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The fungus *Lewia* sp. alleviates the oxidative stress in *F. arundinacea* during the endophyte-assisted phytoremediation of hydrocarbons

El hongo *Lewia* sp. mitiga el estrés oxidante en *F. arundinacea* durante la fitorremediación de hidrocarburos asistida por endófitos

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are mutagenic and carcinogenic compounds, which accumulate in the environment. Endophyte-assisted phytoremediation is a feasible alternative to reduce the concentration of PAHs in soils more efficiently than plants or microorganisms individually. Endophytes improve the growth, fitness, and antioxidant system of their hosts, especially under stress conditions. This study aimed to assess the influence of *Lewia* sp. on the antioxidant defense and hydrocarbon removal by *F. arundinacea* during 45 days of exposure to a hydrocarbon mixture. Plant growth and lipid peroxidation (LPO), as well as superoxide dismutase (SOD), peroxidase (POD) and glutathione S-transferase (GST) activities were determined in a kinetic assay. The association improved the hydrocarbon removal (7-44 units above the non-associated plant) over a 45-day period, while promoting plant growth (about 2-fold) and controlling oxidative damage, particularly in the roots. The root antioxidant system in *F. arundinacea* is itself effective in counteracting the phytotoxicity of direct contact with hydrocarbons. However, the fungus's antioxidant system, which involves the coordinated activity of SOD and POD, helps control LPO in the roots. Once the endophytic association is established, it could be effectively applied to phytoremediate hydrocarbon-polluted soils during a minimum period of 45 days.

Keywords: polycyclic aromatic hydrocarbons; model soil; antioxidant enzymes; tall fescue; endophytic fungi.

Resumen

Los hidrocarburos aromáticos policíclicos (HAP) son compuestos mutagénicos y cancerígenos que se acumulan en el ambiente. La fitorremediación asistida por endófitos es una alternativa para reducir la concentración de HAP en los suelos, de manera más eficiente que cada organismo individualmente. Los endófitos mejoran el crecimiento, la salud y el sistema antioxidante de las plantas, especialmente bajo condiciones de estrés. El objetivo del estudio fue evaluar la influencia de *Lewia* sp. sobre la defensa antioxidante y la remoción de hidrocarburos por *F. arundinacea* durante 45 días de exposición a una mezcla de hidrocarburos. En una cinética, se determinó el crecimiento y la lipoperoxidación (LPO), así como la actividad superóxido dismutasa (SOD), peroxidasa (POD) y glutatión S-transferasa (GST). La asociación mejoró la remoción de hidrocarburos (7-44 unidades arriba de plantas control) y el crecimiento vegetal (cerca del doble) durante 45 días, además de controlar el daño oxidante, particularmente en raíces. El sistema antioxidante de *F. arundinacea* es efectivo para contrarrestar la fitotoxicidad de los hidrocarburos. Sin embargo, el sistema antioxidante del hongo, que implica la actividad coordinada de SOD y POD, ayuda a controlar la LPO en raíces. Una vez establecida la asociación endófita, podría aplicarse para fitorremediar suelos contaminados con hidrocarburos por al menos 45 días.

Palabras clave: hidrocarburos aromáticos policíclicos; suelo modelo; enzimas antioxidantes; festuca; hongos endófitos.

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1 Introduction

Polycyclic aromatic hydrocarbons, compounds usually containing two (naphthalene) to seven (coronene) condensed benzene rings, are considered a major concern due to their wide presence in the environment, their limited biodegradability, their bioaccumulation potential and their mutagenic and carcinogenic effects (Alagic *et al*., 2015; Kadri *et al*., 2017). Biological methods such as endophyte-assisted phytoremediation is a feasible technique to reduce soil pollution, as well as being a more environmentally friendly alternative. In addition to plant growth promoting bacteria, plant endophytes also play an important role in phytoremediation of soil. Endophytes are free-living microorganisms that can inhabit the intercellular space of plant tissues or specific plant structures, without causing negative effects on the host (He *et al*., 2020). Studies about endophytic interactions have shown the key role of these microorganisms in the growth and fitness of plants subjected to stress conditions, including the presence of contaminants (Rho *et al*., 2018). On the other hand, several studies carried out both at the laboratory level and in the field have shown that tall fescue (*Festuca arundinacea*) has an important value for phytoremediation of soils contaminated with organic pollutants, including PAHs (Lu *et al*., 2014; Cruz-Hernández *et al*., 2013; Mendarte-Alquisira *et al*., 2017). This plant species is a common pasture plant for livestock production, and is also used in phytoremediation due to its fibrous root system and large root-specific surface area (Gao *et al*., 2012).

