Environ Sci Pollut Res (2015) 22:1034–1042
DOI 10.1007/s11356-014-3357-y

RESEARCH ARTICLE

Degradation of polycyclic aromatic hydrocarbons in soil by a tolerant strain of *Trichoderma asperellum*

German Zafra · Angélica Moreno-Montaño · Ángel E. Absalón · Diana V. Cortés-Espinosa

Received: 26 May 2014 / Accepted: 17 July 2014 / Published online: 10 August 2014

Abstract *Trichoderma asperellum* H15, a previously isolated strain characterized by its high tolerance to low (LMW) and high molecular weight (HMW) PAHs, was tested for its ability to degrade 3–5 ring PAHs (phenanthrene, pyrene, and benzo[a]pyrene) in soil microcosms along with a biostimulation treatment with sugarcane bagasse. *T. asperellum* H15 rapidly adapted to PAH-contaminated soils, producing more CO_2 than uncontaminated microcosms and achieving up to 78 % of phenanthrene degradation in soils contaminated with 1,000 mg Kg^{-1} after 14 days. In soils contaminated with 1,000 mg Kg^{-1} of a three-PAH mixture, strain H15 was shown to degrade 74 % phenanthrene, 63 % pyrene, and 81 % of benzo[a]pyrene. Fungal catechol 1,2 dioxygenase, laccase, and peroxidase enzyme activities were found to be involved in the degradation of PAHs by *T. asperellum*. The results demonstrated the potential of *T. asperellum* H15 to be used in a bioremediation process. This is the first report describing the involvement of *T. asperellum* in LMW and HMW-PAH degradation in soils. These findings, along with the ability to remove large amounts of PAHs in soil found in the present work provide enough evidence to consider *T. asperellum* as a promising and efficient PAH-degrading microorganism.

Keywords *Trichoderma asperellum* · Polycyclic aromatic hydrocarbons (PAHs) · Soil bioremediation · Laccase · Peroxidase · Dioxygenase

Responsible editor: Robert Duran

G. Zafra · A. Moreno-Montaño · Á. E. Absalón · D. V. Cortés-Espinosa (✉)
Instituto Politécnico Nacional, Centro de Investigación en Biotecnología Aplicada, Carretera Estatal Santa Ines Tecuexcomac-Tepetitla Km 1.5, Tepetitla, Tlaxcala, México C.P. 70900
e-mail: dcortes@ipn.mx

Introduction

Degradation of polycyclic aromatic hydrocarbons (PAHs) in soils has become an environmental priority, mainly because of their elevated persistence and potential harmful effects on human and animal health. PAHs are recalcitrant organic compounds with potential cytotoxic, carcinogenic, genotoxic, and mutagenic effects, characterized by a high hydrophobicity and low aqueous solubility (US-EPA 2008). Low molecular weight (LMW), high molecular weight (HMW) PAHs as well as their toxic intermediary products can be absorbed and accumulated in diverse organisms. Microbial degradation is thought to be the main natural method of degradation of PAHs in soils and biochemical degradation pathways are well documented; several fungal, bacterial, and algal species have been reported as PAH-degrading organisms (Cerniglia and Sutherland 2010; Seo et al. 2009; Todd et al. 2002), making bioremediation an effective and promising technology to remove pollutants from soils.

Fungi belonging to *Trichoderma* genus are worldwide ubiquitous organisms commonly found in soils, known to possess a versatile and powerful enzymatic machinery (e.g. cellulases, hemicellulases, chitinases, proteases, glucanases) useful for the degradation of a wide range of substrates in soils, but specially, cellulosic material (Jaklitsch 2009). *Trichoderma* is one of the biological control agents more commonly used against plant pathogens mainly due to its production of hydrolytic enzymes and secondary metabolites, besides interacting through antibiosis, competing for space and resources, and improving growth and resistance to biotic and abiotic stress (Chernin and Chet 2002). Within the *Trichoderma* genus, *T. asperellum* stands out as a species with a wide range of substrate utilization, high production of antimicrobial compounds and an ability for environmental opportunism through saprotrophic, biotrophic, and mycoparasitic interactions (Chutrakul et al. 2008; Ding et al. 2012;

Druzhinina et al. 2011). *Trichoderma asperellum* is used as a biological control agent against a wide range of plant pathogens including *Colletotrichum gloeosporioides*, *Phytophthora megakarya*, other pathogenic fungi, and nematodes (de los Santos-Villalobos S et al. 2013; Sharon et al. 2007; Slusarski and Pietr 2009; Tondje et al. 2007).

