



## Role of arbuscular mycorrhizal fungus *Rhizophagus custos* in the dissipation of PAHs under root-organ culture conditions

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### ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common contaminants in soil. Arbuscular mycorrhizal (AM) fungi make host plants resistant to pollutants. This study aims to evaluate the impact of anthracene, phenanthrene and dibenzothiophene on the AM fungus *Rhizophagus custos*, isolated from soil contaminated by heavy metals and PAHs, under monoxenic conditions. We found a high level of tolerance in *R. custos* to the presence of PAHs, especially in the case of anthracene, in which no negative effect on AM-colonized root dry weight (root yield) was observed, and also a decrease in the formation of anthraquinone was detected. Increased PAH dissipation in the mycorrhizal root culture medium was observed; however, dissipation was affected by the level of concentration and the specific PAH, which lead us to a better understanding of the possible contribution of AM fungi, and in particular *R. custos*, to pollutant removal.

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## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) contaminate soil and water all over the world, and originate from both natural and industrial sources. Soil pollution by PAHs has a significant adverse effect on plants and soil microorganisms in addition to the well-known carcinogenic and teratogenic effects on humans (Delistraty, 1997; Pickering, 1999). The toxicity of these compounds stems from their high lipophilicity and low water solubility. Their lipophilic nature negatively affects membranes and cell structures; thus, PAHs attach themselves to the lipid membrane compounds (Sikkema et al., 1994), can then readily go through membranes into lipophilic compartments of living cells and be captured by the plant root (Harvey et al., 2002). Their high degree of insolubility means that they have limited bioavailability and consequently difficult to bioremediate; however, PAHs have different distribution patterns according to their molecular size, and their lipophilicity and insolubility increase with the number of aromatic rings (Busbee et al., 1990).

Arbuscular mycorrhiza (AM) fungi belong to a wide spectrum of soil microorganisms which establish symbiotic associations with 90% of terrestrial plants (Smith and Read, 2008). They are considered to be the main components of the ecosystem as they can significantly increase ecosystem efficiency due to the beneficial effects of AM fungi on plant growth, yield and the alleviation of stress caused by pollutants (Smith and Read, 2008). AM fungi are known to be indirectly associated with bioremediation processes due to the so-called mycorrhizosphere effect which stimulates soil microbial activity, improves soil structure and contributes to overall bioremediation of pollutants (Meharg and Cairney, 2000; Joner and Leyval, 2003). The bioremediation of PAHs has therefore been extensively studied in soil experiments (Joner et al., 2001; Joner and Leyval, 2003; Chiapusio et al., 2007; Liu and Dalpé, 2009). Nonetheless, there are very few studies on the effect of these compounds on AM fungi growth under monoxenic conditions. Experiments performed by Verdin et al. (2006) using *Rhizophagus irregulare* demonstrated the effect of this AM fungus on anthracene dissipation and its ability to store this compound in arbuscular vesicles. In addition, although recent studies have revealed the contribution of this fungus to benzopyrene dissipation, this aromatic compound adversely affects AM's lipid membranes (Debiane et al., 2011).

*Rhizophagus custos* was isolated from metal-contaminated soil on the banks of the river Tinto in Huelva (Spain) (Cano et al., 2009).

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This area is not only chronically affected by mining waste but also by the presence of PAHs in sediments (Jiménez-Tenorio et al., 2007). AM fungi can have a different effect on their hosts depending on environmental conditions and the particular species of fungi (Newsham et al., 1995).

Contaminated soils are usually characterized by limited diversity and low overall number of indigenous AM fungi which could therefore develop better strategies to guarantee their survival (Khan, 2005). For this reason, the use of microorganisms isolated from contaminated soils often has advantages over other strains obtained directly from collections of cultures which can lose their ability to metabolize specific compounds. Little is known about the ability of *R. custos* to dissipate soil contaminants and the various strategies this fungus uses to combat the physiological stress caused by pollutants. Other species studied can respond to different stresses by altering their morphology, modifying their external environment and by altering their internal metabolism, although the degree of phenotypic plasticity may vary (Finlay et al., 2008).

The aim of this study is to analyse the specific role played by *R. custos* in PAH uptake and/or dissipation as a potential phytoremediation technique. In particular, we evaluated the resistance of pure *R. custos* cultures to PAHs such as anthracene and phenanthrene – two benzenoid hydrocarbons – and dibenzothiophene, a sulphur-containing aromatic heterocycle.

## 2. Materials and methods

### 2.1. Chemicals

Anthracene, phenanthrene, dibenzothiophene and anthraquinone were obtained from Sigma–Aldrich (Madrid, Spain), 99% purity grade. The HPLC grade solvents (dichloromethane and acetonitrile) were purchased from VWR International (Barcelona, Spain).

### 2.2. Biological material

The isolated AM fungus *Rhizophagus custos* was grown in vitro in a co-culture with Ri T-DNA-transformed carrot roots (*Daucus carota* L.) on a minimal (M) medium (Bécard and Fortin, 1988). The fungus was provided by Mycovitro S.L. (Granada, Spain), collection number O10MYCO-HSP. Root cultures were subcultured every 3 weeks on White's medium (MW) (Bécard and Fortin, 1988), with the AM fungal cultures being subcultured every 3 months in Petri dishes incubated in inverted position in the dark at 25 °C.