Recently, the enhanced efficiency of PAHs phytoremediation by the association between *F. arundinacea* and the endophytic fungus *Lewia* sp. was demonstrated (Cruz-Hernández *et al*., 2013; Mendarte-Alquisira *et al*., 2017). However, contact of an organism with pollutants inevitably leads to a metabolic imbalance that modifies the balance between the production and elimination of reactive oxygen species (ROS) which, if not controlled, can cause oxidative stress that significantly affects the plant health. Thus, to avoid such a condition, any organism used for the purpose of phytoremediation must possess the metabolic machinery to tightly control the intracellular concentration of ROS (Peralta-Pérez and Volke-Sepúlveda, 2012). To counteract the harmful effects of ROS, aerobic organisms have a network of antioxidant mechanisms - both enzymatic and non-enzymatic - to scavenge ROS. The main non-enzymatic antioxidants include glutathione, ascorbate, tocopherol, flavonoids, and carotenoids, while the main antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione S-transferases (GST), peroxiredoxin, and various peroxidases (Mittler, 2002). Several studies have shown that endophytic fungi produce numerous antioxidants in response to biotic and abiotic stress, which play key roles in improving stress tolerance in host plants. In fact, a significant impact of endophytes on the antioxidant activity of colonized hosts compared to non-colonized hosts has been demonstrated, especially under stress conditions (Hamilton *et al*., 2012; Bacon and White, 2016; Mendarte-Alquisira *et al*., 2017). Related to this, the positive role of *Lewia* sp. in promoting growth and alleviating oxidative stress in *F. arundinacea* exposed to hydrocarbons was recently demonstrated (Mendarte-Alquisira *et al*., 2017). Considering the aforementioned background, the aim of this study was to assess the influence of *Lewia* sp. on antioxidant defense and the hydrocarbon removal by *F. arundinacea* during 45 days of exposure to a hydrocarbon mixture. This kinetic study can help predict the time required for endophyte-assisted phytoremediation of a hydrocarbon-contaminated site.

2 Materials and methods

2.1 Plant material

Seeds of *Festuca arundinacea* Schreb. (Titan Ultra, Smith Seed Services) were purchased at the central food market in Mexico City. Seeds were surface-sterilized for which, they were placed into a filter paper (Whatman 40), submerged in 2% commercial anionic surfactant (20 min) with agitation, and rinsed with tap water. Under aseptic conditions, the seeds were immersed in 70% ethanol (30 s), and in a 1.8% NaClO solution added with 0.01% Tween-20 (30 min), and then repeatedly rinsed with sterile deionized water. Seeds were aseptically sown in Magenta boxes (Sigma) containing sterile Murashige-Skoog (MS) medium (Sigma) with sucrose (10 g L^{-1}, Sigma) and Phytagel (1.8 g L^{-1}, Sigma). The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving (121°C, 15 min).

Sixteen seeds per box were grown for 15 days at 25 ± 2°C under a 16 h photoperiod (light

intensity of 60 μmol m^{-2} s^{-1}). Two groups of plants were produced for the assays described below: one corresponding to endophyte-free plants (EF), and the other to endophyte-colonized plants (EC). The plants, inoculated or not, were grown for 15 days more at 25 ± 2 °C (16 h photoperiod). After a total growth period of 30 days, plants were harvested either for analysis (initial time, t_0) or for their transplant to flasks containing the model soil.

2.2 *Fungal inoculum and induction of the endophytic association*

The ascomycete fungus *Lewia* sp. was previously isolated from plants of *F. arundinacea* growing under *in vitro* conditions (Cruz-Hernández *et al*., 2013). The fungal inoculum was prepared by inoculating mycelium discs (5 mm) on Petri dishes containing MS medium with sucrose (10 g L^{-1}) and Phytagel (pH 5.7 ± 0.1) and incubated at 30°C for 15 days. Mycelium disks (5 mm diameter) were cut from the periphery of colonies, and the adhered culture medium was removed with a sterile scalpel. The recovered biomass was aseptically disaggregated by agitation in tubes containing glass beads (3 mm) and isotonic solution (0.9% NaCl) (Mendarte-Alquisira *et al*., 2017). Based on previous results (Mendarte-Alquisira *et al*., 2017), a biomass suspension containing 10.1 ± 0.1 mg mL^{-1} was inoculated around the roots of each plant (2 mL per plant) to establish endosymbiosis in a set of plants (EC).

2.3 *Treatments and culture conditions*

Assays were carried out in glass flasks containing the model soil contaminated with 800 mg kg^{-1} (dry soil) of a hydrocarbon mixture (HCM) consisting of hexadecane (HXD), phenanthrene (PHE), and pyrene (PYR) in a proportion 2:1:0.5 (by weight), respectively. This hydrocarbon mixture was selected since HXD is a major component in diesel, while PAHs are ubiquitous pollutants in water, sediments, soil, and vegetation (Dubrovskaya *et al*., 2017). Model soil consists of a mixture (1:1, v/v) of perlite (Dicalite, Mexico) and silica sand, which were sieved (0.4-2.8 mm, and 0.4-2.0 mm, respectively), washed, and dried (60°C, 72 h). The HCM was dissolved in acetone before being added to 84 g of model soil (dry weight, DW) contained in each flask. Acetone was allowed to evaporate (24 h), and then the soil was moistened with 40 mL of MS medium with sucrose (16 g kg^{-1} DW); the flasks were then autoclaved at 121°C for 20 min.

The thirty-day-old plants grown as indicated above were transferred to the contaminated flasks and separated into two treatments: EF plants and EC plants. Each treatment consisted of three replicates (three flasks) with 12 plants each. Plants were incubated for 45 days at 25 ± 2°C (with a 16 h photoperiod), and harvested after 7, 14, 21, 28 and 45 days for analyses. At each sampling time, 36 plants of each treatment were harvested, and separated into roots and shoots to destructively analyze them as follows: (i) 12 plants (four plants from three independent replicates) were oven-dried (60 °C, 48 h) for biomass determination on a DW basis; (ii) 24 plants were treated fresh: 12 to determine (by triplicate) the content of malondialdehyde (MDA), and 12 to obtain crude extracts (by triplicate) for enzyme activity analyses.