Although *Trichoderma* species are commonly used for the commercial production of lytic enzymes and as biological control agents, their use in pollutant bioremediation is limited. Several studies have shown the ability of *Trichoderma* to biotransform heavy metals (Atagana 2009; Su et al. 2011) and hydrocarbons (Matsubara et al. 2006). In fact, it is known that several species of the genus *Trichoderma* possess the ability to degrade and metabolize PAHs such as naphthalene, phenanthrene, pyrene, and benzo[a]pyrene, even in the presence of heavy metals (Atagana 2009; Verdin et al. 2004). The species reported as metabolizers include *T. hamatum*, *T. harzianum*, *T. koningii*, *T. viride* and *T. virens* (Argumedo-Delira et al. 2012; Cerniglia and Sutherland 2010). However, there are no reports involving *T. asperellum* as a hydrocarbon or PAH-degrading organism. The use of *T. asperellum* as bioremediation agent on PAH-polluted soils may present additional advantages over the use of other soil microorganisms, such as its high growth rate, wide range of substrates, growth-promoting effects on plants, and the production of oxidizing hydrolytic enzymes including laccases, peroxidises, and dioxygenases (Cazares-Garcia et al. 2013; Hadibarata et al. 2007). Thus, the aim of this work was to evaluate the degradation capability of a strain of *T. asperellum* tolerant to LMW and HMW-PAHs in solid culture, for the bioremediation of PAH-polluted soils.

Material and methods

Fungal strain and inoculum preparation

T. asperellum H15 is a strain previously isolated from a heavy crude oil-contaminated soil, showing increased tolerance levels to 3, 4, and 5-ring PAHs and the ability to use them as sole carbon source (Zafra et al. 2014). This strain has been deposited in the Agricultural Research Service (ARS) patent culture collection with registration number NRRL50869. *T. asperellum* H15 was maintained on potato dextrose agar (PDA) plates at 30 °C. Production of spores was carried out in 250-mL flasks containing 30 mL of PDA, inoculated with strain H15 and incubated at 30 °C. Spores were collected on day 4 with the addition of 20 mL of 0.1 % Tween 80 solution, sterile glass beads and gently shaking the flasks for 2 min. The spore suspension concentration was quantified in a Neubauer haematocytometer chamber using a optical microscope.

Degradation of PAHs by T. asperellum H15 in solid culture

Degradation ability of analytical grade phenanthrene (Phe) and a mixture of Phe, pyrene (Pyr), and benzo[a]pyrene (BaP) by *T. asperellum* H15 was evaluated in microcosm solid culture systems using sugarcane bagasse (34.34 % carbon, 0.18 % nitrogen, 0.00343 % phosphorus) as fungal growth support, texturizing agent and alternative carbon source. Sterile sugarcane bagasse (0.35 g dry weight) was placed in 50-mL glass flasks with Czapeck medium (g L^{-1}: sucrose, 30; sodium nitrate, 3; dipotassium phosphate, 1; magnesium sulfate, 0.5; potassium chloride, 0.5; ferrous sulphate, 0.01; pH 7.3) to reach 30 % moisture content, inoculated with a concentration of 2×10^7 spores of strain H15 per gram of contaminated soil and incubated for 5 days at 30 °C. The inoculated sugarcane bagasse was then mixed with 6.65 g of sterile soil (sandy loam with 2.4 % organic matter, 1.4 % total organic carbon, 0.063 % nitrogen, 0.0023 % phosphorus and pH of 8.41) spiked with 1,000 mg Kg^{-1} of Phe or 1,000 mg Kg^{-1} of a mixture of Phe, Pyr, and BaP (1:1:1 ratio). Soil/sugarcane bagasse mixture was incubated at 30 °C for 14 (Phe-contaminated soil) or 18 days (PAH mixture-contaminated soil). Control samples were prepared by inoculating a non-contaminated soil under the same culture conditions. Abiotic controls, consisting of sterile non-inoculated microcosms treated under the same conditions as those of *Trichoderma*-inoculated systems, were included to confirm that the disappearance of PAHs was caused by biodegradation and not by abiotic factors such as absorption or volatilization. Assays were carried out in triplicate.

Heterotrophic activity measurements

Headspace in each of the microcosms flasks was flushed every 48 h for 10 min with sterile and moistened air, to preserve aerobic conditions and avoid carbon dioxide accumulation. CO_2 evolution in the microcosms was measured every 48 h using an Agilent 6890 Series Gas Chromatograph equipped with a thermal conductivity detector and a GS-CarbonPLOT column. Instantaneous and accumulated CO_2 was reported as milligrams of CO_2 per gram of initial dry matter (IDM).

