculum. The degradation time was set to 8 weeks, with samples collection once a week. Assays were done at room temperature, in the dark and in duplicate.

PAHs extraction and UHPLC analyses

Acetonitrile was used to extract PAHs from soil. In short, 1.5 mL of it was added to 0.5 g of soil and extraction was carried out using a rotating shaker at

Table 1 Laccase production (U/g) by SSF on kiwi peels (KP) and peanut shells (PS) by *T. viride*, *P. chrysogenum*, *A. aegerita* and their co-cultures

Fungi + substrate	SSF at pH 5 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	0.89 ± 0.01	0.71 ± 0.02	1.45 ± 0.01	**1.78 ± 0.06**	0.93 ± 0.01	0.18 ± 0.07
T. viride (PS)	0.20 ± 0.06	1.14 ± 0.09	1.52 ± 0.05	**1.78 ± 0.06**	0.81 ± 0.03	0.17 ± 0.02
P. chrysogenum (KP)	0.42 ± 0.08	0.71 ± 0.02	**1.39 ± 0.02**	0.64 ± 0.01	0.53 ± 0.01	0.18 ± 0.07
P. chrysogenum (PS)	0.20 ± 0.02	0.47 ± 0.02	**0.82 ± 0.01**	0.21 ± 0.09	0.25 ± 0.02	0.07 ± 0.07
A. aegerita (KP)	0.51 ± 0.09	1.38 ± 0.04	**6.69 ± 0.04**	6.20 ± 0.05	5.99 ± 0.01	2.39 ± 0.07
A. aegerita (PS)	0.28 ± 0.01	1.26 ± 0.01	**4.71 ± 0.09**	3.75 ± 0.03	2.22 ± 0.02	1.80 ± 0.06
A. aegerita + *T. viride* (KP)	0.21 ± 0.04	1.86 ± 0.03	3.38 ± 0.02	3.61 ± 0.07	**3.92 ± 0.07**	2.39 ± 0.07
A. aegerita + *T. viride* (PS)	0.18 ± 0.01	1.72 ± 0.05	**3.97 ± 0.05**	3.24 ± 0.07	3.75 ± 0.03	2.22 ± 0.02
T. viride + *P. chrysogenum* (KP)	0.89 ± 0.05	**1.48 ± 0.01**	1.39 ± 0.02	0.97 ± 0.02	0.57 ± 0.09	0.54 ± 0.01
T. viride + *P. chrysogenum* (PS)	1.03 ± 0.07	0.33 ± 0.07	**2.26 ± 0.03**	1.20 ± 0.03	0.59 ± 0.03	0.52 ± 0.01
A. aegerita + *P. chrysogenum* (KP)	1.45 ± 0.02	2.55 ± 0.06	**2.68 ± 0.01**	2.67 ± 0.08	2.63 ± 0.02	2.48 ± 0.08
A. aegerita + *P. chrysogenum* (PS)	1.46 ± 0.07	2.77 ± 0.09	**2.93 ± 0.03**	2.65 ± 0.08	2.46 ± 0.01	2.28 ± 0.09
Fungi + substrate	SSF at pH 7 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	0.27 ± 0.02	0.22 ± 0.05	0.77 ± 0.05	0.52 ± 0.02	**1.24 ± 0.05**	0.74 ± 0.02
T. viride (PS)	0.56 ± 0.01	**2.18 ± 0.07**	2.15 ± 0.02	1.93 ± 0.05	1.04 ± 0.03	0.60 ± 0.06
P. chrysogenum (KP)	n.d	n.d	0.48 ± 0.03	0.58 ± 0.05	**1.89 ± 0.09**	0.89 ± 0.03
P. chrysogenum (PS)	n.d	n.d	0.98 ± 0.04	1.16 ± 0.02	**1.17 ± 0.01**	1.19 ± 0.05
A. aegerita (KP)	2.15 ± 0.02	**7.43 ± 0.06**	6.27 ± 0.06	5.80 ± 0.05	4.66 ± 0.07	4.36 ± 0.07
A. aegerita (PS)	2.33 ± 0.07	**4.80 ± 0.05**	3.30 ± 0.01	3.66 ± 0.07	3.99 ± 0.04	2.92 ± 0.01
A. aegerita + *T. viride* (KP)	1.45 ± 0.05	**4.49 ± 0.02**	3.11 ± 0.04	3.75 ± 0.01	2.99 ± 0.01	2.42 ± 0.03
A. aegerita + *T. viride* (PS)	0.37 ± 0.05	1.21 ± 0.05	2.62 ± 0.03	**3.65 ± 0.02**	3.56 ± 0.04	2.68 ± 0.03
T. viride + *P. chrysogenum* (KP)	0.53 ± 0.02	0.54 ± 0.01	1.01 ± 0.09	**1.06 ± 0.02**	0.98 ± 0.01	0.97 ± 0.05
T. viride + *P. chrysogenum* (PS)	0.75 ± 0.03	1.15 ± 0.08	**2.06 ± 0.03**	1.44 ± 0.07	0.96 ± 0.06	0.96 ± 0.02
A. aegerita + *P. chrysogenum* (KP)	1.81 ± 0.04	3.56 ± 0.04	**3.68 ± 0.03**	4.20 ± 0.08	3.83 ± 0.02	3.48 ± 0.08
A. aegerita + *P. chrysogenum* (PS)	1.80 ± 0.05	3.49 ± 0.05	3.59 ± 0.01	**3.87 ± 0.06**	3.86 ± 0.01	3.16 ± 0.09

n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

160 rpm for 30 min and a ultrasound bath for another 10 min. The samples were centrifuged at 8000×*g* and the supernatant was filtered through 25 mm syringe filter and transferred to 2 mL vials for UHPLC analyses.

The quantification of the selected PAHs was performed by ultra-high-performance liquid chromatography, using a Shimadzu Nexera X2 (Shimadzu, USA) with one multi-channel pump (LC-30AD), an autosampler (SIL30AC), an oven (CTO-20AC), a diode array detector (M-20A) and a system controller (CBM-20A) with built-in software (LabSolutions). For PAHs quantification, a Kinetex PAH C18 column (Phenomenex, Inc. CA, USA) was used. The column oven was set at 35 °C. The mobile phase was ultrapure water (pump A) and acetonitrile (pump B). The starting mobile phase composition was 51% A, decreased to 4.5% A in 12.03 min, remaining in this percentage for 16.3 min, increased again to 51% (17.25 min) and remaining in this percentage for 2.35 min. The injection volume was 15 µL at a flow rate of 0.6 mL/min. Samples were monitored by a diode array detector from 190 to 400 nm and chromatograms were extracted at 252 nm.

Table 2 Manganese peroxidase production (U/g) by SSF on kiwi peels (KP) and peanut shells (PS) by *T. viride*, *P. chrysogenum, A. aegerita* and their co-cultures

Fungi + substrate	SSF at pH 5 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	0.07 ± 0.07	0.14 ± .03	0.60 ± 0.04	**3.40 ± 0.03**	2.96 ± 0.02	2.80 ± 0.03
T. viride (PS)	0.07 ± 0.07	0.27 ± 0.05	0.66 ± 0.09	**3.58 ± 0.03**	3.26 ± 0.04	2.92 ± 0.08
P. chrysogenum (KP)	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (PS)	n.d	n.d	n.d	n.d	n.d	n.d
A. aegerita (KP)	0.66 ± 0.11	1.71 ± 0.04	3.46 ± 0.04	**3.58 ± 0.03**	3.00 ± 0.04	2.73 ± 0.02
A. aegerita (PS)	0.62 ± 0.09	1.70 ± 0.05	3.39 ± 0.01	**3.49 ± 0.05**	2.97 ± 0.06	2.93 ± 0.01
A. aegerita + *T. viride* (KP)	0.63 ± 0.01	1.92 ± 0.08	3.51 ± 0.01	**3.58 ± 0.03**	3.26 ± 0.04	2.92 ± 0.08
A. aegerita + *T. viride* (PS)	0.62 ± 0.00	1.90 ± 0.11	**3.46 ± 0.04**	3.39 ± 0.04	3.08 ± 0.04	2.90 ± 0.01
T. viride + *P. chrysogenum* (KP)	0.28 ± 0.04	0.33 ± 0.02	0.65 ± 0.08	**1.29 ± 0.01**	0.74 ± 0.03	0.60 ± 0.07
T. viride + *P. chrysogenum* (PS)	0.28 ± 0.07	0.55 ± 0.06	0.72 ± 0.06	1.10 ± 0.07	**1.11 ± 0.03**	1.02 ± 0.04
A. aegerita + *P. chrysogenum* (KP)	0.44 ± 0.03	0.36 ± 0.08	0.72 ± 0.06	**1.29 ± 0.04**	1.19 ± 0.09	0.89 ± 0.02
A. aegerita + *P. chrysogenum* (PS)	0.31 ± 0.11	0.55 ± 0.04	1.19 ± 0.09	**1.59 ± 0.04**	1.35 ± 0.02	0.76 ± 0.04
Fungi + substrate	SSF at pH 7 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	0.21 ± 0.17	0.41 ± 0.06	0.66 ± 0.08	**0.96 ± 0.01**	0.80 ± 0.03	0.61 ± 0.02
T. viride (PS)	0.29 ± 0.19	0.45 ± 0.07	0.90 ± 0.02	**1.02 ± 0.09**	0.98 ± 0.07	0.70 ± 0.06
P. chrysogenum (KP)	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (PS)	n.d	n.d	n.d	n.d	n.d	n.d
A. aegerita (KP)	0.77 ± 0.11	3.02 ± .02	5.78 ± 0.04	**7.21 ± 0.02**	6.71 ± 0.01	6.12 ± 0.08
A. aegerita (PS)	0.66 ± 0.03	2.83 ± 0.03	5.61 ± 0.07	**6.88 ± 0.08**	5.45 ± 0.01	5.03 ± 0.06
A. aegerita + *T. viride* (KP)	0.63 ± 0.14	1.92 ± 0.08	3.51 ± 0.11	**3.58 ± 0.03**	3.26 ± 0.04	2.92 ± 0.08
A. aegerita + *T. viride* (PS)	0.62 ± 0.02	1.90 ± 0.11	**3.46 ± 0.04**	3.39 ± 0.06	3.08 ± 0.04	2.90 ± 0.01
T. viride + *P. chrysogenum* (KP)	0.25 ± 0.07	0.39 ± 0.02	0.81 ± 0.01	**1.04 ± 0.04**	0.90 ± 0.01	0.68 ± 0.06
T. viride + *P. chrysogenum* (PS)	0.65 ± 0.03	0.38 ± 0.01	0.72 ± 0.08	**1.00 ± 0.04**	0.96 ± 0.07	0.85 ± 0.06
A. aegerita + *P. chrysogenum* (KP)	0.71 ± 0.04	1.72 ± 0.02	2.17 ± 0.03	**2.22 ± 0.04**	2.17 ± 0.01	1.71 ± 0.02
A. aegerita + *P. chrysogenum* (PS)	0.63 ± 0.04	1.33 ± 0.01	2.06 ± 0.01	**2.08 ± 0.01**	1.85 ± 0.01	1.53 ± 0.02