2.4 *Preparation of crude extracts*

About 100 mg of fresh roots or shoots were ground separately with liquid nitrogen and then homogenized in 1 ml of cold sodium phosphate buffer (50 mM, pH 7) with 1% polyvinylpyrrolidone, and 5 μL of protease inhibitor cocktail (P8215, Sigma). Homogenates were treated in a cell disruptor (Mikro-Dismembrator U, Sartorius) for 2 min at 2000 rpm and then centrifuged (4°C, 10 min, and 10629 x g). The resulting supernatants were used as crude extracts (CE) for enzymatic assays, and the protein concentration was determined by the Lowry method in a microplate spectrophotometer (ELX808, Bio-Tek Instruments), using a commercial kit (Bio-Rad) and bovine serum albumin (BSA, Sigma) as standard.

2.5 *Lipid peroxidation*

Lipid peroxidation (LPO) was quantified by measuring the MDA concentration in crude extracts prepared from about 100 mg of fresh tissues, which were homogenized in Tris-HCl buffer (20 mM, pH 7.4) with 10 μL of butylated hydroxytoluene (500 mM), and centrifuged (4°C, 15 min, 20817 x g). The reaction mixture (200 μL) contained 100 μL of CE added with a solution containing 15% trichloro acetic acid, 0.5% thiobarbituric acid and 0.25 N HCl (Mendarte-Alquisira *et al*., 2017). The mixture was incubated at 90°C for 25 min, and the chromogen produced was measured at 535 nm. The MDA concentration was estimated considering an extinction coefficient (ε) of 155 $mM^{-1}cm^{-1}$ (Buege and Aust, 1978).

2.6 Antioxidant enzymes activity

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined with a commercial kit (19160, Sigma), which measures the decrease in absorbance (450 nm) due to reduction of a water-soluble tetrazolium salt with the superoxide radical ($\cdot O_2^-$). The rate of reduction of the tetrazolium salt is linearly related to xanthine oxidase activity, which is inhibited by SOD. The measurements are compared with a standard curve of SOD. One unit (U) of SOD activity is defined as the amount of enzyme that inhibits in 50% the reduction of $\cdot O_2^-$ per minute at 25°C and pH 7.

Non-specific peroxidases (POD, EC 1.11.1.7) activity was assayed by measuring the oxidation of guaiacol to tetra-guaiacol catalyzed by POD in the presence of H_2O_2, which was monitored at 450 nm for 4 min. The reaction mixture contained 10 μL of CE, 200 μL of sodium phosphate buffer (50 mM, pH 7), and 10μL of 1% guaiacol. The activity was calculated considering ε = 26.6 mM^{-1} cm^{-1} (Chance and Maehly, 1955). One unit of POD activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of tetra-guaiacol per minute at 25°C and pH 7.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was quantified using a commercial kit (CS0410, Sigma), which measures the increase in absorbance (340 nm) due to the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST (Habig and Jakoby, 1981). The reaction mixture contained 20 μL of CE and 180 μL of substrate solution (200 mM GSH and 100 mM CDNB in Dulbecco's buffer at pH 7). The activity was calculated from the initial reaction rate considering ε = 9.6 mM^{-1} cm^{-1} (Habig and Jakoby, 1981). One unit of GST activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of the GS-DNB conjugate per minute at 25 °C and pH 7.

2.7 Extraction and quantification of hydrocarbons

In order to analyze their removal, residual hydrocarbons after each established sampling time were recovered from the model soil by microwave-assisted solvent extraction (MARS Xpress, CEM). For that, 5 g of dry soil were added with 30 mL of a mixture (1:1, v/v) prepared with dichloromethane (DCM)/acetone, and processed at 150°C (175-200 psig) for 30 min. The solvent mixture was completely evaporated in a rotary evaporator (V-800, Büchi), and the residual HCM was dissolved in 10 mL of DCM for chromatographic analysis.

The residual hydrocarbons content in each flask was measured by gas chromatography (GC, GC-2010 Plus Shimadzu) with a flame ionization detector (FID), using and a DB-H1T column (15 m, 0.25 mm of intern diameter x 0.10 μm of film thickness, Agilent) with nitrogen as carrier gas (2 mL min^{-1}). The injection volume was 1 μL. GC was performed using an initial temperature of 100°C, which was increased to 200°C (20°C min^{-1}), and then held for 1 min. The injector and detector temperature was set at 300°C. The hydrocarbon removal was calculated by comparing the sample peak height with the peak of the corresponding hydrocarbon from a standard curve prepared in DCM. The detection limits were 0.89, 0.45 and 0.22 mg kg^{-1} for HXD, PHE and PYR, respectively. The available initial concentration of each hydrocarbon in the HCM (about 270 mg kg^{-1}) after sterilization was as follows (mg kg^{-1}): HXD, 156.7 ± 6.1; PHE, 50.5 ± 1.0; PYR, 59.3 ± 3.8.