Enzymatic assays

Activity of *T. asperellum* H15 extracellular laccase and peroxidase enzymes was screened in agar plates by means of the oxidation of chromogenic dyes ABTS (Saparrat et al. 2000), *o*-anisidine (OA) (Conesa et al. 2000) and azure B (AB) (Archibald 1992), respectively, in the presence and absence of 1,000 mg L^{-1} of a mixture of Phe, Pyr, and BaP. Laccase screening was performed in minimal medium plates (g L^{-1}: glucose, 2; $(NH_4)_2\,C_4H_4O_6$, 1; KH_2PO_4, 0.26; $NaHPO_4$, 0.26; $MgSO_4\cdot7H_2O$, 0.5; $CuSO_4\cdot5H_2O$, 0.5; $CaCl_2\cdot5H_2O$, 0.01;

$FeSO_4$, 0.005; $ZnSO_4$ $7H_2O$, 0.005; $NaMoO_4$ $7H_2O$, 0.0002; $MnCl_2 \cdot H_2O$, 0.00009; H_3BO_3, 0.0007; malt extract, 2; ABTS, 0.2; agar, 16. pH 5.5); OA-oxidizing peroxidase activity was evaluated in plates with modified Kirk medium (g L^{-1}: glucose, 10; KH_2PO4, 2; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.1; 2,2-dimethylsuccinate, 2,2; $(NH_4)_2$ $C_4H_4O_6$, 0.5; yeast extract, 0.2; *o*-anisidine, 0.3; agar, 16. pH 5.0), and AB-oxidizing peroxidase activity was evaluated in plates with 20 ml of Czapeck medium supplemented with azure B (0.0066 g L^{-1}). Plates were inoculated with PDA discs (5 mm diameter), containing 3-day-old active mycelia. Plates were incubated at 30 °C for 10 days. Determination of specific enzymatic activities was carried out in liquid culture. Glass flasks with 50 mL of minimal medium contaminated with a mixture of Phe (25 mg L^{-1}) and Pyr (25 mg L^{-1}) were inoculated with 1×10^6 spores mL^{-1} of strain H15 and incubated at 30 °C for 10 days. Enzymatic activities were assessed every 48 h from culture supernatants. Laccase extracellular activity was determined spectrophotometrically by the oxidation of ABTS (Nagai et al. 2002). Cationic radical formation was detected by measuring the increase in absorbance at 420 nm (e420=36,000 M^{-1} cm^{-1}). Catechol 1,2 dioxygenase extracellular activity was determined spectrophotometrically by the formation of *cis*, *cis*-muconic acid at 260 nm (e260= 16,800 M^{-1} cm^{-1}) (Wojcieszynska et al. 2011). Catechol 2,3 dioxygenase extracellular activity was determined by the formation of 2-hydroxymuconic semialdehyde at 375 nm (e375=36,000 M^{-1} cm^{-1}) (Wojcieszynska et al. 2011). Phenol red (PSP)-oxidizing peroxidase activity was determined spectrophotometrically at 37 °C by the oxidation of phenol red at 610 nm (Kuwahara et al. 1984). Veratryl alcohol (VA)-oxidizing peroxidase activity was determined spectrophotometrically at 37 °C by the oxidation of veratryl alcohol to verytraldehyde at 310 nm (Tien and Kirk 1988). One unit of enzyme activity (U/l) was defined as the amount of enzyme required to generate 1 μmol of each reaction product in 1 min. Protein concentrations of the culture supernatants were determined by the bicinchoninic acid method (BCA) using bovine serum albumin as standard (Smith et al. 1985).

PAH measurements

Residual PAHs were extracted from 1 g of initial dry matter (for solid culture) with the addition of 25 mL of a dichloromethane-acetone solution (7:3 ratio) using a Multiwave 3000 SOLV apparatus (Anton Paar) for 20 min, according to EPA method 3546. The resulting extracts were evaporated, suspended in 2 mL of acetonitrile and analyzed in an HP Agilent 1100 HPLC system equipped with a C18 reverse-phase column, with an UV absorbance detector set at 245–360 nm under an isocratic ambient in acetonitrile:water (90:10) and a flow rate of 1 ml min^{-1}. For liquid culture, residual PAHs were extracted from mycelium and liquid medium; first the mycelium was filtered from 50 mL medium through cellulose filter paper with medium retention (Whatman grade 1) and resuspended in 10 mL acetone, then, it was sonicated for 10 min, and the organic phase was recovered by filtration with the same type of filter paper. Residual PAHs were extracted from the filtered liquid medium by stirring with 50 mL of ethyl acetate for 30 min, then, this organic phase was mixed with acetone fraction obtained from the mycelium, the mixture were evaporated, resuspended in acetonitrile, and quantified by HPLC as described above.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by a multiple comparison test (LSD) with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value <0.05.

Results

Heterotrophic activity of strain H15 in soil microcosms

Figures 1 and 2 show the growth of *T. asperellum* H15 during the PAH biodegradation process in microcosms in the presence of Phe and a mixture of Phe, Pyr and BaP, respectively. CO_2 production by strain H15 in uncontaminated microcosms increased gradually throughout the process, reaching the highest value on day 2 (6.914 mg CO_2 g^{-1} IDM) and a marked and constant decrease from day 4 to 6, with a lower but relatively constant production of CO_2 from day 8 to the end of the process. The presence of a single hydrocarbon (Phe) in soil initially delayed the growth of strain H15 but after day 4, Phe-contaminated microcosms produced CO_2 levels higher than those in uncontaminated microcosms (Fig. 1) with an accumulated CO_2 production of 27.615 versus 22.063 mg CO_2 g^{-1} IDM, respectively. On the other hand and as observed with Phe, the presence of a three-PAH mixture in soil also delayed the initial growth of strain H15, although from day 4, it produced accumulated CO_2 levels higher than those obtained in uncontaminated microcosms (Fig. 2). CO_2 production differences between PAH-contaminated and PAH-uncontaminated microcosms were not significant.