### 2.3. Root-organ culture conditions

The in vitro experiments were carried out in sterile glass Petri dishes (90 mm in diameter) in order to avoid PAH absorption. Dishes were oven-sterilized at 170 °C for 3 h before use. Each dish was filled with 35 mL of M medium with and without the single PAH anthracene, phenanthrene, and dibenzothiophene, respectively. Prior to selecting the PAH diluting solvent, we tested mycorrhizal root tolerance using acetone, hexane, acetonitrile and chloroform as solvents and obtained the most effective root development in the following order: acetone > hexane > acetonitrile > chloroform (data not shown). Thus, to prepare the PAH-enriched culture medium, we added these organic compounds dissolved in acetone. The different PAH solutions were immediately added to the culture medium after autoclaving and cooling to RT in order to avoid evaporation occurring during the sterilization process, to reach the final concentrations of 60, 120 and 240 µM for each compound. The medium was then stirred for 15 min under sterile conditions in order to evaporate the solvent. Small losses of 10% due to evaporation were detected (analysed by HPLC as described below). Then, 35 mL of this medium was added per Petri dish. M medium with no PAHs as well as M medium containing PAH-free acetone were used as controls.

The monoxenic culture (*R. custos* and carrot roots) was produced according to the technique described by Chabot et al. (1992) using a 1 cm<sup>2</sup> piece of Gel-Gro medium containing carrot roots colonized by *R. custos* (M). In the non-inoculated treatment (NM = non-mycorrhizated control root), we used a piece of carrot root (70 mm in length). A root-free plate was used as control for PAH quantification. There were ten replicates for each experimental treatment.

### 2.4. Analysis of extraradical mycorrhization parameters

Firstly, a non-destructive analysis of the replicates was carried out at the end of the experiments after 7 weeks. Total extraradical mycelium (ERM) length, branch absorbance structures (BAS) and number of spores were assessed using the

quantification method described by Bago et al. (1998). The total number of hyphae per gridline intersection was used to estimate the total length of hyphae in a given area (Newman, 1966; see Giovannetti and Mosse, 1980) with  $R = \pi NA/2H$ , where  $N$  is the number of intersections,  $A$  the area within which the hyphae lies,  $H$  the total length of the straight lines and  $R$  the total length of hyphae in the Petri dish. Spores and BAS were also counted in each cell formed by the gridlines and totalled for each Petri dish.

Secondly, the Petri dish was divided into four quarters. One section was used to determine root colonization, the second for PAH analyses, the third for root dry weight estimation and the fourth for fluorescence microscopic visualization.

### 2.5. Estimation of AM root colonization

Roots (M and NM) were separated from the medium by solubilising the solidified media in 2 volumes of Tris–HCl buffer (50 mM, pH 7.5) with EDTA (10 mM) (v/v) and filtering roots using a sieve (Debiane et al., 2009). Histochemical staining procedures with the non-vital Trypan Blue stain were used to determine total root colonization according to the method described by Phillips and Hayman (1970). Stained roots were observed with a light microscope and the intensity of root cortex colonization by the AM fungus was determined as described by Trouvelot et al. (1986) using MYCOCALC software (<http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html>). The parameters measured according to this method were mycorrhization frequency ( $F$ ), mycorrhization intensity in the radical system ( $M$ ), and mycorrhization intensity per fragment of root cortex colonization ( $m$ ), and arbuscular abundance in the mycorrhizal root cortex ( $A$ ) and in the colonized site ( $a$ ). The data were expressed as percentages. In addition, mycorrhiza development was evaluated using the gridline intersect method described by Giovannetti and Mosse (1980).

### 2.6. PAH extraction and HPLC analysis

PAHs from the second section of the Petri dishes were quantified. Anthracene, phenanthrene and dibenzothiophene were extracted with dichloromethane from the medium, the root surface (extraradical content) as well as from the internal tissue of the carrot root (intraradical content). After sub-sampling, the roots were separated from the medium by solubilising the solidified media as described above. Extraradical PAH content from the dry root was measured by washing with dichloromethane (0.05 mg mL<sup>-1</sup>) and vortexing for 15 min. The dichloromethane extract was evaporated to dryness under N<sub>2</sub>; the residue was resuspended in acetonitrile and analysed by HPLC. The same roots were then homogenized and extracted with dichloromethane using three sonication cycles (15 min at 60 °C) to obtain the intraradical PAH content. Results were expressed as µmoles PAH mg<sup>-1</sup> dry root.

The remaining anthracene in the medium (free-root control, NM control and M treatments) was analysed using a hollow punch 6 mm in diameter. This piece was transferred to a glass tube and the solid medium was solubilised using 0.5 mL of sodium citrate buffer 10 mM pH 6.0 (Verdin et al., 2006). After complete solubilisation, the PAHs from the sample were extracted three times with the same volume of dichloromethane. The organic fraction was evaporated under N<sub>2</sub> and resuspended in acetonitrile for HPLC analysis.