2.8 Statistical analysis

Significant differences between treatments were tested by one-way ANOVA and the comparison of means was done by a Tukey-B test (α = 0.05). Statistical differences among treatments are indicated with different letters or asterisks. The results are expressed as mean values obtained from at least three independent replicates with their corresponding standard deviation (SD). The correlation analysis was tested between hydrocarbon removal, lipid peroxidation (MDA), and the enzyme activity (SOD, POD, and GST) in EF and EC plants. Analyses were performed with SPSS, version PASW 18 (IBM SPSS-IBM Corp. 2009).

3 Results

3.1 Hydrocarbon removal

About 50% of the hydrocarbons contained in the HCM were removed during the first 7 days of incubation in both EF and EC plants. With the exception of HXD in EF plants, the HCM removal increased significantly over time in the two plant groups, reaching more than 83% removal of each individual hydrocarbon in EC plants, and up to 81% in EF plants (Table 1). Except for PHE, *Lewia* sp. significantly improved the removal

Table 1. Hexadecane (HXD), phenanthrene (PHE) and pyrene (PYR) removal by endophyte-free (EF) and *Lewia* sp.-colonized (EC) plants of *F. arundinacea* grown for 45 days in a model soil polluted with a hydrocarbon mixture⋆.

Time (days)	HXD (%)		PHE (%)		PYR (%)	
	EF	EC	EF	EC	EF	EC
7	55.0±0.4	66.3±3.5*	62.1±1.3	49.3±4.1*	58.5±1.0	63.9±1.3*
14	49.0±2.3	76.8±3.3*	70.0±2.0	49.3±1.4*	57.3±0.5	60.8±0.1*
21	48.0±2.5	79.9±2.7*	71.6±2.0^{c}	50.3±1.3*	63.5±0.7	68.9±0.3*
28	51.8±1.3	77.3±4.7*	72.8±0.7	63.9±1.3*	71.5±1.1	85.1±0.4*
45	51.2±1.2	95.4±0.8*	76.2±1.0	83.1±2.5*	81.0±0.5	91.4±0.1*
Initial available (mg kg^{-1})	156.7±6.1		50.5±1.0		59.3±3.8	

⋆Significant differences between EF and EC plants are indicated with an asterisk (p<0.001, n=3)

Table 2. Pearson correlation coefficients between hydrocarbon (HCM) removal, lipid peroxidation (MDA), and antioxidant enzyme activity (SOD, POD, and GST) in shoots and roots of endophyte-free (EF), and endophyte-colonized (EC) plants of *F. arundinacea*.

Variable	Endophyte-free (EF)					Endophyte-colonized (EC)				
	HCM	MDA	SOD	POD	GST	HCM	MDA	SOD	POD	GST
					Roots					
HCM	1					1				
MDA	0.86**	1				-0.79**	1			
SOD	-0.93**	-0.83*	1			-0.87**	0.72*	1		
POD	-0.74*	-0.61	0.94**	1		-0.89**	0.75*	0.98**	1	
GST	-0.76*	-0.63	0.82*	0.89**	1	0.09	-0.34	0.19	0.30	1
					Shoots					
HCM	1					1				
MDA	0.82**	1				0.92**	1			
SOD	-0.05	-0.23	1			0.70	0.62	1		
POD	-0.77*	-0.43	0.4	1		-0.68	-0.80*	-0.68	1	
GST	-0.26	-0.29	0.87**	0.72	1	-0.90**	-0.89**	-0.74*	0.90**	1

* Indicates a significant correlation at p <0.001; ** Indicates a significant correlation at p <0.0001.

of HXD and PYR from 7 to 45 days of culture, reaching more than 90% removal at the end of the culture. Contrary to the above, the removal of PHE by EF plants was more efficient than by EC until 28 days; however, subsequently, removal by EC plants increased slightly (7 units) with respect to EF.

3.2 Plant growth and lipid peroxidation

Compared to EF plants, *Lewia* sp. significantly improved (>2-fold) total biomass production in *F. arundinacea* since the seedling production period (i.e. 15 days before transplant), obtaining healthier plants from the start of exposure to HCM in the model soil. Particularly, the root biomass production was significantly increased (>2.7-fold) due to the endophytic association compared to EF plants (Fig. 1a).

Regarding oxidative stress, measured in terms of LPO (MDA production), *Lewia* sp. decreased (2.3 - 6.0 times) the MDA content in the roots compared to the EF roots after 14 days (Fig. 1b), which was significantly (p <0.0001) negative correlated with the HCM removal (Table 2). In EF roots, the MDA content increased (up to 6 times with respect to t_0) as a function of the exposure time to the HCM, registering a significant (p <0.0001) positive correlation (Table 2). Although the MDA content in the shoots of EC plants was initially lower than in EF plants, after 14 days of exposure to the HCM, the LPO was significantly increased in both plant groups after 45 days, reaching