n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

Enzyme extraction and assays

Each week, 18 g of soil were taken from each flask of which 3 g were placed into 15 mL centrifuge tubes with 8 mL of buffer, according to the extracted enzyme. Laccase extraction buffer contained 100 mM sodium acetate, at pH 5 and pH 7, 5 mM $CaCl_2$, 0.05% tween 80 and 1% polyvinylpolypyrrolidone. For manganese peroxidase (MnP) extraction, 50 mM phosphate buffer adjusted to pH 5 and pH 7 was used; and for lignin peroxidase (LiP) extraction 50 mM sodium acetate buffer, pH 5 and pH 7 with 1% polyvinylpolypyrrolidone. Samples were placed on a rotary shaker (100 rpm at 25 °C) for an hour and centrifuged at 7500×*g* for 15 min. Supernatant was transferred to fresh tubes and analysed for each enzyme activity (D'Annibale et al. 2006).

Laccase activity was measured by the oxidation of 2,2-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) in 100 mM sodium acetate buffer, pH 5 or 7, according to the extraction buffer, at 30 °C (Bourbonnais and Paice 1990). MnP reaction mixture contained 100 µM succinate buffer (adjusted at pH 5 or 7) with 0.1 µM of 3-methyl-2-benzthaiazolinone

Table 3 Lignin peroxidase production (U/g) by SSF on kiwi peels (KP) and peanut shells (PS) by *T. viride*, *P. chrysogenum, A. aegerita* and their co-cultures

Fungi + substrate	SSF at pH 5 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (PS)	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (KP)	0.07 ± 0.07	0.15 ± 0.06	0.49 ± 0.03	**0.66 ± 0.09**	0.57 ± 0.07	0.49 ± 0.04
P. chrysogenum (PS)	0.06 ± 0.06	0.13 ± 0.03	0.59 ± 0.04	**0.62 ± 0.05**	0.51 ± 0.03	0.49 ± 0.03
A. aegerita (KP)	0.69 ± 0.02	1.38 ± 0.02	1.61 ± 0.07	2.16 ± 0.09	**2.24 ± 0.09**	2.13 ± 0.08
A. aegerita (PS)	0.45 ± 0.09	0.72 ± 0.06	0.99 ± 0.05	1.24 ± 0.07	**1.75 ± 0.04**	1.52 ± 0.02
A. aegerita + *T. viride* (KP)	0.70 ± 0.06	1.10 ± 0.08	**1.17 ± 0.06**	0.98 ± 0.07	0.77 ± 0.05	0.62 ± 0.08
A. aegerita + *T. viride* (PS)	0.63 ± 0.04	1.02 ± 0.09	**1.07 ± 0.05**	0.88 ± 0.01	0.65 ± 0.03	0.52 ± 0.06
T. viride + *P. chrysogenum* (KP)	0.63 ± 0.04	0.98 ± 0.03	**1.16 ± 0.02**	1.13 ± 0.02	1.10 ± 0.07	1.80 ± 0.07
T. viride + *P. chrysogenum* (PS)	0.62 ± 0.04	0.98 ± 0.07	**1.11 ± 0.01**	1.02 ± 0.02	1.12 ± 0.02	1.73 ± 0.07
A. aegerita + *P. chrysogenum* (KP)	0.63 ± 0.04	0.98 ± 0.03	**2.10 ± 0.08**	1.98 ± 0.07	1.78 ± 0.07	1.65 ± 0.05
A. aegerita + *P. chrysogenum* (PS)	0.62 ± 0.01	0.96 ± 0.05	**2.11 ± 0.01**	1.99 ± 0.02	1.83 ± 0.06	1.79 ± 0.09
Fungi + substrate	SSF at pH 7 (weeks)					
	1	2	3	4	5	6
T. viride (KP)	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (PS)	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (KP)	0.06 ± 0.06	0.28 ± 0.04	0.48 ± 0.02	0.54 ± 0.07	**0.64 ± 0.04**	0.54 ± 0.08
P. chrysogenum (PS)	0.06 ± 0.06	0.42 ± 0.03	0.55 ± 0.06	**0.72 ± 0.06**	0.65 ± 0.03	0.65 ± 0.03
A. aegerita (KP)	0.06 ± 0.06	0.36 ± 0.04	0.54 ± 0.07	**0.69 ± 0.02**	0.51 ± 0.04	0.51 ± 0.04
A. aegerita (PS)	0.44 ± 0.02	0.69 ± 0.02	0.72 ± 0.06	2.01 ± 0.04	**2.08 ± 0.04**	1.69 ± 0.09
A. aegerita + *T. viride* (KP)	0.69 ± 0.02	0.99 ± 0.05	1.09 ± 0.07	**1.79 ± 0.05**	1.39 ± 0.01	2.64 ± 0.01
A. aegerita + *T. viride* (PS)	0.69 ± 0.02	0.72 ± 0.06	0.95 ± 0.01	**1.70 ± 0.05**	1.38 ± 0.02	0.94 ± 0.05
T. viride + *P. chrysogenum* (KP)	0.54 ± 0.07	0.62 ± 0.08	0.89 ± 0.02	**1.16 ± 0.02**	1.04 ± 0.01	0.83 ± 0.06
T. viride + *P. chrysogenum* (PS)	0.28 ± 0.04	0.48 ± 0.02	0.64 ± 0.04	**0.90 ± 0.03**	0.60 ± 0.07	0.60 ± 0.07
A. aegerita + *P. chrysogenum* (KP)	0.42 ± 0.03	0.55 ± 0.06	0.72 ± 0.06	**1.43 ± 0.09**	0.89 ± 0.02	0.62 ± 0.08
A. aegerita + *P. chrysogenum* (PS)	0.36 ± 0.04	0.54 ± 0.07	0.69 ± 0.02	**1.09 ± 0.02**	0.76 ± 0.04	0.52 ± 0.06

n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

hydrazone hydrochloride (MBTH), 5 µl of 3-dimethylaminobenzoic acid (DMAB), 0.2 µM of $MnSO_4$ and 0.1 µM H_2O_2. LiP assay was performed with similar procedure for MnP assay except that H_2O_2 was omitted (D'Annibale et al. 2006; Pawar 2015). Enzyme activities were measured using a spectrophotometer (BioTek Synergy HT) at 420 nm (laccase), 590 nm (MnP) and 401 nm (LiP). One unit of enzyme activity was defined as the amount of enzyme that produces 1 µmol of product per min under the assay conditions.

Results and discussion

Evaluation of SSF enzyme production

SSF mimics the conditions under which fungi grow naturally (Singh Nee Nigam and Pandey 2009), as lignocellulosic residues promote a better fungal growth due to the polysaccharides and lignin present in these materials (Couto and Toca-Herrera 2007).

A screening of fungi that produce lignin-modifying enzymes was performed, aiming to evaluate the production of laccase, lignin peroxidase and