Chromatographic analysis was carried out using a HP 1050 HPLC system (Agilent®, Hewlett–Packard) equipped with a 190–700 nm diode array detector (DAD). Separations were carried out on a Waters RP 80A C18 reversed phase column (4 µm, 3.9 × 150 mm). Acetonitrile and phosphoric acid (15 mM; pH < 2) were used in isocratic elution mode with 85% acetonitrile for up to 6 min. The flow rate was 0.8 mL min<sup>-1</sup>. Eluted substances were detected in the wavelength range from 210 to 280 nm.

### 2.7. Root dry weight estimation

The third section of the Petri dishes was used for root dry weight estimation. Roots were separated from the medium by solubilising the solidified media as described above. The root material obtained was lyophilized and its dry weight measured.

### 2.8. Microscopic visualization of the structures

For PAH fluorescence visualization in NM and M roots, the DMI600B inverted epifluorescence microscope (Leica Microsystems, Bensheim, Germany) equipped with a digital camera and controlled by Leica Imaging software was used. Fluorescence was monitored by excitation at 450–490 nm (BP). The wavelength of fluorescence emitted was detected at 515 nm (LP).

### 2.9. Statistical analysis

Statistical analysis was carried out using Statgraphics Centurion XVI. The data were analysed using ANOVA and the means compared using Tukey's HSD (honestly significant difference) post-hoc test at  $p < 0.05$ . All homoscedasticity assumptions were confirmed using Levene's tests.

### 3. Results and discussion

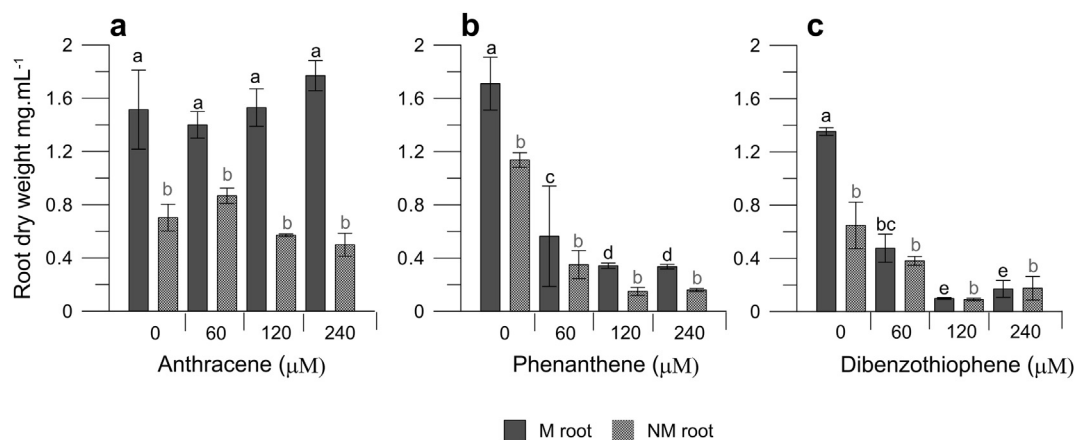
#### 3.1. Effect of PAHs on root growth

Mycorrhizal carrot roots typically showed an increase in dry weight compared to non-mycorrhizal roots of 30–40% when grown in the absence of PAHs (Fig. 1). In our experiments, different patterns of root development were detected according to the PAHs used. Interestingly, the different concentrations of anthracene used did not affect root growth in either mycorrhizal or non-mycorrhizal roots (Fig. 1a). However, the presence of phenanthrene and dibenzothiophene had a pronounced negative effect on mycorrhizal root growth, showing a reduction of around 60% at 60  $\mu\text{M}$  of the initial concentration of the aromatic compounds in the culture media. By doubling the PAH concentration, 80% and 92% reductions, respectively, in root dry weight were observed which was also found at 240  $\mu\text{M}$  initial concentration for these two aromatics (Fig. 1b, c). The non-mycorrhizal roots in the presence of anthracene experienced a drastic reduction in root growth as compared with AM colonized roots. Thus, *R. custos* mitigates the toxic effect of this PAH. However, a decrease in root growth was detected in both mycorrhizal and non-mycorrhizal roots in the presence of phenanthrene and dibenzothiophene. Studies carried out on AM chicory roots (*Cichorium intybus* L.) also showed differences in toxicity depending on the PAH structures involved: anthracene was found to be less toxic than benzopyrene since the toxicity of PAHs increases with ring number (Debiane et al., 2011). In our experiments, phenanthrene, which has the same ring number of anthracene, affected root growth more negatively than anthracene, probably due to differences in its chemical properties such as the bay effect (Gomes and Mallion, 2001). The improvement in the nutritional status of the host plants caused by the presence of mycorrhiza fungi increases the resistance to pollutants (Smith and Read, 2008). In soil experiments, several studies have actually shown that AM fungus can enhance plant growth in the presence of PAH-spiked soil (Wu et al., 2011). Our results demonstrate that the beneficial effect of *R. custos* we observed enhances root growth in the presence of anthracene and also at low concentrations of phenanthrene. Our results reveal a clear pollutant–plant interaction as the toxic compound interacts more directly with the root when grown in root organ culture than when grown in soil, and no organic matter or polymeric substances were present in the medium to retain the contaminant or to make it less available to the plants (Hatzinger and Alexander, 1995).