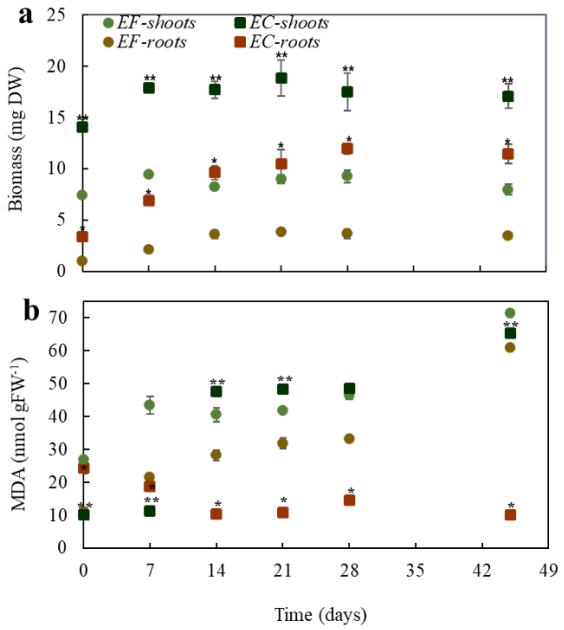

Figure 1. Biomass production (a), and malondialdehyde (MDA) concentration (b) in roots and shoots of endophyte-free (EF) and endophyte-colonized (EC) plants of *F. arundinacea* grown for 45 days under *in vitro* conditions in the presence of a hydrocarbon mixture. Significant differences regarding EF plants are indicated with * for roots and with ** for shoots (P < 0.0001; n = 12 for biomass; n = 3 for MDA).

up to 6 times more than the initial time. For both EF and EC shoots, a significant (p <0.0001) positive correlation was obtained between HCM removal and MDA content (Table 2).

3.3 Antioxidant enzyme activity

The presence of *Lewia* sp. stimulated SOD activity in the roots, particularly during the first 14 days of culture, contrary to that observed in the shoots, in which the activity in EF plants was greater (1.6 to 3.0-fold) than that registered in EC plants during the same period; after 21 days, SOD activity in shoots and roots remained without significant changes among EC and EF plants (Fig. 2a). The endophytic fungus significantly promoted POD activity in both shoots and roots throughout the culture time and, particularly during the first 7 days in the shoots (>2-fold regarding EF shoots) (Fig. 2b). SOD and POD activity in the roots shows significant negative correlations with the HCM in both EF and EC plants; likewise, the activity of both enzymes was positively correlated (p <0.001) with the MDA content in EC roots. Both enzymes were also positively correlated (> 0.94) with each other in the roots of both plant groups, but not in the shoots (Table 2). SOD activity in the shoots only showed significant correlation with GST, being positive in EF plants and negative EC plants. GST activity was the only one that remained at an almost constant level throughout the growing period in the roots of EC plants (Fig. 2c). In the shoots, GST activity registered an increase at 7 days in both EF and EC plants, being significantly higher (1.5-fold) in the former compared to that of EC plants up to 21 days. After 28 days, this activity decreased in both groups of plants, remaining at low levels until 45 days. In EC plants, while GST in the shoots presents negative correlation (p <0.001) with the HCM, MDA, and SOD, in the roots, this enzyme activity was not correlated with any variable (Table 2).

4 Discussion

This work describes the beneficial effect of the endophytic association between *F. arundinacea* and *Lewia* sp., in terms of plant growth, as well as in the hydrocarbon removal - particularly HXD - and in the control of oxidative stress generated by the presence of a hydrocarbon mixture (HCM), which was reflected in a significant decrease in LPO (MDA), especially in the roots. It should be noted that about 66% of the initial HCM concentration was not bioavailable in the model soil, obtaining about 35, 22 and 53% bioavailable of HXD, PHE (3 rings) and PYR (4 rings), respectively, regarding the initial concentration in each case. Of the three hydrocarbons in the mixture, PYR was the most bioavailable for the plant in the studied system.

4.1 Hydrocarbon removal

Unlike metals, there are no specific transporters in plant roots for organic pollutants uptake. That is, they are taken up passively together with water and nutrients from the soil, with root hairs being the most active tissue in this process (Alagic *et al.*, 2015). During phytoremediation of organic pollutants, endophytes can substantially improve biotransformation through different metabolic traits and enzyme systems, leading to greater process efficiency, further reducing phytotoxicity and pollutant concentration compared to using plant or soil microbes