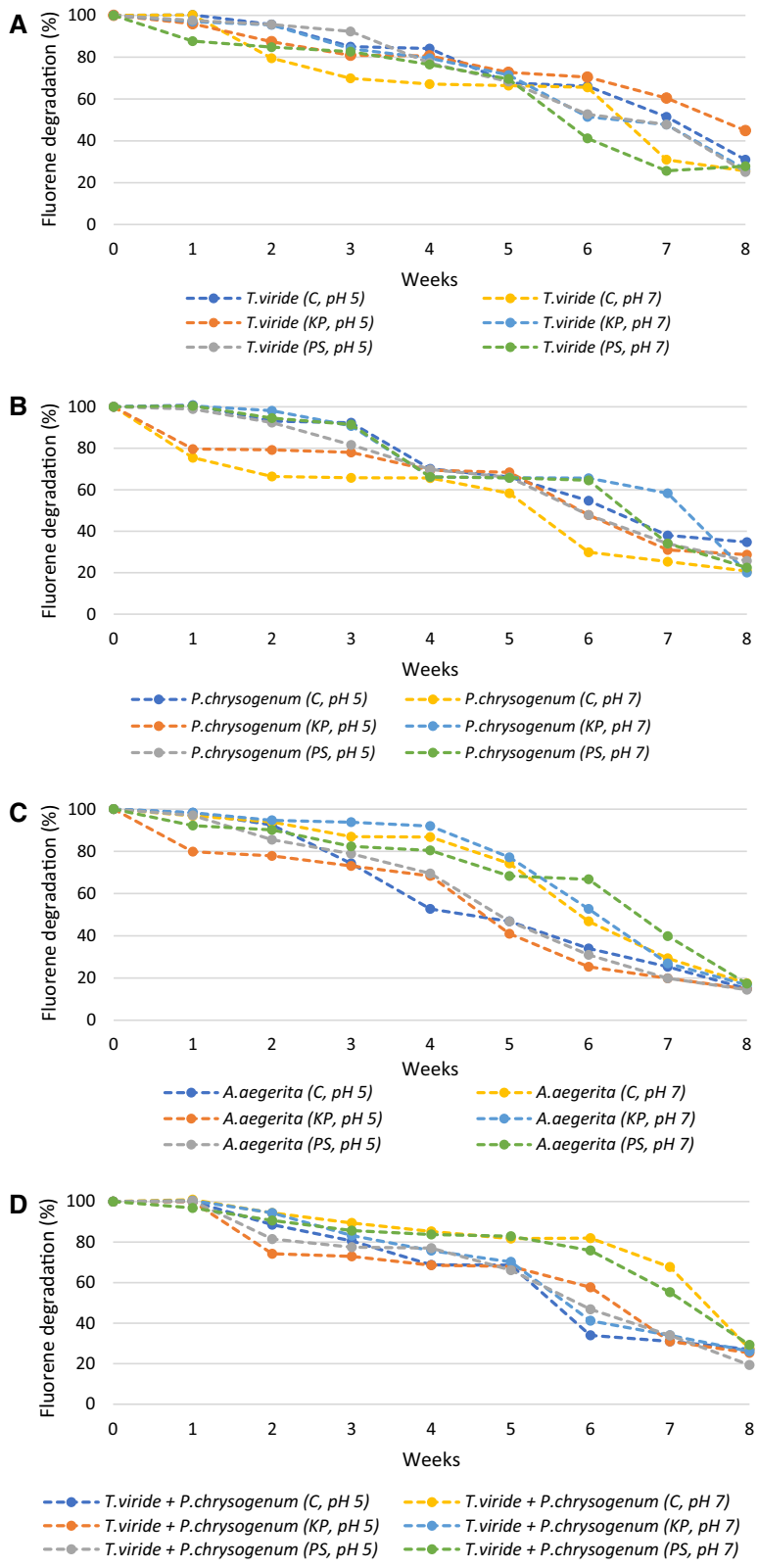
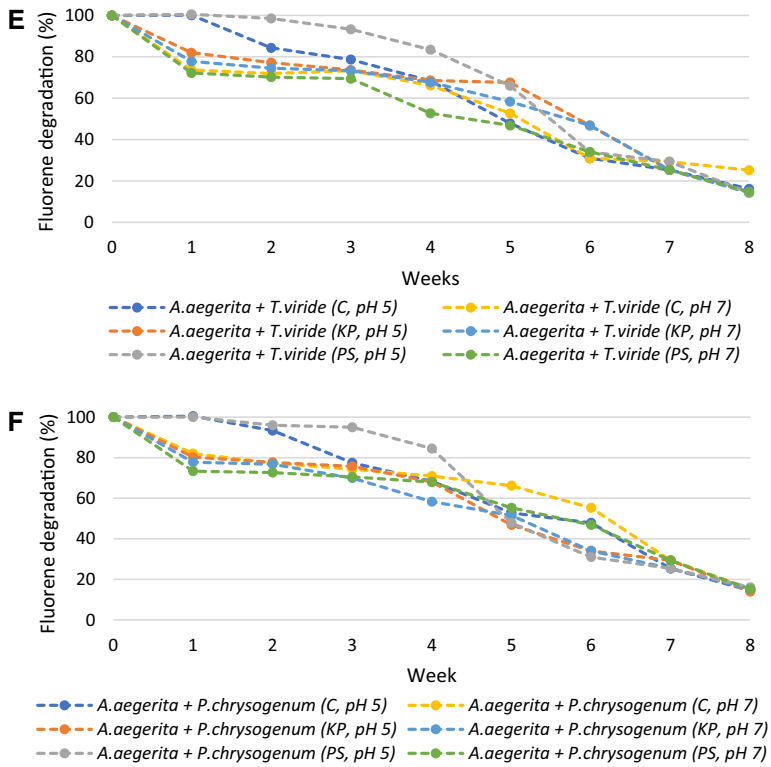


Fig. 1 Fluorene degradation by the fungal strains: *T. viride* (**A**), *P. chrysogenum* (**B**), *A. aegerita* (**C**), and their co-culture: *T. viride* + *P. chrysogenum* (**D**), *A. aegerita* + *T. viride* (**E**) and *A. aegerita* + *P. chrysogenum* (**F**), in soil with a mixture of selected PAHs, at pH 5 and pH 7, using the substrates kiwi peels (KP) and peanut shells (PS), and without substrate (**C**)

Fig. 1 continued



manganese peroxidase (Tables 1, 2, 3). For that, three fungal strains were evaluated through SSF, using kiwi peels and peanut shells as substrates at pH 5 and 7. *Penicillium chrysogenum* and *T. viride* were selected for this study as they are normally present in soil with different organic pollutants, despite not reaching such high production of enzymes as *A. aegerita*, which belong to the litter decomposing fungi. The highest enzymatic production was obtained for *A. aegerita* grown in kiwi peels, with the highest laccase levels of 7.43 U/g at pH 7, with 2 weeks fermentation (Table 1). For MnP, 7.21 U/g was achieved at pH 7 with 4 weeks fermentation (Table 2), while for LiP 2.24 U/g was obtained at pH 5, with 5 weeks fermentation (Table 3). When peanut shells was used as substrate, the maximum production was reached at the same pH and fermentation time, with 4.8 U/g for laccase and 6.88 U/g for MnP, while for LiP 2.08 U/g was reached at pH 7, after 5 weeks fermentation.

Penicillium chrysogenum and *T. viride* also achieved better laccase production at pH 7, with 2.18 U/g at 2 weeks fermentation on peanut shells for *T. viride,* and 1.89 U/g at 5 weeks fermentation on kiwi peels for *P. chrysogenum*. There was no detection of MnP activity on *P. chrysogenum* extracts, but *T. viride* was able to produce 3.58 U/g at pH 5, after 4 weeks fermentation on peanut shells. *T. viride* was not able to produce LiP, while traces of this activity were detected in the extracts of *P. chrysogenum*. Therefore, both organic residues were effective for the enzymes production, which could be related to their chemical composition. Kiwi peels contain 53.73% of carbohydrates, 3.84% of protein, 6.93% of soluble and 18.92% of insoluble dietary fibres (Soquetta et al. 2016), 25.26% of lignin (Gençer 2015), free sugars and fatty acids (Dias et al. 2020), besides higher mineral content than their respective edible parts (Soquetta et al. 2016). Peanut shells are rich in many functional compounds as cellulose, hemicellulose and lignin (Duc et al. 2019), in which lignin can represent up to 41.3% of the lignocellulose, being this value 2 to 4 times higher than for most common agricultural residues, such as rice straw, wheat straw, sugarcane bagasse and corn cob/stover (Anike et al. 2016).

Co-cultivation of the studied fungi was also investigated in order to improve the production of lignolytic enzymes. Usually, in lab conditions there are no interactions as competition, symbiotic or

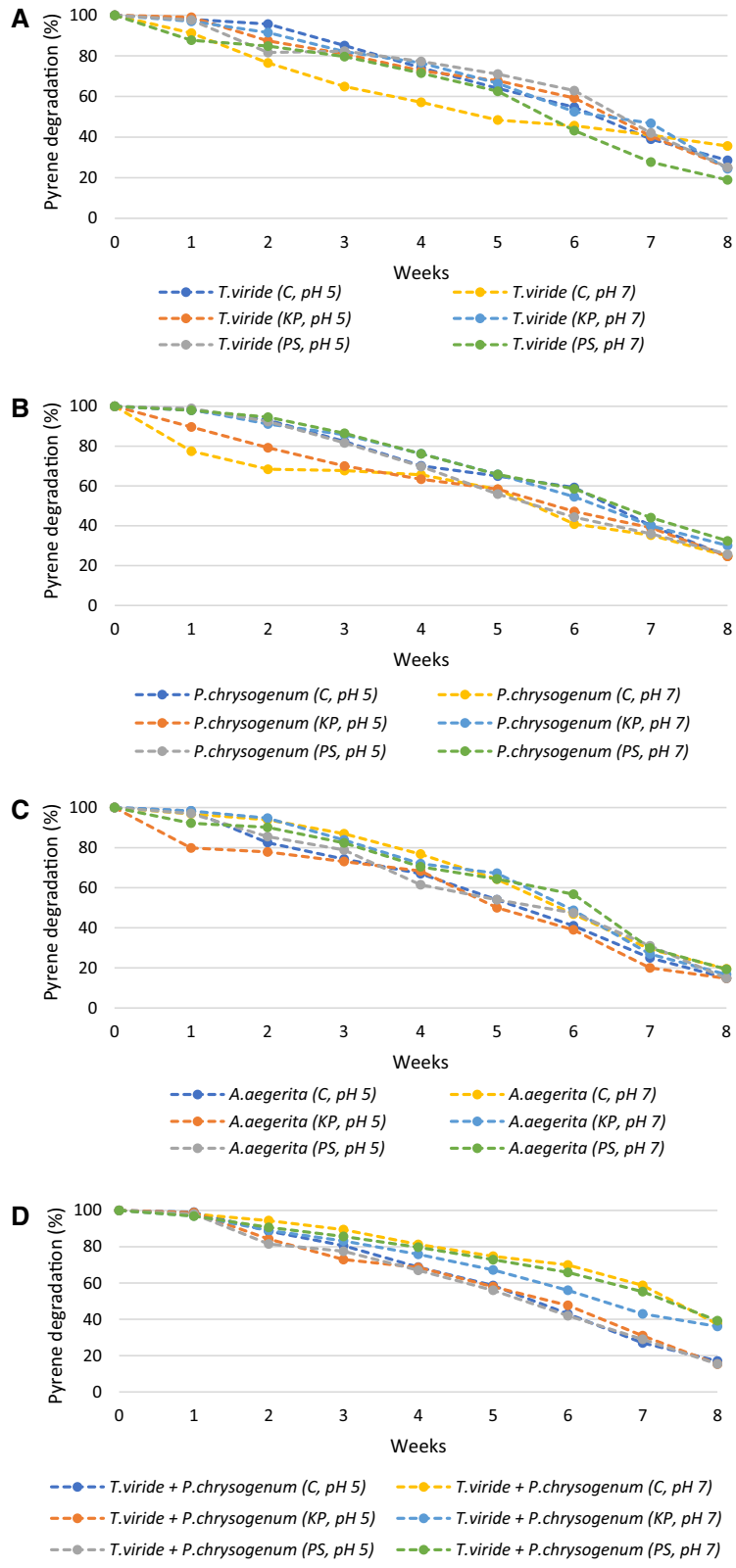
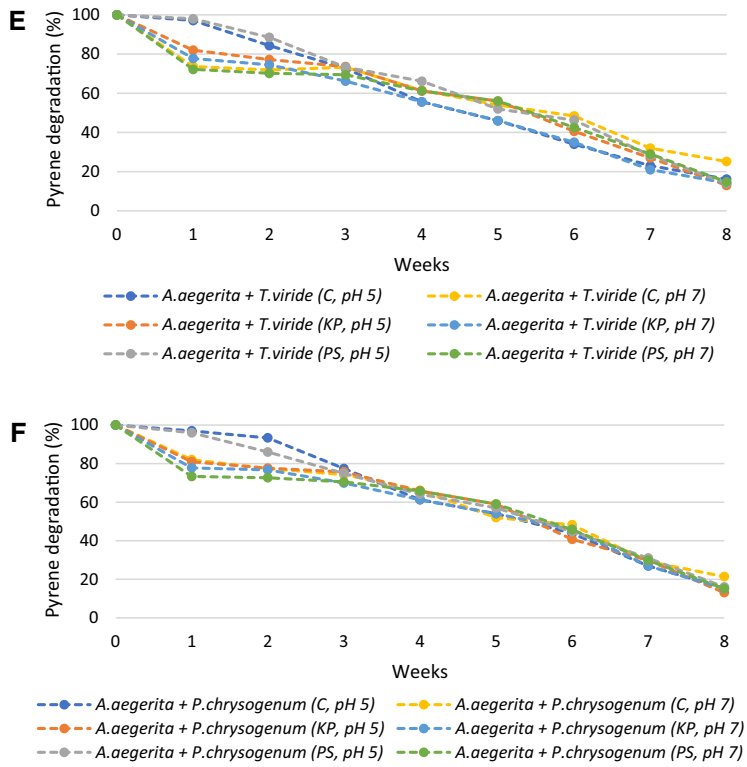