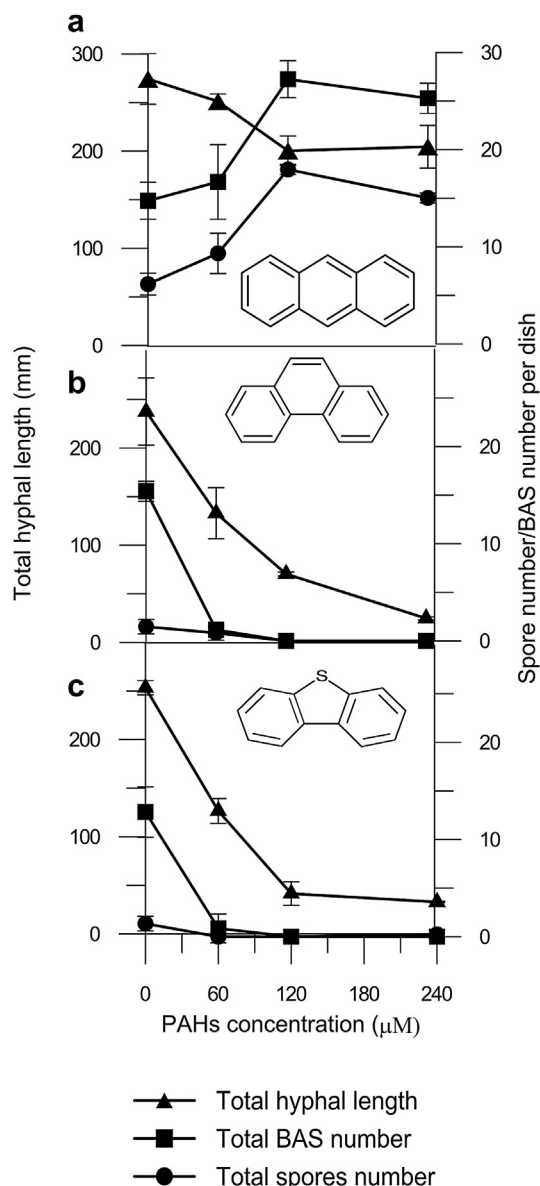
#### 3.2. Intra and extraradical development of AM fungal structures in presence of PAHs

There was approximately a 9-fold and 7-fold reduction in hyphal length during the experiments in the presence of the highest concentrations of phenanthrene and dibenzothiophene, respectively (Fig. 2b, c). However, anthracene did not drastically decrease total hyphal length, which recorded only a one-fold decrease with respect to the control (Fig. 2a). Branched absorbing structures (BAS) were regularly observed along runner and lower order hyphae in the absence of PAHs. In anthracene-supplemented medium (Fig. 2a), we observed an increase in the number of BAS; the opposite effect was detected in our experiments performed in the presence of phenanthrene or dibenzothiophene, where no BAS were observed when concentrations reached 120 and 240  $\mu\text{M}$  (Fig. 2b, c). Spore levels were higher in the media supplemented with anthracene than in the controls, with the number of spores increasing along with anthracene concentration (Fig. 2a). In media supplemented with phenanthrene or dibenzothiophene (Fig. 2b, c), although the number of spores did not change at low initial concentrations, a small decrease in sporulation was observed when the amount of these PAHs was increased. Our results indicate that PAHs exert a negative effect on *R. custos* development, BAS formation and sporulation under root organ culture conditions, as has previously been described for other AM fungi (Franco-Ramírez et al., 2007). However, the presence of anthracene was not markedly hostile to the typical symbiotic development pattern, which was also shown by an increase in the number of spores and BAS (Fig. 2a). In this respect, there is a close relationship between the absence of negative effects on root growth and the development of AM fungal structures detected in the presence of anthracene. It is important to note that different results have been observed in the case of *R. irregulare* grown with anthracene as a pollutant (Debiane et al., 2008). In that study, *R. irregulare* was found to be more sensitive to root growth and hyphal development in chicory roots than *R. custos* in carrot roots in our experiments, which could *R. custos* is adapts to pollutants in a particular way.