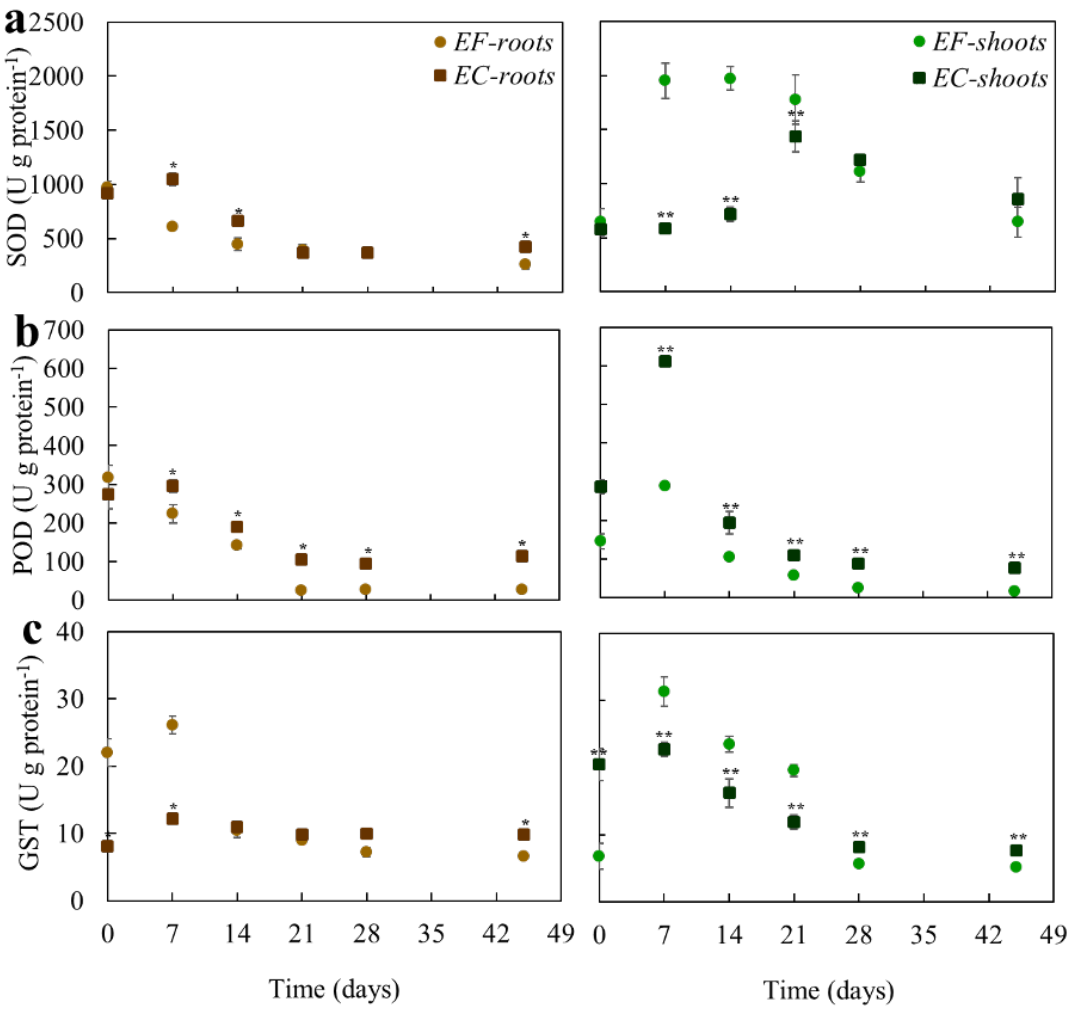

Figure 2. Antioxidant enzyme activity, SOD (a), POD (b) and GST (c), in roots (left) and shoots (right) of endophyte-free (EF) and endophyte-colonized (EC) plants of *F. arundinacea* grown for 45 days in a model soil containing about 280 mg kg^{-1} DW of available hydrocarbons. Significant differences regarding EF plants are indicated with * for roots and with ** for shoots (P<0.0001, n=3).

alone (Muratova *et al*., 2015; He *et al*., 2020). Indeed, the most powerful tool in detoxification and degradation of PAHs has been shown to occur through the synergistic process between plants and associated microorganisms (Alagic *et al*., 2015). In the present study, the HCM removal by EC plants resulted higher than that obtained by EF plants, indicating that *Lewia* sp. possess the metabolic capacity to improve such a process. In a previous study, *Lewia* sp. also stimulated the hydrocarbon removal by *F. arundinacea* in a non-axenic system using perlite contaminated with 1500 mg kg^{-1} of a HCM, reaching 100% degradation of PYR after 45 days of culture (Cruz-Hernández *et al*., 2013). Nonetheless, in such a study, the initial sorption of hydrocarbons in perlite was not considered. The high HCM removal in the previous study, performed in a non-axenic system, can also be attributed to the presence of existing microorganisms subjected to the selective pressure of the presence of contaminants (Dourado *et al*., 2017), thus contributing to degradation. In another study, inoculation of tall fescue with the arbuscular mycorrhizal fungus *Glomus caledonium* improved removal of 4- and 5-ring PAHs (Lu and Lu, 2015).

The high HCM removal during the first seven days of cultivation (Table 1), can be attributed to the sorption in the roots of both EF and EC plants. This was observed in rice plants grown in a polluted soil,

in which more than 60% of the total accumulated PAHs - both adsorbed and absorbed - was found in the root tissues (Jiao *et al*., 2007). In fact, most lipophilic organic compounds, such as hydrocarbons in the HCM, have been shown to be adsorbed on the root surface, which is closely related to the surface area and lipid content in the roots (Gao and Zhu, 2004; Jiao *et al*., 2007). As *Lewia* sp. improves root production by *F. arundinacea*, the increased hydrocarbon removal by EC plants may be due, at least in part and particularly at the beginning of the trial, due to a greater surface area available for sorption of these highly water-insoluble pollutants. Furthermore, fungal metabolism has been shown to contribute significantly to the biodegradation of aromatic compounds (Cisneros-de la Cueva *et al*., 2016).