Fig. 2 Pyrene degradation by the fungal strains: *T. viride* (**A**), *P. chrysogenum* (**B**), *A. aegerita* (**C**), and their co-culture: *T. viride* + *P. chrysogenum* (**D**), *A. aegerita* + *T. viride* (**E**) and *A. aegerita* + *P. chrysogenum* (**F**), in soil with a mixture of selected PAHs, at pH 5 and pH 7, using the substrates kiwi peels (KP) and peanut shells (PS), and without substrate (**C**)

Fig. 2 continued



antagonistic effects with just one species cultivation, while co-cultivation of microorganisms might be a better approach to induce diversity in enzymatic production, as co-cultivations frequently allow exposure to other chemical signals, which could be overlooked in mono species cultivation. Moreover, genomic sequencing analyses even show that some secondary metabolism is not triggered or/and expressed when species are exposed to laboratory conditions (Rutledge and Challis 2015). Thus, co-cultivation may promote the activation of genes that are not detected in single cultivation, which enables a better understanding of cultures in natural environment (Marmann et al. 2014). In general, no synergetic effect was observed when the microorganisms were co-cultivated, except for LiP activity, for which the enzyme levels detected in the co-culture of *P. chrysogenum* and *T. viride* were higher than those measured for each species grown separately. However, and regardless of that, the levels of enzyme activity were always higher for *A. aegerita*.

Many studies have reported the study of several fungi and lignocellulosic residues for the production of ligninolytic enzymes, which resulted in different levels of enzyme production. For example, Balaji et al. (Balaji et al. 2014) isolated 21 potential PAHs-degrading fungi from petroleum hydrocarbons contaminated soil samples, with *T. viride* and *P. chrysogenum* among them. *T. viride* was able to produce 23 ± 0.05 U/mL of laccase, and *P. chrysogenum* produced 79 ± 0.60 U/mL of laccase and 47 ± 0.01 U/mL of peroxidase (Balaji et al. 2014).

Other authors have reported the production of lignin-degrading enzymes by these microorganisms. For example, Cabrera et al. (2020) obtained a maximum production of laccase, 0.22 U/mL, by *T. viride* M5-2 after 48 h in submerged fermentation with wheat bran. The maximum laccase production by *T. viride* isolated from coconut husk was 0.65 U/mL after 96 h of incubation in medium with guaiacol (Divya et al. 2014). Senthivelan and his team (Senthivelan et al. 2019) obtained an optimal laccase production (7.9 U/mL) with 5 days cultivation by *P. chrysogenum* in optimized medium.

Other fungi have also been reported regarding the production of these enzymes, as well as the use of lignocellulosic residues as peanut shells. The fungus *Pycnoporus* SYBC-L3 reached the highest activity of

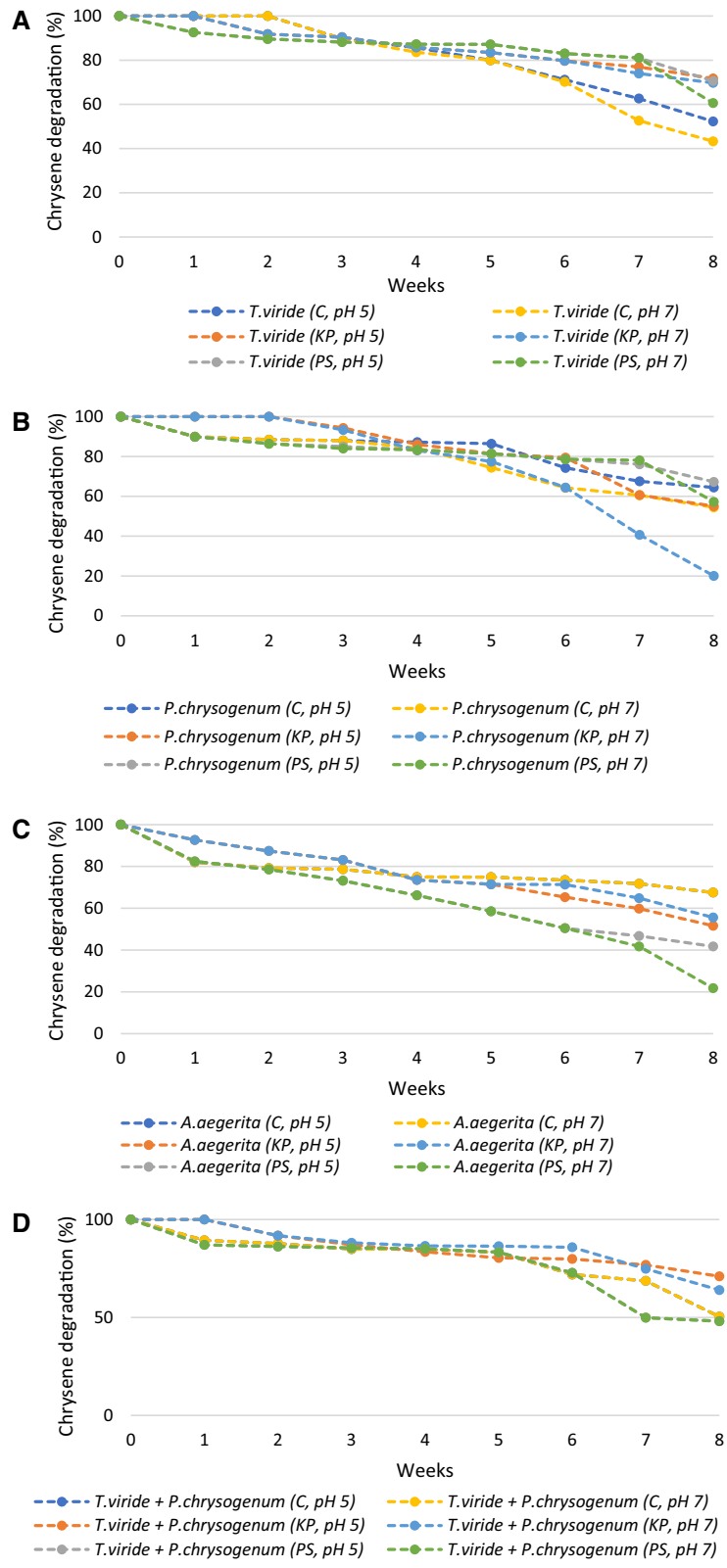
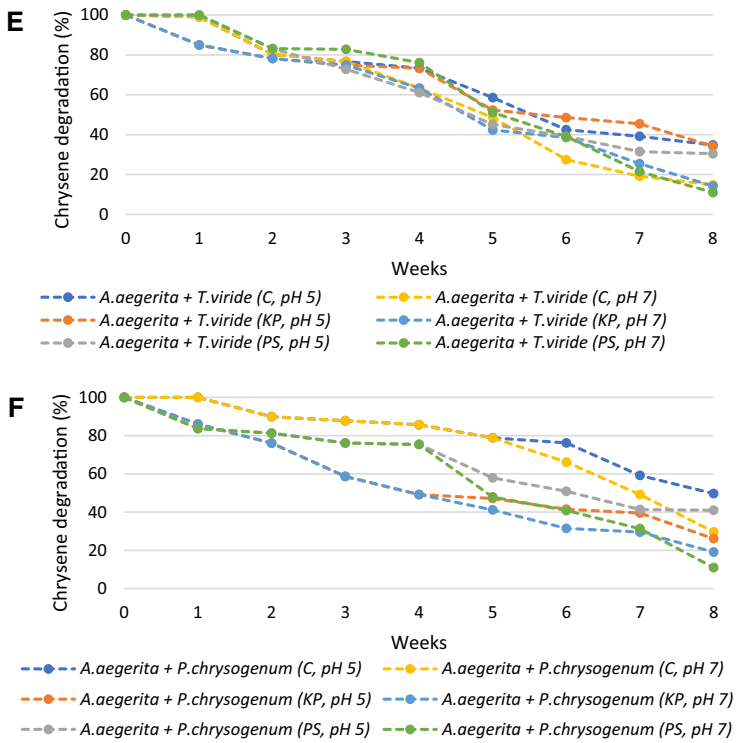