The percentage of root colonization, presented in Fig. 3, show that low concentrations of PAHs did not generally affect AM colonization; however, when PAH concentrations reached values of 240  $\mu\text{M}$ , AM colonization was considerably inhibited, mainly by phenanthrene and dibenzothiophene, in which the percentage of root colonization was  $\sim 7\%$  and  $\sim 21\%$ , respectively. In these cases,



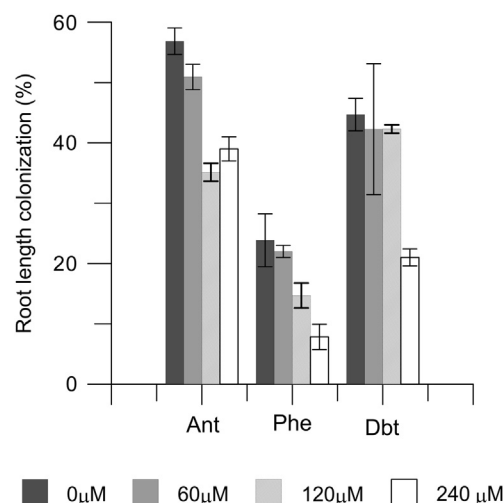
**Fig. 1.** Root dry weight ( $\text{mg mL}^{-1}$ ) of mycorrhiza-transformed carrot roots (grey bars) and non-mycorrhiza-transformed carrot roots (dotted bars) grown in monoxenic culture in the presence of different initial concentrations of anthracene (a), phenanthrene (b), and dibenzothiophene (c). Values are means of three replicates. Bars denote mean  $\pm$  S.E. ( $n = 3$ ). Different letters for each group (mycorrhizal and non-mycorrhizal carrot roots) above bars indicate significant differences according to Tukey's test ( $p < 0.05$ ).



**Fig. 2.** Total hyphal length (mm) (black triangle), spore number (black circle) and branched absorbing structures (BAS) number (black square) of *R. custos* after application of different initial concentrations of anthracene (a), phenanthrene (b) and dibenzothiophene (c). Values are means of three replicates and three repetitions. Bars denote mean  $\pm$  S.E. ( $n = 9$ ).

extraradical hyphae and, to a lesser degree, vesicles were the main fungal structures observed.

More specifically, Table 1 shows notable differences in AM fungal development when different PAH concentrations are applied. As we saw with the percentage of root colonization (Fig. 3), the largest differences in colonization intensity (M%) were due to high concentrations of anthracene, phenanthrene and dibenzothiophene. Arbuscular mycorrhizal fungi abundance (a%) drastically decreased in the presence of phenanthrene, and these fungal structures were found to have degenerated (data not shown) due to the visibly harmful effect of phenanthrene on *R. custos*. The effect of anthracene and dibenzothiophene was less pronounced; however, the latter greatly reduced the frequency of mycorrhization (F%). Several studies have reported that AM fungal colonization is negatively affected by the presence of petroleum hydrocarbons and by a mix of PAHs or a single PAH in soil (Cabello, 1997; Gaspar et al.,



**Fig. 3.** Percentage of carrot root length colonization in the presence of different initial concentrations of anthracene (Ant), phenanthrene (Phe) and dibenzothiophene (Dbt). Bars denote mean  $\pm$  S.E. ( $n = 3$ ).

2002). Such negative effects have been explained by the direct toxic impact of these compounds on AM fungi, as has been shown by certain in vitro experiments (Debiane et al., 2011).

### 3.3. Residual PAHs in the growth medium

Around 20% of PAH abiotic losses were detected in all cases during root cultivation with the aromatic compounds, particularly in treatments with phenanthrene and dibenzothiophene (data not shown). The ability to remove PAHs from the media as compared with the respective controls, was observed both in NM and M roots and expressed as μM of dissipated PAH per mg L<sup>-1</sup> of dry root in the Petri dishes. This elimination of PAHs declined with the increasing concentrations of these aromatic compounds (Fig. 4), as high concentrations of PAHs also affected the functionality of root development and symbiosis, thus decreasing the effect of mycorrhiza on PAHs dissipation.

As shown in Fig. 4a, mycorrhizal roots and, though to a lesser extent, non-mycorrhizal roots contributed to the elimination of anthracene from the growth media at low PAH concentrations. However, in the presence of phenanthrene, dissipation was more evident in non-mycorrhizal roots.

The presence of dibenzothiophene in root growth media produced an effect similar to that observed in the anthracene treatment. This compound was almost totally eliminated from the media when the AM fungus was present. In other words, although dibenzothiophene reduced spore formation in *R. custos*, the root colonized by this fungus may contribute to dibenzothiophene dissipation, mainly through the extraradical mycelium.

Our results indicate that PAH dissipation depends on the nature of the compound involved and on its concentration, with different behaviour patterns being observed for each compound. In the presence of anthracene, mycorrhiza played a protective role by contributing to root growth. However, in the presence of dibenzothiophene, this protective effect was not so evident despite the high level of dissipation observed in the presence of this compound.

Our findings from monoxenic cultures confirm the results of studies carried out on soils with AM fungal plants, in which an important role has been attributed to AM fungi in PAH dissipation (Gao et al., 2010; Wu et al., 2011). Mycorrhizal fungi do not only increase the absorption surface but also the organic surface through their hyphae and spores, which contributes to PAH mobilization



**Table 1**

Mycorrhiza development parameters in carrot roots in the presence of anthracene, phenanthrene and dibenzothiophene determined 42 days after inoculation using MYCOCALC software, expressed in percentages: (F) frequency, (M) intensity of root cortex colonization, (m) intensity of mycorrhization per fragment of root cortex colonization and (A) arbuscular abundance in the mycorrhizal root cortex and (a) the colonized location. Different letters (a, b, c) for each series indicate significant differences according to Tukey's test ( $p < 0.05$ ).