PAH biodegradation in solid culture

The biodegradation efficiency of Phe, Pyr, and BaP by *T. asperellum* H15 in solid culture after 8, 14, and 18 days of incubation is shown in Fig. 3. A high degradation was obtained when Phe was added individually to microcosms, reaching a degradation efficiency of 78.3 % after 14 days of incubation. When a mixture of thee PAHs was added, the degradation efficiency of Phe by strain H15 was relatively

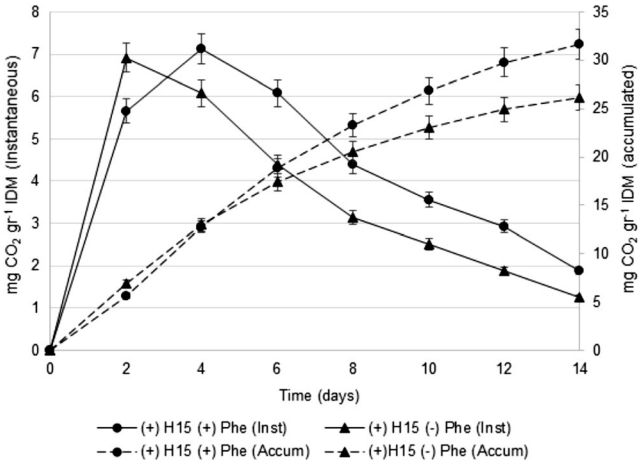


Fig. 1 Microbial activity of *T. asperellum* H15 in microcosms with soil contaminated with 1,000 mg Kg^{-1} of phenanthrene

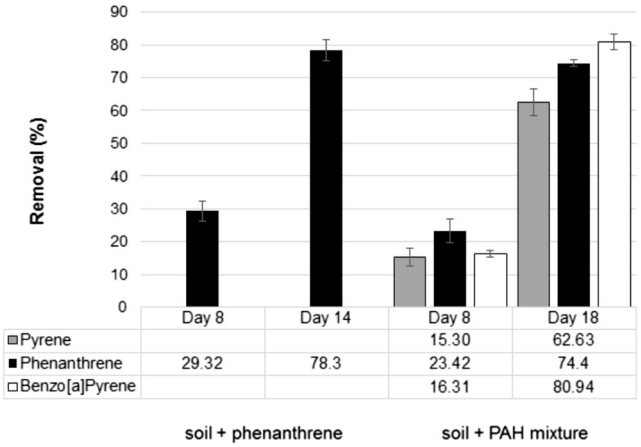


Fig. 3 PAH removal by *T. asperellum* H15 in microcosms with sterile soil contaminated with 1,000 mg Kg^{-1} of Phe or 1,000 mg Kg^{-1} of a mixture of PAHs

low for day 8 (23.4 %), but for day 18 had increased substantially (74.4 %). The same situation was observed for Pyr and BaP; even though there was a lower degradation of Pyr and BaP during the first week of growth (15.3 and 16.31 %, respectively), there was a marked increase by day 18 (62.63 and 80.94 %, respectively). The abiotic losses of PAHs during the biodegradation experiments were similar and negligible.

Enzymatic assays and PAH removal in liquid culture

Plate-based screening of laccase and peroxidase activity by strain H15 is shown in Fig. 4. Extracellular laccase activity was observed from day 2 of incubation, when there was an evident increase in ABTS oxidation in plates containing 1,000 mg L^{-1} of the PAH mixture compared with those without PAHs (Fig. 4a). By day 10, plates with and without PAHs showed a complete oxidation of ABTS. Peroxidase activity, assessed by the oxidation of OA and AB, was detected only from day 7. Strain H15 was able to oxidize OA and AB in plates with no substantially appreciable differences regarding the presence or absence of PAHs in medium, although AB showed a lower intensity of the halo coloration in comparison with OA plates (Fig. 4b, c). By day 10 of incubation, the oxidation of both dyes was clear, but the overall oxidation rate was slower, and the effect of the presence of PAHs was less evident than ABTS plates.