On the other hand, the root PYR concentration is, in general, higher than that of PHE, even in soils with similar concentrations of both PAHs (Gao and Zhu, 2004). This has been demonstrated for different plant species, also finding significantly positive correlations between root concentrations of PHE or PYR and root lipid content (Gao and Zhu, 2004; Gao and Ling, 2006). The results of this study confirm the above, since the removal of PYR was greater than that of PHE in both systems with EF and EC plants. This can be explained by the fact that most lipophilic organic compounds tend to accumulate in the root epidermis, and the degree to which such compounds enter plant roots depends on the octanol/water partition coefficient (K_{ow}). In general, the greater the lipophilicity of a compound, the greater its concentration in the roots (Gao and Ling, 2006). Thus, since PYR is a more lipophilic molecule (log K_{ow} 4.88) than PHE (log K_{ow} 4.46), the former tends to accumulate to a greater extent in the roots of *F. arundinacea*, therefore registering a greater removal from the model soil.

4.2 Oxidative stress and antioxidant defense

Endophytes can facilitate phytoremediation directly or indirectly. In the first case, they intervene directly in the biodegradation processes (by producing several types of enzymes), as well as in the extraction and accumulation of contaminants. They can also enhance plant growth by synthetizing enzymes and various bioactive compounds, such as phytohormones, by increasing nutrient supply and relieving oxidative stress, indirectly promoting phytoremediation (He *et al*., 2020). It is well known that under unfavorable conditions, such as the presence of contaminants, the equilibrium between the production and the scavenging of ROS in plants may be disturbed, which can lead to oxidative stress. When the ROS level exceeds the threshold, LPO increases in cell membranes, exacerbating oxidative stress through the production of lipid-derived radicals that can react with and damage proteins and DNA. The level of LPO has been widely used as an indicator of ROS-mediated damage to cell membranes under stress conditions, with MDA being one of the end products of the peroxidation of unsaturated fatty acids in the cells and responsible for to cell membrane damage (Mittler, 2002). Several studies have shown that exposure to PAHs induces lipid peroxidation (MDA content) in species such as *Arabidopsis thaliana* (Liu *et al*., 2009), *Solanum lycopersicum* (Ahammed *et al*., 2012), *Bruguiera gymnorrhiza* (Song *et al*., 2012), and *F. arundinacea* (Mendarte-Alquisira *et al*., 2017). In this study, the HCM significantly stimulated the MDA production in *F. arundinacea*, except in the EC roots (Table 2), suggesting that the presence of *Lewia* sp. avoided the LPO during 45 days of culture, even considering that this organ is the first one directly exposed to HCM. This indicates that this endophytic fungus plays a key role in controlling LPO in tall fescue roots, which could also be related to improved growth (Fig. 1).

In fact, a key role of endophytes in terms of phytoremediation is their contribution in the control of oxidative stress in plants growing in the presence of toxic compounds, consequently improving plant growth. The increase in biomass production in *F. arundinacea* exposed to hydrocarbons was also recorded in plants associated with the endophytic fungi *Neotyphodium coenophialum* (Soleimani *et al*., 2010), and *Lewia* sp. (Cruz-Hernández *et al*., 2013; Mendarte-Alquisira *et al*., 2017) and with *G. caledonium* (Lu and Lu, 2015). Several studies have also shown an increase in the antioxidant activity of endophyte-colonized plants compared to non-colonized plants, especially when exposed to stress conditions, thus leading to counteract oxidative stress (Hamilton *et al*., 2012; Bacon and White, 2016). A key tolerance mechanism under abiotic stress conditions in endophytic associations, includes protection against oxidative stress by increasing the production of antioxidants either by the endophyte or by the host plant in response to the endophyte (Bacon and White, 2016).

It has been consistently shown that inoculating plants with mycorrhizal or endophytic fungi reduces the phytotoxicity of organic xenobiotic pollutants,

such as PAHs, alleviating oxidative stress as a result of antioxidant enzymes such as SOD, POD, CAT, and GST. In addition to their action eliminating ROS, several of these enzymes are also directly involved in degradation-detoxification reactions of xenobiotic, including various types of POD, and GSTs (Gao *et al*., 2012; Lenoir *et al*., 2016). Many studies indicate that fungi - both mycorrhizal and endophytic - intervene in two key aspects of xenobiotic phytoremediation: (i) improving the establishment and growth of plants in polluted soils; (ii) increasing the removal or degradation of pollutants (Lenoir *et al*., 2016).

In EC roots, the positive correlation MDA-SOD and MDA-POD (Table 2) suggests that LPO in this organ is controlled, at least in part, by the joint activity of both enzymes, among which a high positive correlation (0.98, $p < 0.0001$) was also found (Table 2). This response may be influenced by the fungus's own antioxidant activity, since in EF roots there was no correlation between these variables. The strong positive correlation between the two antioxidant enzymes in the roots of EF and EC plants, suggests their coordinated activity to control ROS produced during the photosynthetic electron transport chain. In fact, the balance between SOD and POD or CAT activity in cells is crucial to maintaining a stable level of superoxide radicals ($\cdot O_2^-$) and H_2O_2 (Mittler, 2002). Similar to our results, SOD and APX (ascorbate peroxidase) were positively correlated with each other in all tissues of *B. gymnorrhiza* plants grown in the presence of PYR (Song *et al*., 2012).