	Anthracene				Phenanthrene				Dibenzothiophene			
	0	60	120	240	0	60	120	240	0	60	120	240
F (%)	79 a	75 a	70 ab	73 ab	78.2 a	63.2 a	59.7 b	47.7 b	75.6 a	45.0 ab	56.2 ab	36.6 b
M (%)	37.9 a	33.9 b	23.4 d	26.0 c	15 a	10.78 a	4.01 b	0.47 b	17.6 a	16.8 a	13.6 a	6.2 b
m (%)	38.1 a	34.3 b	23.6 d	26.1 c	12.98 a	8.44 ab	5.94 ab	1 b	17.5 a	15.9 a	14.2 a	6.2 b
A (%)	21.4 a	21.9 a	13.5 b	14.7 b	0.81 a	3.37 a	1.45 a	0.02 a	0.4 a	0.4 a	0.7 a	0.1 a
a (%)	27.06 a	26.05 a	21.03 b	24.3 a	11.5 a	2.9 b	9.3 ab	2.0 b	5.83 a	2.4 ab	8.75 a	5 a

and binding to the root, and consequently to a more complete removal of PAHs from the medium.

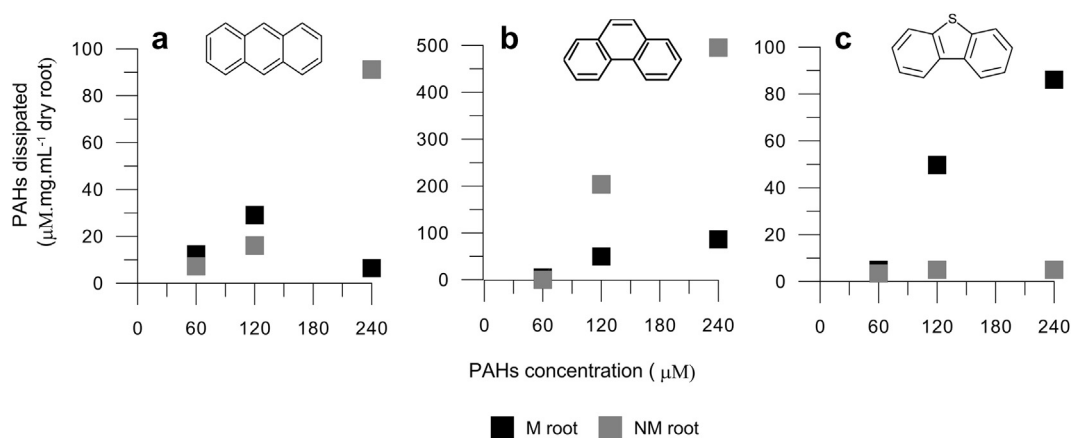
It is worth pointing out that in experiments carried out on anthracene, we detected the presence of anthraquinone, the first oxidation metabolite from this aromatic compound (Hammel, 1995) in both mycorrhizal and non-mycorrhizal roots (data not shown). Small amounts of this metabolite were also detectable in root-free controls (0.36, 0.95, 0.8  $\mu\text{M}$  in 60, 120 and 240  $\mu\text{M}$  treatments, respectively) probably due to abiotic oxidation and taking into account the absence of this metabolite in the standard, which could also explain the anthracene loss detected on the dishes. Higher concentrations of anthraquinone were found in the surrounding medium of non-mycorrhizal roots (0.6, 3.53, 12.34  $\mu\text{M}$  in 60, 120 and 240  $\mu\text{M}$  treatments, respectively) and mycorrhizal roots (0.09, 0.40, 6.46  $\mu\text{M}$  in 60, 120 and 240  $\mu\text{M}$  treatments, respectively).

The appearance of this metabolite is probably due to oxidation caused by plant peroxidases produced during oxidative stress, by laccases found in the roots, or by free radicals from the root exudates (Wild et al., 2005). AM fungi have been described as obligate symbionts with little or no capacity to degrade organic pollutants (Joner and Leyval, 2003). Nevertheless, our results represent the first evidence of the indirect involvement of AM fungi in PAH conversion by roots in the absence of a rhizosphere effect. In treatments with phenanthrene and dibenzothiophene, the absence of metabolites could be due to the high level of chemical stability of these aromatic compounds (Poater et al., 2006). Moreover, plant peroxidases have a limited effect on bioremediation processes due to their low substrate oxidation potential (+1.12 V). They are therefore able to oxidize anthracene and, to a lesser extent, phenanthrene (Kersten et al., 1990; Hammel, 1995).