Figures 5 and 6 show the specific activities of five PAH-oxidizing enzymes, as well as Phe and Pyr degradation in liquid medium. The presence of PAHs in the liquid cultures led to an increase in the specific activity of laccase from day 4 to 8 in comparison to controls without PAHs, maintaining relatively constant values from day 4 to 8. VA-oxidizing peroxidase activity was also higher by day 6 in comparison to controls without PAHs, reaching its highest value (23.94 U mg^{-1}), although by day 8, enzyme activity decreased (20.4 U mg^{-1}) and controls without PAHs reached their maximum (25.29 U

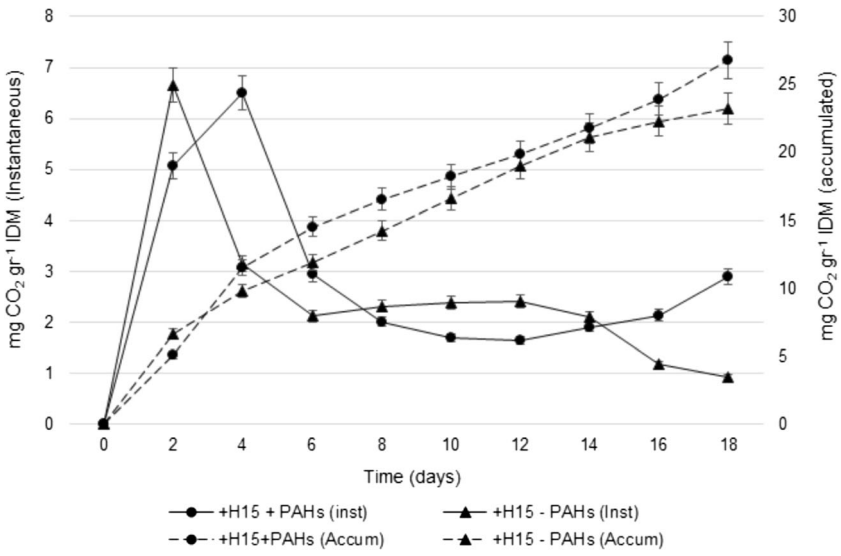


Fig. 2 Microbial activity of *T. asperellum* H15 in microcosms with soil contaminated with 1,000 mg Kg^{-1} of a mixture of three PAHs

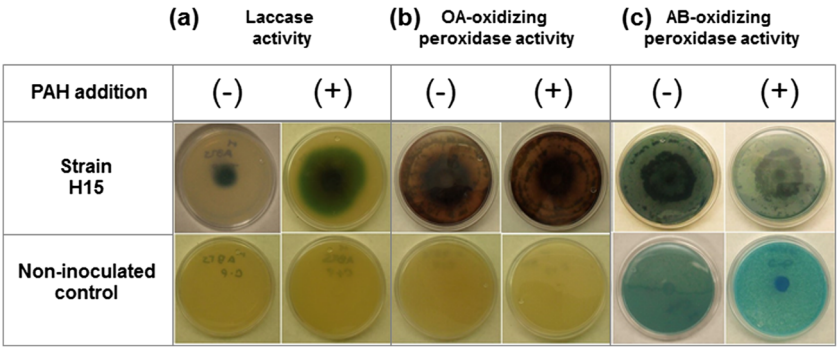


Fig. 4 Plate-based enzymatic screening of *T. asperellum* H15. **a** ABTS oxidation after 2 days of growth in minimal medium; **b** *o*-anisidine oxidation after 6 days of growth in modified Kirk medium; **c** azure B decoloration after 10 days of growth in Czapeck medium

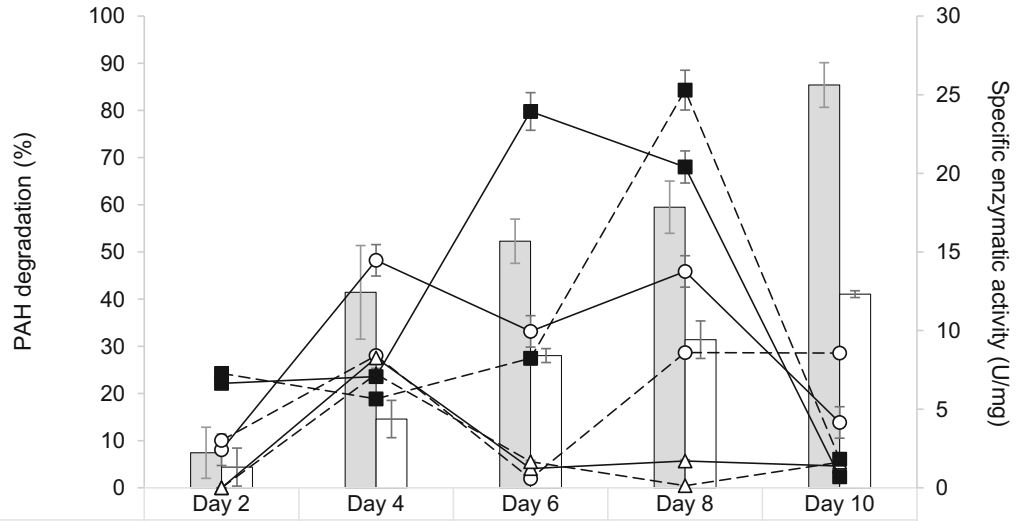
mg^{-1}). In contrast, PSP-oxidizing peroxidase activity was identical in cultures with and without PAHs, showing low or basal activity except at day 4 (Fig. 5). The presence of PAHs in the medium also produced an evident initial increase in the specific activity of catechol 1,2 dioxygenase (from day 2 to 6) and catechol 2,3 dioxygenase (from day 2 to 6) in comparison with controls without PAHs, although the enzyme activity in the latter was higher in medium without PAHs from day 6 (Fig. 6). Maximum degradation values of PAHs were 85.39 % for Phe and 41.06 % for Pyr at day 10. Phe degradation was significantly higher than Pyr degradation from day 2 until the last day of evaluation.