In addition to their action in controlling H_2O_2 levels in cells, plant PODs have a broad substrate specificity and therefore can degrade a wide range of organic compounds (Dubrovskaya *et al*., 2017). Several studies have confirmed that PODs produced by species such as sorghum, alfalfa, and tall fescue, are involved in the rhizosphere degradation of PAHs and PAHs-derivatives. For instance, PODs in sorghum oxidize PHE and anthracene, whereas in alfalfa (Dubrovskaya *et al*., 2017) and *F. arundinacea* (Gao *et al*., 2012) PODs participate directly in the oxidation of PHE. Nonetheless, similar to the results here obtained for POD (Fig. 2), a decline in the activity was also found in extracellular extracts of alfalfa plants grown in the presence of PHE (Dubrovskaya *et al*., 2017), and in tissues of *B. gymnorrhiza* plants exposed to PYR (Song *et al*., 2012). It has been proposed that PAHs-derivatives and products of their microbial degradation can inhibit the activity of PODs, which could indicate a greater availability for these substances in the active centers of these enzymes (Dubrovskaya *et al*., 2017). Based on the above, in relation to EF plants, the lower POD activity in EC plants, as well as the greater PAHs removal and the improved biomass production and plant fitness after 45 days, could be related to the joint activity of plant and fungal PODs to metabolize PHE and PYR. Indeed, it is well known that interactions between plant roots and associated microorganisms can biotransform organic xenobiotics into non-toxic or less toxic forms (Alagic *et al*., 2015). Many fungi produce extracellular PODs that are responsible for the initial oxidation of PAHs (Kadri *et al*., 2017). Although the peroxidases produced by basidiomycete fungi (lignin- and manganese-PODs) have been extensively studied in relation to biotransformation of xenobiotics, the potential roles of ascomycete fungi - such as *Lewia* sp. - for that purpose, as well as the catabolic pathways involved, remain poorly understood. Nonetheless, ascomycetes have been identified as the dominant phylum in polluted environments, where they can transform or remove PAHs (Aranda, 2016).

On the other hand, the positive correlation (> 0.82) between POD-SOD, GST-SOD and GST-POD in EF roots suggests that: (i) GST activity could be involved in antioxidant defense and (ii) the root antioxidant system could itself be effective in counteracting the negative effect of direct contact with the HCM. In addition to their roles in endogenous metabolism, GSTs are induced as an evolutionarily conserved cellular response to oxidative stress (Moons, 2005). In contrast to EF, in EC roots, there was no correlation of GST with any of the antioxidant enzymes (Table 2), which suggests that, in the presence of *Lewia* sp., this enzyme does not participate in antioxidant defense and could rather be involved in the biotransformation of hydrocarbons through conjugation reactions. As part of the conjugation enzymes in cells, GSTs increase the solubility of xenobiotics through the formation of conjugates, and are also involved in antioxidant defense through their peroxidase and thioltransferase activity (Aranda, 2016). With respect to parental xenobiotics, the toxicity of conjugates is reduced due to their binding to non-toxic cellular compounds, such as proteins, peptides, amino acids, and polysaccharides, among others (Alagic *et al*., 2015).

In EC shoots, the high (-0.89) negative correlation of MDA with GST could indicate the participation of this enzyme also in conjugation reactions (both in fungal and plant cells), particularly with PAHs, thus contributing to their detoxification and the subsequent decrease in LPO and increased plant growth. The

high and positive correlation between GST and POD could be related to the formation of less toxic PAHs-conjugates by GSTs, thus favoring POD activity in this organ.

Conclusions

This work confirms the beneficial effect of the endophytic association *F. arundinacea-Lewia* sp. in the plant growth and hydrocarbon removal, as well as in the control of LPO, particularly in the roots, due to the presence of a HCM. The root antioxidant system in *F. arundinacea* is itself effective in counteracting the negative effect of direct contact with the HCM. However, the fungus helps control LPO in the roots, which may be a result of its own antioxidant system, involving the coordinated SOD and POD activities. Decreased POD activity in both shoots and roots may be due to the presence of PAHs in the HCM, which in turn may be related to the joint activity of plant and fungal PODs to metabolize PHE and PYR in associated plants.

Rapid initial removal of hydrocarbons from the soil may result from sorption in the *F. arundinacea* root system, whose weight (on a dry basis) was significantly increased (more than twice) by *Lewia* sp. while increasing the surface area available for hydrocarbons uptake in associated plants. The fungus possess the metabolic capacity to improve the hydrocarbon removal by *F. arundinacea* in a model soil, reaching between 83 and 95% removal of all hydrocarbons in the mixture in 45 days. Therefore, once the endophytic association has been established, it could be applied to efficiently phytoremediate hydrocarbon-polluted soils for a minimum period of 45 days.

While in non-associated plants, GST activity seems to be primarily involved in antioxidant defense, there is evidence that in associated plants, both fungal and plant GSTs could be related to PAHs detoxification through conjugation reactions, thus contributing to improve growth in the latter plants. This study shows the ability of *F. arundinacea* to tolerate the phytotoxicity of xenobiotics, such as PAHs present in HCM, confirming that this species is a good alternative for use in phytoremediation. However, induction of endophytic association with *Lewia* sp. significantly improves the plant's potential to phytoremediate a hydrocarbon-polluted solid matrix and, promisingly, to reduce PAHs contamination.

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