Fig. 3 Chrysene degradation by the fungal strains: *T. viride* (**A**), *P. chrysogenum* (**B**), *A. aegerita* (**C**), and their co-culture: *T. viride* + *P. chrysogenum* (**D**), *A. aegerita* + *T. viride* (**E**) and *A. aegerita* + *P. chrysogenum* (**F**), in soil with a mixture of selected PAHs, at pH 5 and pH 7, using the substrates kiwi peels (KP) and peanut shells (PS), and without substrate (**C**)

Fig. 3 continued



∼5 U/g in peanut shells after 25 days of assays (Liu et al. 2018). *Funalia trogii* IFP0027 grown on peanut shells was able to produce 11.9 U/mL of laccase after 144 h of fermentation (Li et al. 2017). *Ganoderma lucidum* cultivated on peanut shells produced on the 4th day a maximum amount of 332 U/mL of MnP, and with 70% (w/w) of moisture MnP production increased to 545 U/mL in 5 days fermentation. At the same time, the highest production of LiP (95 U/mL) was reported within 9 days and laccase (44 U/mL) within 5 days of fermentation (Nisa et al. 2014). In liquid basal medium *A. aegerita* was capable of producing 30.80 ± 0.51 U/L of laccase, 52.29 ± 0.11 U/L of MnP and 18.63 ± 0.05 U/L of LiP after 14 days cultivation at 27 °C (Shantaveera and Ramalingappa 2015). A different combination of solid waste, wheat straw and millet was used as cultivation substrate for *A. aegerita* 156 (Isikhuemhen et al. 2009). Laccase production varied between 0.1 and 6.2 U/g and MnP activity between 0.3 and 0.9 U/g. However, after fruiting (beyond 78 days), laccase and peroxidases activity decreased.

PAHs degradation

The distribution of PAHs in soil depends mainly on the PAHs hydrophobicity and their affinity towards microcompartments of the aggregates (Styrishave et al. 2012). PAH molecules with higher hydrophobicity and more aromatic rings present stronger sorption affinity (Zhang et al. 2014). In the current work, artificially contaminated soil was used. So, the desorption rate of PAHs in soil is expected to be higher than naturally contaminated soil, as observed by Huesemann et al. (2004) for anthracene degradation in freshly spiked soil (72% degradation) and aged soil (34% degradation).

The potential of the studied fungi, as well as the influence of the cultivation strategy (axenic culture or co-culture), of soil amendment (kiwi peels and peanut shells) and of soil pH (5 and 7) in the degradation of PAHs in soil were evaluated. In general, *A. aegerita* was the most promising fungus for PAHs degradation and the co-cultivation did not improve the degradation of the pollutants, with exception of chrysene. Also, pH has a higher influence on PAHs degradation than either amendment.

Fluorene (Fig. 1A) was more degradable in soil at pH 5 as *A. aegerita* was able to degrade around 85% of this pollutant after 8 weeks degradation, using peanut shells or kiwi peels as soil amendment. Similar degradation percentages were achieved with the co-cultivation of *A. aegerita* and *P. chrysogenum* (86.04%) and *A. aegerita* and *T. viride* (85.76%). Degradation of pyrene (Fig. 1B) after 8 weeks ranged between 61 and 87%. In all combinations better degradation results were obtain at soil pH 5, with the highest degradation by co-cultivation of *A. aegerita* and *T. viride,* and of *A. aegerita* and *P. chrysogenum* on kiwi peels, 87% and 86.94%, respectively. Axenic culture of *A. aegerita* was able to degrade 85.22% of pyrene.

There was less chrysene remaining (Fig. 1C) at soil pH 7, with the highest degradation obtained by co-cultivation of *A. aegerita* and *T. viride,* and *A. aegerita* and *P. chrysogenum* on peanuts shells, 89%. Chrysene degradation by *A. aegerit*a corresponded to 78%. A different pattern of degradation was observed with benzo[a]pyrene (Fig. 1D), as one of most persistent PAHs. *Trichoderma viride* was the least efficient at the benzo[a]pyrene degradation, being only capable of 4.32–30.65% of degradation. *Agrocybe aegerita* alone achieved the highest degradation at soil pH 5, 86.83%, without soil amendment. With kiwi peels as soil amendment, 86.52% degradation was observed, and with peanut shells 81.31%. *Penicillium chrysogenum* grew better at soil pH 5, with a degradation ranging between 57.8 and 71.3% (Figs. 2, 3, 4).

In this study a higher degradation rate is observed at soil pH 5 for fluorene, pyrene, and benzo[a]pyrene, and in the case of chrysene, a higher degradation was observed at soil pH 7. Pawar (Pawar 2015) observed higher and faster degradation by consortium of bacterial population, at pH 7.5, at which the nutrient availability is greater. However, fungi grew actively at acidic pH 5 and 5.5, which also indicates that fungi are more tolerant to acidic soil than bacteria (Pawar 2015).

The distribution of PAHs in soil depends mainly on the PAHs hydrophobicity and their affinity towards microcompartments of the aggregates (Styrishave et al. 2012). In situ degradation of PAHs is often slow, as PAHs get more and more persistent the longer they stay in soil. Their persistence may be affected also by nutrients (greater availability at neutral pH), by their bioavailability (sorption on particles), temperature, oxygen, pH and by the presence of PAHs-degrading microorganisms. According to Pawar (Pawar 2015), there are different dominant fungal species depending on the soil pH. *Penicillium* species predominated at acidic soil pH and with fewer *Aspergillus* populations, whereas at alkaline conditions (pH 8.0 and 8.5) *Aspergillus* was predominant and *Penicillium* was not detected. However, that study indicated that fungi play an active role in the degradation at acidic pH and bacteria play role in degradation at neutral soil pH. Chupungars et al. (2009) reported that *Agrocybe* sp. CU-43, isolated from Thailand soil was able to degrade 100 ppm of fluorene in N-limiting medium (pH 4.5, 130 rpm at 28 °C) in 6 days. By increasing concentration to 250 and to 500 ppm, the time needed to complete degradation increased to 3 and 4 weeks, respectively.

Evaluation of enzyme production during PAHs degradation

Enzymatic activities in the soil were assessed considering that they depend on environmental factors and on co-existing chemicals as reported by Bonomo et al. (2001), supporting that enzyme activities are affected by the soil pH. The obtained results of the current study are presented in Tables 4, 5 and 6. The highest laccase levels were detected in the extracts of *A. aegerita* (Table 4), being the laccase activity levels higher in soil at neutral pH than at pH 5. During PAHs degradation by *A. aegerita* with kiwi peels as soil amendment, 2.15 U/g of laccase were detected after the 1st week, increasing to 6.43 U/g after the 2nd week, which was the highest value attained starting then to decrease slowly. At the same time, it was also capable of producing of MnP (Table 5), reaching 3.02 U/g after the 2nd week, increasing up to 7.21 U/g at the end of the 4th week and decreasing very slowly to 5.88 U/g at the end of the process (8 weeks). Lower production was observed with peanut shells as soil amendment, with a maximum production of laccase of 4.80 U/g and 6.88 U/g of MnP. LiP activity was also detected in the extracts of *A. aegerita*, being that of 3.39 U/g with kiwi peels as soil amendment and 2.57 U/g on peanut shells, both in soil at pH 7.

On the other hand, laccase and MnP levels reached with *T. viride* were higher at soil pH 5, with 1.78 U/g at the 5th week and 1.17 U/g at the 4th week, respectively, both with kiwi peels. Around 1.69 U/g of laccase were detected on *P. chrysogenum* extracts