Discussion

In this study, we investigated the ability of *T. asperellum* H15, a strain previously isolated from a heavy crude oil-contaminated soil, to degrade several LMW and HMW PAHs in soil as well as to produce several PAH-oxidizing enzymes in the presence of PAHs associated with the degradation in liquid culture. Few data on PAH degradation by *Trichoderma* species are available; however, studies have shown that several *Trichoderma* species are capable of tolerating and metabolizing hydrocarbons including LMW and HMW PAHs (Argumedo-Delira et al. 2012; Atagana 2009; Hadibarata et al. 2007; Ravelet et al. 2000; Saraswathy and Hallberg 2002; Verdin et al. 2004). Previous work in our group showed remarkably high tolerance levels of *T. asperellum* H15 (up to 6,000 mg Kg^{-1}) toward the same LMW and HMW PAHs tested in this work, as well as the ability to use them as sole carbon source (Zafra et al. 2014).

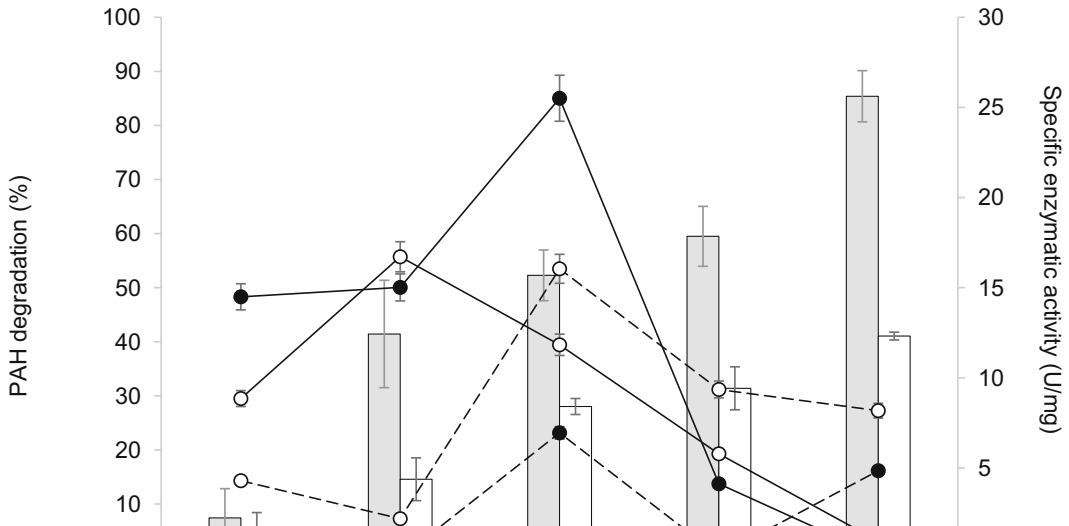
Many fungal species possess the ability to degrade PAHs and the potential to remediate polluted soils. However, one limiting factor in the success of these organisms is the inability to adapt and grow properly on extensively contaminated soils (Tabak et al. 2003). Solid-state fermentation in microcosms showed that although the presence of PAHs initially delayed the growth of strain H15 in soil, CO_2 production remained higher in contaminated microcosms from day 4 when compared to uncontaminated microcosms. This suggests a rapid and successful adaption of strain H15 in PAH-polluted soils, as well as an ability to use the sugarcane bagasse and PAHs for growth. Although CO_2 production does not correlate with PAH degradation levels, strain H15 produced remarkably higher amounts of CO_2, at least twice, in comparison with other reported native and transformant PAH-degrading fungi when growing in microcosm at comparable PAH concentrations (Cortes-Espinosa et al. 2006, 2011; Reyes-Cesar et al. 2014). Unlike some reports indicating low tolerance levels to PAHs in several *Trichoderma* members such as *T. harzianum*, *T. viride* or *Trichoderma* sp. strains (Argumedo-Delira et al. 2012; Matsubara et al. 2006; Verdin et al. 2004), the elevated tolerance levels showed by *T. asperellum* H15 could facilitate its adaptation to polluted soils and thus improve the rate of removal/degradation of PAHs.