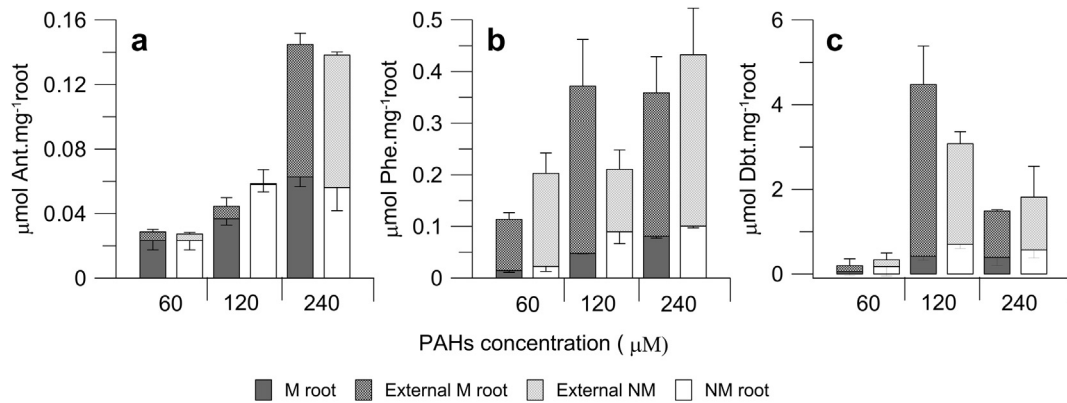
### 3.4. Intraradical and superficially bound radical PAH content

Fig. 5 shows the PAH content bound to the root surface and within the root tissue. As has been previously described, PAHs are absorbed by roots and mainly experience an apoplastic flow and, to a lesser extent, a symplastic flow (Wild et al., 2005). On the whole, we detected smaller amounts of PAHs in the mycorrhizal roots than in the non-mycorrhizal roots in relation to all the aromatic compounds tested. These results suggest that AM fungi may limit PAH accumulation by roots that are exposed to high levels of PAHs in soil through their accumulation in extraradical hyphae and spores. The amount of superficially root-bound PAHs was slightly greater in mycorrhizal than in non-mycorrhizal roots, especially at a concentration of 120  $\mu\text{M}$ . Some studies show that all PAHs are rapidly adsorbed on the root surface although root absorption is extremely limited and highly variable depending on the plant species and environmental conditions (Trapp et al., 1990). Roots are known to be capable of absorbing and adsorbing aromatic compounds, with fungal hyphae contributing to this process (Jiao et al., 2007). Some studies have actually shown that AM fungi can act as a filtration barrier and have attributed this behaviour to the increased heavy metal biosorption by outer and inner components of the mycelium (Krupa and Kozdrój, 2007). In addition, AM fungi have a large surface area, which enables mycorrhizal fungi to adsorb considerable amounts of pollutants from soil (Rajkumar et al., 2012).

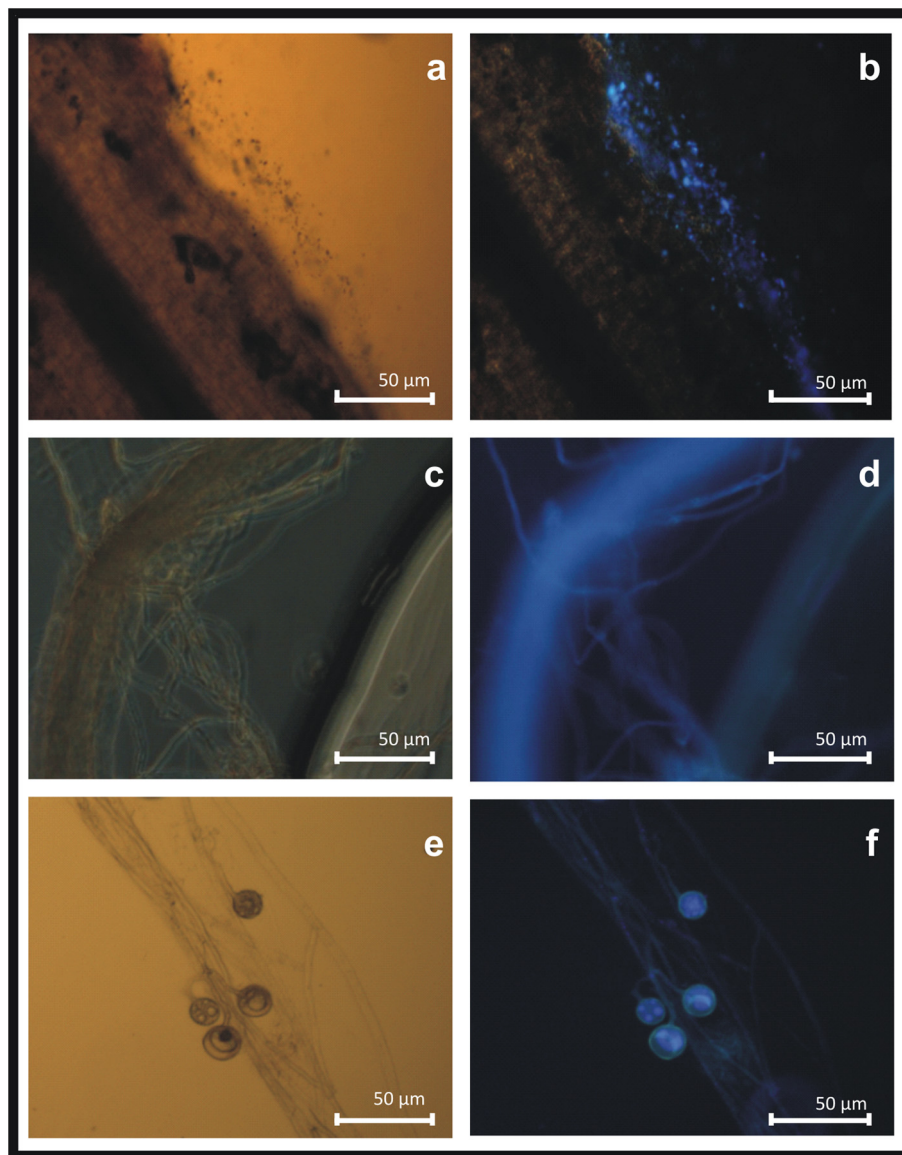
Our results demonstrate that, despite their similar structures, anthracene and phenanthrene showed very different dissipation behaviours, which could be due to the higher chemical stability level of phenanthrene as compared with anthracene on the basis of quantum mechanics (Watson et al., 2001; Poater et al., 2006). This