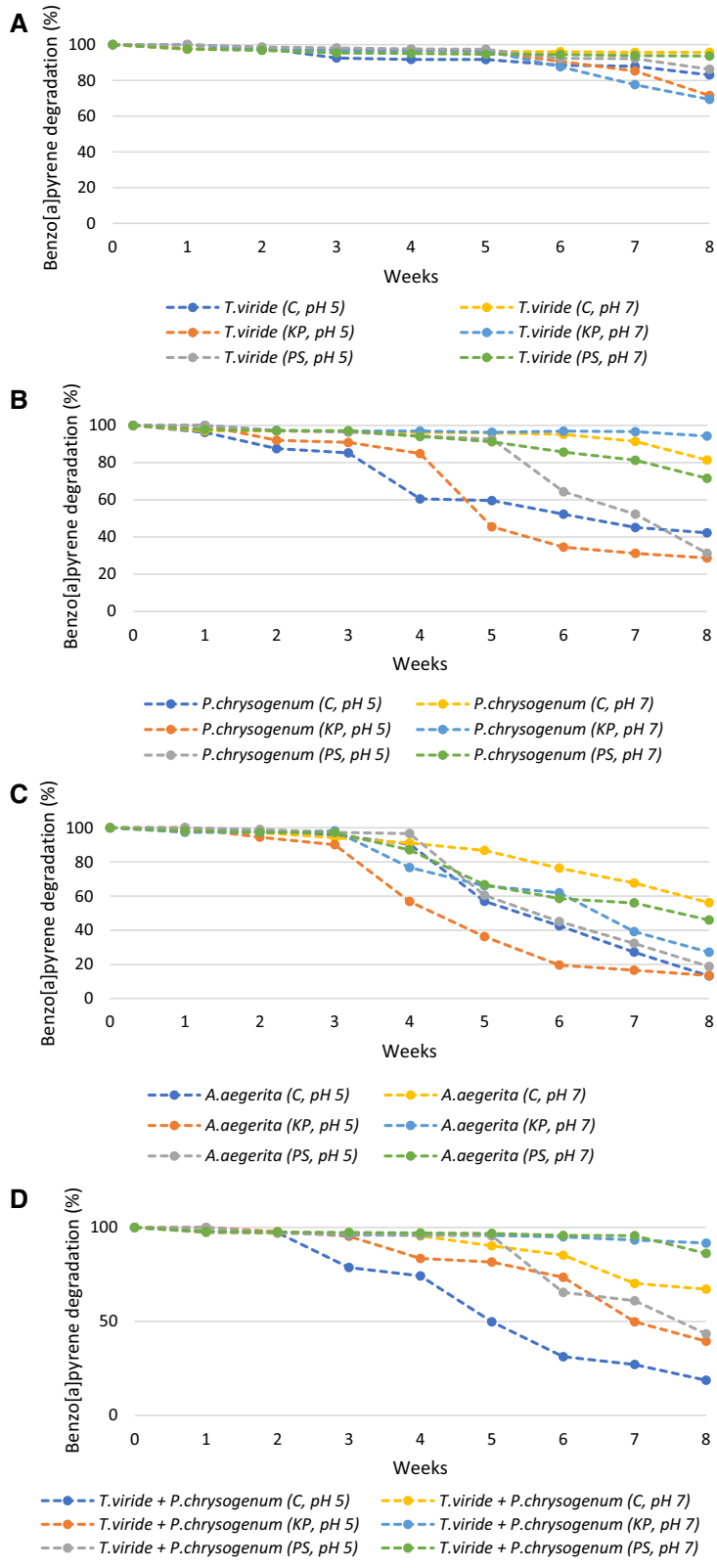
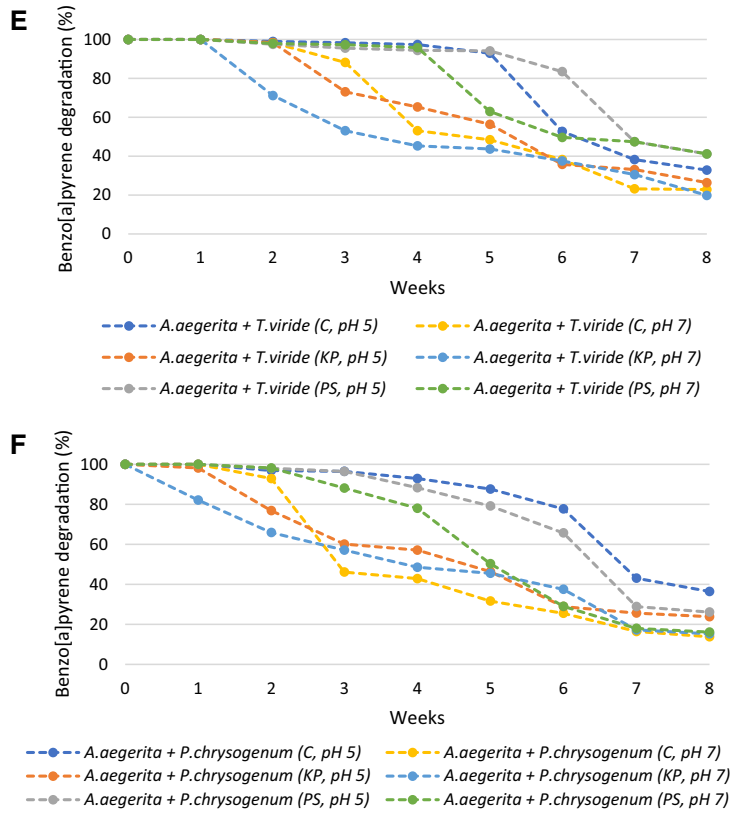


Fig. 4 Benzo[a]pyrene degradation by the fungal strains: *T. viride* (**A**), *P. chrysogenum* (**B**), *A. aegerita* (**C**), and their co-culture: *T. viride* + *P. chrysogenum* (**D**), *A. aegerita* + *T. viride* (**E**) and *A. aegerita* + *P. chrysogenum* (**F**), in soil with a mixture of selected PAHs, at pH 5 and pH 7, using the substrates kiwi peels (KP) and peanut shells (PS), and without substrate (**C**)

Fig. 4 continued



from soil at pH 5 with kiwi peels (4th week), while no MnP activity was observed. Despite, the highest PAHs degradation potential was observed in soil at pH 5 by *A. aegerita*, simultaneous production of enzymes was more modest at this pH, being maximum in soil at pH 7. However, the highest levels of enzymes produced by *T. viride* and *P. chrysogenum* were detected in soil at pH 5, showing in these cases that PAHs degradation appear to be correlated with enzyme production.

In natural soil, the enzyme activities are impaired due to absorption and immobilization between soil particles and organic matter depending on the soil type (Naseby and Lynch 1997). In the study of Ting et al. (2011) the laccase production by *G. lucidum* was enhanced when PAHs were present. Laccase activity peaked on the 6th day, and then dropped slowly in cultures. The maximum mean laccase activity, 670 U/L, was detected in the cultures with 20 mg/L of PAHs (phenanthrene and pyrene). When the concentrations of PAHs in cultures were elevated to 50 and to 100 mg/L, laccase activities were reduced to 450 and 220 U/L, respectively. At the same time, the highest PAHs degradation and laccase production with *G. lucidum* in liquid medium was observed at pH 4, on 6th day with 812 U/L (Ting et al. 2011). In Cao and co-workers work (Cao et al. 2020), during the benzo[a]-pyrene degradation in liquid culture by the *L. theobromae,* 123 U/L of LiP and 424.9 U/L of laccase were detected at 4th day. The LiP and laccase activities increased during the initial period and started decreasing after the peak on the 4th day, when benzo[a]pyrene degradation started. The changing enzyme activities agreed with the benzo[a]pyrene degrading rates, which suggested that these enzymes were involved in its degradation (Cao et al. 2020).

Conclusions

Three fungal strains were evaluated in this study. All combinations were able to degrade fluorene, pyrene, chrysene, and benzo[a]pyrene. The degradation potential was affected by pH, co-cultivation and soil amendment. Agricultural residues used as soil

Table 4 Laccase activity (U/g) during PAH degradation in soil at pH 5 and pH 7 by *T. viride*, *P. chrysogenum, A. aegerita* and their co-culture

Fungi + substrate	pH 5 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	0.07 ± 0.07	0.89 ± 0.01	0.7 ± 0.02	1.45 ± 0.04	**1.61 ± 0.02**	0.70 ± 0.07	0.37 ± 0.05	n.d
T. viride (KP)	0.07 ± 0.07	1.20 ± 0.01	1.14 ± 0.09	1.52 ± 0.05	**1.78 ± 0.06**	0.93 ± 0.01	0.18 ± 0.07	n.d
T. viride (PS)	0.07 ± 0.07	0.42 ± 0.08	0.71 ± 0.02	**1.39 ± 0.02**	0.64 ± 0.01	0.25 ± 0.02	0.07 ± 0.07	n.d
P. chrysogenum (C)	0.06 ± 0.06	0.20 ± 0.05	0.47 ± 0.05	**0.82 ± 0.01**	0.21 ± 0.09	n.d	n.d	n.d
P. chrysogenum (KP)	0.06 ± 0.06	0.51 ± 0.09	0.93 ± 0.08	**1.69 ± 0.04**	0.20 ± 0.05	n.d	n.d	n.d
P. chrysogenum (PS)	0.06 ± 0.06	0.28 ± 0.01	0.61 ± 0.02	**0.95 ± 0.04**	0.54 ± 0.01	n.d	n.d	n.d
TV + *PC* (C)	0.12 ± 0.03	0.11 ± 0.03	0.20 ± 0.07	**0.68 ± 0.01**	0.58 ± 0.1	0.55 ± 0.03	n.d	n.d
TV + *PC* (KP)	0.12 ± 0.03	1.04 ± 0.01	**1.48 ± 0.01**	1.39 ± 0.03	0.97 ± 0.02	0.57 ± 0.09	0.54 ± 0.01	n.d
TV + *PC* (PS)	0.12 ± 0.03	1.03 ± 0.07	1.33 ± 0.07	**2.26 ± 0.03**	1.20 ± 0.03	0.59 ± 0.03	0.52 ± 0.01	n.d
A. aegerita (C)	0.17 ± 0.02	1.75 ± 0.01	2.31 ± 0.03	2.63 ± 0.09	**2.69 ± 0.04**	2.34 ± 0.06	1.72 ± 0.05	1.53 ± 0.02
A. aegerita (KP)	0.21 ± 0.04	1.86 ± 0.02	3.38 ± 0.02	3.61 ± 0.07	**3.92 ± 0.07**	2.39 ± 0.07	1.83 ± 0.06	1.00 ± 0.01
A. aegerita (PS)	0.18 ± 0.01	1.72 ± 0.05	**3.97 ± 0.05**	3.24 ± 0.07	**3.75 ± 0.03**	2.22 ± 0.02	1.80 ± 0.06	1.00 ± 0.09
AA + *TV* (C)	0.25 ± 0.01	0.75 ± 0.06	0.97 ± 0.05	1.21 ± 0.0	**1.11 ± 0.03**	1.02 ± 0.04	1.00 ± 0.01	0.92 ± 0.04
AA + *TV* (KP)	0.35 ± 0.02	0.55 ± 0.03	0.84 ± 0.03	1.63 ± 0.02	**2.05 ± 0.06**	1.58 ± 0.05	1.52 ± 0.07	1.50 ± 0.02
AA + *TV* (PS)	0.35 ± 0.02	0.43 ± 0.02	0.82 ± 0.03	**1.11 ± 0.03**	1.02 ± 0.04	0.70 ± 0.04	0.61 ± 0.01	0.57 ± 0.01
AA + *PC* (C)	0.11 ± 0.03	0.12 ± 0.01	0.37 ± 0.02	**1.29 ± 0.01**	1.02 ± 0.04	0.82 ± 0.04	0.80 ± 0.04	0.78 ± 0.01
AA + *PC* (KP)	0.17 ± 0.01	0.27 ± 0.05	0.48 ± 0.05	**2.24 ± 0.09**	1.29 ± 0.04	1.35 ± 0.12	1.15 ± 0.12	1.11 ± 0.01
AA + *PC* (PS)	0.12 ± 0.03	0.25 ± 0.02	0.43 ± 0.01	**1.75 ± 0.04**	1.59 ± 0.04	1.19 ± 0.09	1.09 ± 0.09	1.28 ± 0.02
Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	0.07 ± 0.07	0.21 ± 0.02	0.60 ± 0.04	0.66 ± 0.09	**0.70 ± 0.07**	0.59 ± 0.08	0.48 ± 0.09	0.21 ± 0.02
T. viride (KP)	0.07 ± 0.07	0.46 ± 0.05	0.73 ± 003	0.87 ± 0.02	**0.93 ± 0.01**	0.56 ± 0.05	0.56 ± 0.01	0.55 ± 0.01
T. viride (PS)	0.07 ± 0.07	0.32 ± 0.06	0.72 ± 0.06	0.79 ± 0.01	**0.96 ± 0.06**	0.96 ± 0.02	0.74 ± 0.02	0.60 ± 0.06
P. chrysogenum (C)	n.d	0.06 ± 0.06	0.55 ± 0.03	**0.67 ± 0.05**	0.53 ± 0.02	n.d	n.d	n.d
P. chrysogenum (KP)	n.d	0.06 ± 0.06	0.66 ± 0.01	**0.75 ± 0.04**	0.69 ± 0.02	0.60 ± 0.07	0.43 ± 0.04	n.d
P. chrysogenum (PS)	n.d	0.06 ± 0.06	0.65 ± 0.08	**0.69 ± 0.07**	0.61 ± 0.09	0.53 ± 0.02	0.41 ± 0.04	n.d
TV + *PC* (C)	0.12 ± 0.03	0.23 ± 0.02	0.62 ± 0.04	0.68 ± 0.09	**0.71 ± 0.07**	0.69 ± 0.02	0.59 ± 0.08	0.48 ± 0.09
TV + *PC* (KP)	0.12 ± 0.03	0.47 ± 0.05	0.79 ± 003	0.91 ± 0.02	**0.98 ± 0.01**	0.97 ± 0.05	81 ± 0.01	0.69 ± 0.07
TV + *PC* (PS)	0.12 ± 0.03	0.31 ± 0.06	0.78 ± 0.06	0.86 ± 0.01	**0.96 ± 0.06**	0.96 ± 0.02	0.84 ± 0.02	0.77 ± 0.02
A. aegerita (C)	2.05 ± 0.04	4.31 ± 0.03	4.63 ± 0.09	**4.69 ± 0.04**	2.34 ± 0.06	1.82 ± 0.05	1.03 ± 0.08	0.93 ± 0.02