Biodegradation assays showed that soils contaminated with *T. asperellum* H15 led to a rapid degradation of considerable amounts of Phe, Pyr, and Bap. Although low Phe biodegradation took place by day 8 (29.32 % for Phe-contaminated soil and 23.42 % for PAH mixture-contaminated soils), by the end of the process, it had increased importantly (78.3 and 74.4 %, respectively). The same situation was observed in microcosms contaminated with a mixture of three PAHs, where scarce Pyr and BaP degradation was observed during the first week of growth (15.3 and 16.31 %) but by the end of the second week also increased substantially (62.63 and 80.94 %). This notorious shift in the PAH degradation rate could be associated with the induction of enzymes involved in the degradation of PAHs at different stages. HMW PAHs, particularly BaP, are barely metabolized substrates or used as a sole carbon and energy source and are poor inducers of their own degradative enzymes (Bouchez et al. 1995). The presence of alternative substrates, including LMW-PAHs, favors microbial growth and induces the expression of PAH catabolic enzymes (Juhasz and Naidu 2000). Our results are consistent with previous reports showing an increased cometabolic degradation of HMW PAHs in response to the presence of LMW PAHs and their corresponding degradative pathways (Juhasz and Naidu 2000; Yuan et al. 2003). PAH degradation by strain H15, particularly of HMW PAHs, showed to be higher and faster than with other reported non-ligninolytic and ligninolytic degrading fungi. For example, *Irpex lacteus*, *Coprinus cinereus*, and *Pleurotus ostreatus* degraded 65–80 % Phe and 30–65 % Pyr out of 400 mg Kg^{-1} after 28 days of incubation (Matsubara et al.



	Day 2	Day 4	Day 6	Day 8	Day 10
Phenanthrene degradation (%)	7.42	41.44	52.28	59.49	85.39
Pyrene degradation (%)	4.39	14.58	28.03	31.40	41.06
Laccase (+) PAHs (U/mg)	2.42	14.47	9.95	13.76	4.16
Laccase (-) PAHs (U/mg)	3.01	8.41	0.58	8.60	8.57
PSP-oxidizing perox (+) PAHs (U/mg)	0.00	8.26	1.22	1.70	1.39
PSP-oxidizing perox (-) PAHs (U/mg)	0.00	7.23	1.66	0.14	1.63
VA-oxidizing perox (+) PAHs (U/mg)	6.64	7.07	23.93	20.40	0.70
VA-oxidizing perox (-) PAHs (U/mg)	7.27	5.65	8.24	25.29	1.82

Fig. 5 Laccase and peroxidase specific activity of *T. asperellum* H15 in liquid culture with 100 mg L^{-1} of a mixture of phenanthrene and pyrene



	Day 2	Day 4	Day 6	Day 8	Day 10
Phenanthrene degradation (%)	7.42	41.44	52.28	59.49	85.39
Pyrene degradation (%)	4.39	14.58	28.03	31.40	41.06
1,2 dioxygenase (+) PAHs (U/mg)	14.49	15.02	25.51	4.12	0.11
1,2 dioxygenase (-) PAHs (U/mg)	0.00	0.00	6.95	0.14	4.85
2,3 dioxygenase (+) PAHs (U/mg)	8.85	16.71	11.83	5.79	0.94
2,3 dioxygenase (-) PAHs (U/mg)	4.29	2.18	16.05	9.35	8.18

Fig. 6 Catechol dioxygenase specific enzymatic activity of *T. asperellum* H15 in liquid culture with 100 mg L^{-1} of a mixture of phenanthrene and pyrene

2006), while *Anthracophyllum discolor* degraded 75 % out of 45 mg Kg^{-1} BaP in soil after 60 days of incubation (Acevedo et al. 2011). The same was observed with strains of *Fusarium* sp., *Monilinia* sp., *Aspergillus terreus* and *Talaromyces spectabilis* strains in soil microcosms and solid culture when using similar LMW and HMW-PAH mixtures, even in the presence of concentrations below 100 mg Kg^{-1} of individual PAHs (Reyes-Cesar et al. 2014; Thion et al. 2013; Wu et al. 2008;) and regarding the degradation of 10 mg Kg^{-1} of Pyr in liquid culture by *Trichoderma harzianum* (Ravelet et al. 2000). This depicts the high potential of strain H15 to remediate PAH in contaminated soils.

The metabolism of PAHs by fungi has been extensively studied, especially in basidiomycetes. Most fungi metabolize PAHs with enzymes that include LiP, MnP, laccases, cytochrome P450 monooxygenases, and epoxide hydrolases (Cerniglia and Sutherland 2010). In contrast, aromatic-ring-hydroxylating dioxygenases are more commonly found in bacteria and greatly contribute to the initial ring cleavage of aromatic compounds, including LMW PAHs (Hadibarata and Tachibana 2010). Probable mechanisms for PAH degradation in *T. asperellum* could include the production of laccases (Cazares-Garcia et al. 2013), peroxidases (Cristica et al. 2010), and dioxygenases (Hadibarata et al. 2007) among others. We observed that the presence of PAHs in liquid cultures led to a significant increase in the activity of catechol 1,2 and 2,3 dioxygenases during the initial 4 days of incubation, reaching a maximum production at day 6 and a subsequent decrease from day 8. This could indicate an involvement of these enzymes in the early stages of degradation, which play a key role in the initial oxidation of aromatic compounds in other microorganisms (Seo et al. 2009). Hadibarata et al. (2007) reported a direct implication of catechol 1,2 and 2,3 dioxygenases in the degradation of Phe by *Trichoderma* sp. 109, a strain similar to *T. asperellum* H15 that possesses the ability to grow with PAHs as sole carbon source. 1-Hydroxy-2-naphthoic acid, salicylic acid, and catechol were identified as major intermediaries, indicating a Phe degradation pathway in *Trichoderma* via dioxygenation at positions 3 and 4 and subsequent meta-cleavage, leading to PAH mineralization. A high production of extracellular catechol 1,2 and 2,3 dioxygenases associated with PAH metabolism have been also observed in the degradation of chrysene by *Fusarium* sp. (Hidayat et al. 2012), as well naphthalene and BaP by ligninolytic fungi *Armillaria* sp. and *Polyporus* sp. (Hadibarata et al. 2012; Hadibarata and Kristanti 2012). In contrast, laccase activity was nearly undetectable during the initial 2 days of degradation but increased notoriously from day 4, with a higher production in response to the presence of PAHs. It is likely that *T. asperellum* laccases are involved in the oxidation of aromatic rings and play a crucial role in the degradation of PAHs by *T. asperellum*, as they are strongly linked to aromatic hydrocarbon degradation in other fungi (Baldrian 2006; Haritash and Kaushik 2009). Three different *T. asperellum* laccase genes have been identified *in silico* (Cazares-Garcia et al. 2013), two of them being extracellular enzymes with probable PAH-oxidizing capacity. On the other hand, there is no evidence of classic fungal peroxidases such as MnP and LiP in *T. asperellum* genome, nor in the secretome of this fungus grown in sugarcane bagasse (Marx et al. 2013). However, we found evidence suggesting that strain H15 produced peroxidases with OA/PSP-oxidizing activity (related with MnP activity) and AB/VA-oxidizing activity (related with LiP activity) in solid and liquid culture. Increased activity of VA-oxidizing peroxidase activity was observed in treatments with soil contaminated with PAHs; this result suggests that this enzyme is involved in the degradation of PAHs; in contrast, PSP-oxidizing peroxidase activity, although detectable during the first days, did not appear to be involved in the degradation of PAHs by *T. asperellum*. Identified *T. asperellum* peroxidases include cytochrome C peroxidases, catalase peroxidases, glutathione peroxidise, and dye decolorizing (DyP-type) peroxidases (Fawal 2014). Although peroxidase activity in *T. asperellum* has been described mainly as a response against oxidative stress (Fawal et al. 2013), VA-oxidizing peroxidase activity detected in strain H15 could in fact be related to DyP-type peroxidase (TaspDyPrx01, PeroxiBase ID 12842). DyP peroxidases possess a broad substrate specificity and have been previously described as LiP in *Actinobacteria*, having a significant role in bacterial lignin degradation (Ahmad et al. 2011) as well as a role in fungal degradation of lignin by basidiomycetes (Liers et al. 2011).

Sugarcane bagasse, in addition to serving as soil texturizer, could have also contributed with carbohydrates that could have been utilized by *T. asperellum* as carbon source. In fact, *T. asperellum* produces a potent lignocellulolytic cocktail when grown on sugarcane bagasse (Marx et al. 2013), favoring the use of alternative carbon sources (including PAHs). This is particularly relevant in soil, where a complex mixture of substrates can be found and greatly favors the use of biostimulation in conjunction with *T. asperellum* for the bioremediation of soils.

The results of this study indicate that *T. asperellum* H15 possesses the ability to degrade high amounts of LMW and HMW PAHs from contaminated soils and has a great potential for use in soil remediation processes. Based on our findings, we suggest that PAH degradation mechanisms in *T. asperellum* H15 could be mediated by dioxygenase enzymes, which could contribute to the initial degradation of LMW-PAHs (Phe) and subsequently, laccase, peroxidise, and dioxygenase enzymes continue the degradation process of the remaining LMW and HMW-PAHs.

Conclusions

Our results show that *T. asperellum* H15 is an efficient PAH degrader in soil and is able to tolerate high amounts of PAHs and that the presence of PAHs induces the production of enzymes involved in the oxidation of PAH aromatic rings at

different stages. This, along with its great ability to grow in soil, make *T. asperellum* a microorganism with great potential for use in the bioremediation of PAH-contaminated soils. To our knowledge, this is the first report on the biodegradation of LMW and HMW PAHs by *T. asperellum*. Further studies testing the bioremediation of impacted soils at field scale, as well as intermediate production are necessary to better address the degradation mechanisms and bioremediation potential of this microorganism.

Acknowledgments This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT) project CB2008-105643, Instituto Politécnico Nacional project SIP20144071 and CONACYT grant 269828.

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