Table 4 continued

Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
A. aegerita (KP)	2.15 ± 0.02	**6.43 ± 0.06**	6.27 ± 0.06	5.80 ± 0.05	3.99 ± 0.04	2.92 ± 0.01	1.94 ± 0.08	1.38 ± 0.07
A. aegerita (PS)	2.33 ± 0.07	**4.80 ± 0.05**	4.30 ± 0.15	4.66 ± 0.07	2.80 ± 0.02	1.93 ± 0.08	1.82 ± 0.01	1.03 ± 0.08
AA + TV (C)	0.55 ± 0.07	1.73 ± 0.03	2.99 ± 0.01	**3.42 ± 0.03**	2.86 ± 0.01	2.80 ± 0.01	2.00 ± 0.04	2.00 ± 0.01
AA + TV (KP)	0.76 ± 0.01	1.81 ± 0.04	3.56 ± 0.04	**3.68 ± 0.03**	3.20 ± 0.08	2.83 ± 0.02	2.48 ± 0.08	2.26 ± 0.04
AA + TV (PS)	0.72 ± 0.09	1.80 ± 0.05	3.49 ± 0.05	**3.59 ± 0.01**	2.87 ± 0.06	2.86 ± 0.01	2.16 ± 0.09	2.05 ± 0.03
AA + PC (C)	0.55 ± 0.07	1.73 ± 0.03	2.99 ± 0.01	**3.42 ± 0.03**	2.86 ± 0.01	2.80 ± 0.01	2.00 ± 0.04	2.00 ± 0.01
AA + PC (KP)	0.44 ± 0.01	0.99 ± 0.05	1.09 ± 0.07	1.72 ± 0.01	**1.93 ± 0.08**	1.03 ± 0.08	1.00 ± 0.06	0.85 ± 0.09
AA + PC (PS)	0.31 ± 0.01	0.72 ± 0.08	0.95 ± 0.01	**1.82 ± 0.01**	1.43 ± 0.09	1.11 ± 0.02	1.03 ± 0.08	0.86 ± 0.05

TV: *T. viride*, PC: *P. chrysogenum*, AA: *A. aegerita*, n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

Table 5 Manganese peroxidase activity (U/g) during PAH degradation in soil at pH 5 and pH 7 by *T. viride*, *P. chrysogenum, A. aegerita* and their co-culture

Fungi + substrate	pH 5 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	0.06 ± 0.06	0.41 ± 0.06	0.63 ± 0.04	**0.93 ± 0.09**	0.83 ± 0.09	0.64 ± 0.05	0.45 ± 0.03	0.44 ± 0.03
T. viride (KP)	0.06 ± 0.06	0.45 ± 0.07	1.10 ± 0.08	**1.17 ± 0.06**	0.98 ± 0.07	0.77 ± 0.05	0.51 ± 0.04	0.48 ± 0.02
T. viride (PS)	0.06 ± 0.06	0.43 ± 0.04	1.02 ± 0.09	**1.17 ± 0.05**	0.88 ± 0.01	0.65 ± 0.03	0.48 ± 0.06	0.44 ± 0.03
P. chrysogenum (C)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (KP)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (PS)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
TV + PC (C)	0.70 ± 0.06	0.96 ± 0.01	1.71 ± 0.01	**1.90 ± 0.06**	1.87 ± 0.01	0.95 ± 0.03	0.44 ± 0.05	0.63 ± 0.02
TV + PC (KP)	0.25 ± 0.07	0.98 ± 0.03	**1.16 ± 0.02**	1.13 ± 0.02	1.10 ± 0.07	0.80 ± 0.07	0.71 ± 0.09	0.58 ± 0.04
TV + PC (PS)	0.25 ± 0.07	0.98 ± 0.07	**1.02 ± 0.09**	1.02 ± 0.02	0.92 ± 0.02	0.73 ± 0.07	0.68 ± 0.06	0.56 ± 0.03
A. aegerita (C)	0.71 ± 0.05	1.05 ± 0.03	**2.17 ± 0.01**	2.17 ± 0.11	2.14 ± 0.02	2.10 ± 0.07	2.00 ± 0.09	1.75 ± 0.01
A. aegerita (KP)	0.93 ± 0.09	1.12 ± 0.08	3.21 ± 0.02	**3.72 ± 0.08**	2.59 ± 0.02	2.20 ± 0.01	2.17 ± 0.01	1.82 ± 0.06
A. aegerita (PS)	0.77 ± 0.01	1.08 ± 0.06	3.41 ± 0.01	**3.61 ± 0.07**	2.72 ± 0.04	2.35 ± 0.09	2.23 ± 0.09	1.97 ± 0.02
AA + TV (C)	0.70 ± 0.06	0.96 ± 0.01	1.21 ± 0.01	**2.90 ± 0.06**	2.87 ± 0.05	1.63 ± 0.02	1.43 ± 0.02	1.02 ± 0.02
AA + TV (KP)	0.63 ± 0.04	0.98 ± 0.03	1.16 ± 0.02	**2.13 ± 0.02**	2.10 ± 0.07	1.80 ± 0.07	1.71 ± 0.09	1.58 ± 0.04
AA + TV (PS)	0.62 ± 0.04	0.98 ± 0.07	1.71 ± 0.01	**2.02 ± 0.02**	1.92 ± 0.02	1.73 ± 0.07	1.68 ± 0.06	1.56 ± 0.03
AA + PC (C)	0.41 ± 0.01	0.66 ± 0.01	**1.98 ± 0.03**	1.90 ± 0.02	1.71 ± 0.08	1.60 ± 0.02	1.52 ± 0.03	1.40 ± 0.03
AA + PC (KP)	0.63 ± 0.04	0.98 ± 0.03	**2.10 ± 0.08**	1.98 ± 0.07	1.78 ± 0.07	1.65 ± 0.05	1.65 ± 0.05	1.48 ± 0.05
AA + PC (PS)	0.62 ± 0.01	0.96 ± 0.05	**2.11 ± 0.01**	1.99 ± 0.02	1.83 ± 0.06	1.79 ± 0.09	1.64 ± 0.05	1.44 ± 0.03
Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	0.21 ± 0.07	0.41 ± 0.06	0.66 ± 0.08	**0.96 ± 0.01**	0.80 ± 0.03	0.61 ± 0.02	0.56 ± 0.01	0.31 ± 0.08
T. viride (KP)	0.29 ± 0.09	0.45 ± 0.07	0.90 ± 0.02	**1.02 ± 0.09**	0.98 ± 0.07	0.70 ± 0.06	0.61 ± 0.02	0.40 ± 0.03
T. viride (PS)	0.27 ± 0.01	0.43 ± 0.04	0.71 ± 0.02	**0.88 ± 0.01**	0.73 ± 0.07	0.65 ± 0.05	0.59 ± 0.05	0.38 ± 0.09
P. chrysogenum (C)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (KP)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (PS)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
TV + PC (C)	0.25 ± 0.04	0.77 ± 0.01	**1.08 ± 0.06**	1.07 ± 0.02	0.96 ± 0.01	0.74 ± 0.07	0.61 ± 0.05	0.52 ± 0.01
TV + PC (KP)	0.25 ± 0.07	0.79 ± 0.01	1.05 ± 0.08	**1.15 ± 0.02**	0.92 ± 0.08	0.71 ± 0.09	0.45 ± 0.07	0.32 ± 0.06
TV + PC (PS)	0.25 ± 0.07	0.79 ± 0.02	0.81 ± 0.01	**1.04 ± 0.04**	0.90 ± 0.01	0.68 ± 0.06	0.41 ± 0.06	0.32 ± 0.05
A. aegerita (C)	0.65 ± 0.03	2.79 ± 0.01	4.12 ± 0.08	**5.04 ± 0.04**	4.96 ± 0.07	4.85 ± 0.06	3.79 ± 0.09	3.69 ± 0.05

Table 5 continued

Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
A. aegerita (KP)	0.77 ± 0.01	3.02 ± 0.02	5.78 ± 0.01	**7.21 ± 0.02**	6.71 ± 0.01	6.12 ± 0.08	5.93 ± 0.03	5.88 ± 0.07
A. aegerita (PS)	0.66 ± 0.03	2.83 ± 0.03	5.61 ± 0.07	**6.88 ± 0.08**	5.45 ± 0.01	5.03 ± 0.06	4.80 ± 0.08	4.61 ± 0.07
AA + TV (C)	0.58 ± 0.04	1.74 ± 0.01	3.38 ± 0.08	**3.40 ± 0.03**	2.96 ± 0.01	2.80 ± 0.03	2.63 ± 0.04	2.53 ± 0.09
AA + TV (KP)	0.63 ± 0.04	1.92 ± 0.08	3.51 ± 0.01	**3.58 ± 0.03**	3.26 ± 0.04	2.92 ± 0.08	2.71 ± 0.04	2.66 ± 0.01
AA + TV (PS)	0.62 ± 0.04	1.90 ± 0.01	**3.46 ± 0.04**	3.39 ± 0.02	3.08 ± 0.04	2.90 ± 0.01	2.70 ± 0.05	2.62 ± 0.09
AA + PC (C)	0.45 ± 0.07	1.63 ± 0.03	2.96 ± 0.01	**3.32 ± 0.03**	2.96 ± 0.01	2.70 ± 0.01	2.03 ± 0.04	2.05 ± 0.01
AA + PC (KP)	0.66 ± 0.01	1.71 ± 0.04	3.46 ± 0.04	**3.58 ± 0.03**	3.00 ± 0.08	2.73 ± 0.02	2.38 ± 0.08	2.16 ± 0.04
AA + PC (PS)	0.62 ± 0.09	1.70 ± 0.05	3.39 ± 0.05	**3.49 ± 0.01**	2.97 ± 0.06	2.93 ± 0.01	2.06 ± 0.09	2.05 ± 0.03

TV: *T. viride*, PC: *P. chrysogenum*, AA: *A. aegerita*, n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

Table 6 Lignin peroxidase activity (U/g) during PAH degradation in soil at pH 5 and pH 7 by *T. viride, P. chrysogenum, A. aegerita* and their co-culture

Fungi + substrate	pH 5 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (KP)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (PS)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (C)	0.06 ± 0.06	0.13 ± 0.03	0.59 ± 0.04	**0.63 ± 0.01**	0.51 ± 0.03	0.49 ± 0.03	0.25 ± 0.07	0.14 ± 0.02
P. chrysogenum (KP)	0.06 ± 0.06	0.18 ± 0.01	0.51 ± 0.04	**0.74 ± 0.02**	0.57 ± 0.05	0.51 ± 0.03	0.32 ± 0.06	0.32 ± 0.02
P. chrysogenum (PS)	0.06 ± 0.06	0.19 ± 0.02	0.49 ± 0.01	**0.60 ± 0.09**	0.58 ± 0.03	0.56 ± 0.03	0.31 ± 0.05	0.30 ± 0.08
TV + PC (C)	0.12 ± 0.03	0.24 ± 0.07	0.48 ± 0.03	**0.60 ± 0.05**	0.56 ± 0.04	0.54 ± 0.08	0.41 ± 0.02	0.24 ± 0.06
TV + PC (KP)	0.12 ± 0.03	0.20 ± 0.01	0.42 ± 0.06	0.72 ± 0.06	**1.25 ± 0.08**	0.90 ± 0.04	0.81 ± 0.04	0.67 ± 0.01
TV + PC (PS)	0.12 ± 0.03	0.17 ± 0.09	0.27 ± 0.05	0.57 ± 0.06	**0.70 ± 0.04**	0.66 ± 0.09	0.59 ± 0.03	0.29 ± 0.08
A. aegerita (C)	0.65 ± 0.08	0.72 ± 0.06	1.31 ± 0.03	1.63 ± 0.09	**2.13 ± 0.08**	1.93 ± 0.08	1.69 ± 0.04	0.72 ± 0.05
A. aegerita (KP)	0.69 ± 0.02	1.38 ± 0.02	1.61 ± 0.07	2.16 ± 0.09	**2.24 ± 0.09**	2.13 ± 0.08	1.73 ± 0.06	1.41 ± 0.01
A. aegerita (PS)	0.45 ± 0.09	0.72 ± 0.06	0.99 ± 0.05	1.24 ± 0.01	**1.75 ± 0.04**	1.52 ± 0.02	1.10 ± 0.06	0.83 ± 0.09
AA + TV (C)	0.28 ± 0.07	0.55 ± 0.06	0.72 ± 0.06	1.10 ± 0.07	**1.11 ± 0.03**	1.02 ± 0.04	0.70 ± 0.04	0.61 ± 0.01
AA + TV (KP)	0.41 ± 0.01	0.65 ± 0.08	1.02 ± 0.04	1.63 ± 0.02	**2.05 ± 0.06**	1.58 ± 0.05	1.17 ± 0.04	0.74 ± 0.03
AA + TV (PS)	0.34 ± 0.02	0.54 ± 0.07	0.99 ± 0.05	**1.19 ± 0.09**	0.80 ± 0.04	0.57 ± 0.06	0.55 ± 0.04	0.36 ± 0.08
AA + PC (C)	0.28 ± 0.04	0.33 ± 0.02	0.65 ± 0.08	**1.29 ± 0.01**	0.74 ± 0.01	0.60 ± 0.07	0.34 ± 0.07	0.28 ± 0.09
AA + PC (KP)	0.44 ± 0.01	0.36 ± 0.08	0.72 ± 0.06	**1.29 ± 0.04**	1.19 ± 0.09	0.89 ± 0.02	0.42 ± 0.06	0.30 ± 0.08
AA + PC (PS)	0.31 ± 0.01	0.55 ± 0.04	1.18 ± 0.09	**1.59 ± 0.04**	1.35 ± 0.12	0.76 ± 0.04	0.43 ± 0.08	0.37 ± 0.05
Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
T. viride (C)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (KP)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
T. viride (PS)	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
P. chrysogenum (C)	0.06 ± 0.06	0.28 ± 0.04	0.48 ± 0.02	0.54 ± 0.07	**0.64 ± 0.04**	0.54 ± 0.08	0.41 ± 0.02	0.24 ± 0.06
P. chrysogenum (KP)	0.06 ± 0.06	0.42 ± 0.03	0.55 ± 0.06	**0.72 ± 0.06**	0.65 ± 0.03	0.57 ± 0.07	0.46 ± 0.08	0.27 ± 0.06
P. chrysogenum (PS)	0.06 ± 0.06	0.36 ± 0.04	0.54 ± 0.07	**0.69 ± 0.02**	0.51 ± 0.04	0.50 ± 0.01	0.44 ± 0.04	0.24 ± 0.09
TV + PC (C)	0.12 ± 0.03	0.21 ± 0.06	0.48 ± 0.02	0.57 ± 0.06	**0.60 ± 0.03**	0.57 ± 0.08	0.55 ± 0.01	0.32 ± 0.06
TV + PC (KP)	0.12 ± 0.03	0.29 ± 0.03	0.54 ± 0.08	0.70 ± 0.02	**0.71 ± 0.01**	0.63 ± 0.02	0.42 ± 0.05	0.31 ± 0.05
TV + PC (PS)	0.12 ± 0.03	0.24 ± 0.07	0.53 ± 0.05	**0.66 ± 0.04**	0.65 ± 0.07	0.54 ± 0.09	0.50 ± 0.01	0.30 ± 0.04
A. aegerita (C)	0.44 ± 0.07	0.69 ± 0.02	0.72 ± 0.06	2.01 ± 0.04	**2.08 ± 0.04**	1.69 ± 0.09	1.09 ± 0.07	0.60 ± 0.07

Table 6 continued

Fungi + substrate	pH 7 (weeks)							
	1	2	3	4	5	6	7	8
A. aegerita (KP)	0.69 ± 0.01	0.99 ± 0.05	1.09 ± 0.07	2.79 ± 0.05	**3.39 ± 0.01**	2.64 ± 0.01	1.18 ± 0.04	0.76 ± 0.04
A. aegerita (PS)	0.54 ± 0.07	0.72 ± 0.08	0.95 ± 0.01	**2.57 ± 0.05**	2.41 ± 0.06	1.76 ± 0.01	1.13 ± 0.06	0.83 ± 0.06
AA + TV (C)	0.44 ± 0.07	0.52 ± 0.06	0.74 ± 0.02	**1.59 ± 0.07**	1.13 ± 0.07	0.86 ± 0.05	0.69 ± 0.09	0.37 ± 0.05
AA + TV (KP)	0.69 ± 0.02	0.77 ± 0.0	0.95 ± 0.01	**1.70 ± 0.05**	1.38 ± 0.02	0.94 ± 0.05	0.66 ± 0.07	0.46 ± 0.09
AA + TV (PS)	0.54 ± 0.07	0.62 ± 0.08	0.89 ± 0.02	**1.60 ± 0.02**	1.24 ± 0.01	0.83 ± 0.06	0.50 ± 0.09	0.42 ± 0.06
AA + PC (C)	0.28 ± 0.04	0.48 ± 0.02	0.64 ± 0.04	**0.90 ± 0.03**	0.60 ± 0.07	0.42 ± 0.06	0.34 ± 0.07	0.22 ± 0.08
AA + PC (KP)	0.42 ± 0.03	0.55 ± 0.06	0.72 ± 0.06	**1.43 ± 0.09**	0.89 ± 0.02	0.62 ± 0.08	0.42 ± 0.06	0.32 ± 0.08
AA + PC (PS)	0.36 ± 0.04	0.54 ± 0.07	0.69 ± 0.02	**1.09 ± 0.02**	0.76 ± 0.04	0.52 ± 0.06	0.43 ± 0.08	0.31 ± 0.01

TV: *T. viride*, PC: *P. chrysogenum*, AA: *A. aegerita*, n.d. not detected; maximal enzymatic activity of each culture is highlighted in bold

amendment for the fungi growth are known to induce or enhance the activities of ligninolytic enzymes such as laccase, MnP and LiP, and increase the degradation rates of PAHs by fungi. Results obtained suggest the involvement of ligninolytic enzymes in PAHs degradation. Moreover, the results indicated the importance of considering fungi, which are naturally present in soil, for degradation purpose of persistent pollutant.

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