**Fig. 4.** Residual PAHs in the culture medium of mycorrhizal carrot roots (M) (black squares) and non-mycorrhizal carrot roots (NM) (grey squares) of *R. custos* in the presence of different initial concentrations of anthracene (a), phenanthrene (b) and dibenzothiophene (c). Residual concentration after 42 days of growth/incubation is expressed as  $\mu\text{M}$  of the PAH per  $\text{mg mL}^{-1}$  of carrot root in each treatment. Data represents means of 9 values.



**Fig. 5.** Intraradical PAH content (lower part of bar) and extraradical PAH content (upper part of bar) of mycorrhizal carrot roots (M) and non-mycorrhizal carrot roots (NM) expressed as  $\mu\text{moles mg}^{-1}$  root dry weight in the presence of anthracene (a), phenanthrene (b) and dibenzothiophene (b). Data represents means of 6 values. Error bars represent standard deviation ( $n = 3$ ). For clarity, only a positive or a negative direction error bar is shown for each stacked bar.



**Fig. 6.** Panel a, carrot root (*Daucus carota* L.) grown in the presence of 140  $\mu\text{M}$  anthracene (bright field); Panel b, carrot root grown in the presence of 140  $\mu\text{M}$  anthracene (fluorescence microscopy IP UV filter). Panel c, extraradical mycelia of *R. custos* cultured in the presence of 60  $\mu\text{M}$  anthracene (bright field); Panel d, extraradical mycelia of *R. custos* (fluorescence microscopy IP UV filter). Panel e, fungal spores of *R. custos* cultured in the presence of 60  $\mu\text{M}$  anthracene (bright field); Panel f, fungal spores of *R. custos* (fluorescence microscopy IP UV filter).

is corroborated by our study of the effect of this aromatic compound on root growth and mycorrhizal parameters.

Anthraquinone was also detected in the mycorrhizal and non-mycorrhizal roots, although a larger amount of this metabolite was found in non-mycorrhizal roots. One study has shown that some oxygenate metabolites could be more toxic than the original compound (Lundstedt et al., 2007) possibly due to the toxic effect of this oxidated metabolite on root growth. No metabolites from phenanthrene and dibenzothiophene were found in the culture medium or in the roots.

### 3.5. Fluorescence visualization

Autofluorescence visualization with UV excitation was used to localize anthracene, phenanthrene and dibenzothiophene both on and in root tissues, as autofluorescence can be produced by PAHs due to the conjugated aromatic rings in their molecular structure.

As expected, we detected the presence of anthracene, phenanthrene and dibenzothiophene in carrot roots colonized and non-colonized by *R. custos* due to the fluorescence observed in hyphae and root tissue (Fig. 6). Fluorescence microscopy showed the presence of anthracene in non-mycorrhizal root exudates as they could be involved in PAH adsorption (Fig. 6b) (Yoshitomi and Shann, 2001). These results are in line with our chemical analyses where we also observed accumulation of these aromatic compounds in carrot roots and also with other soil experiments that show extraradical mycelia's capacity to take up PAHs (Gaspar et al., 2002; Jiao et al., 2007). It is also interesting to note that we detected fluorescence in spores and in the mycelium in the presence of anthracene (Fig. 6c–f). Recent studies have demonstrated the ability of some AM fungi to compartmentalize the excess of Cu in inactive spores, which represents a possible survival strategy in a polluted environment (Cornejo et al., 2013). In our experiments, the detection of anthracene in spores could be a similar phenomenon and also explain the high root yield in the presence of anthracene and its low penetration in the root. On the other hand, soil studies showing the ability of *Glomus geosporum* to absorb phenanthrene through extraradical mycelium and spores (Gaspar et al., 2002) confirm our findings. The fact that only small amounts of phenanthrene and dibenzothiophene were detected in spores prevented us from reaching any definitive conclusion regarding the absorbance of these compounds by *R. custos*. However, further studies on aromatic compound storage by fungal structures are needed to clarify the role of AM fungi in PAH dissipation.

## 4. Conclusion

In our study, significant differences in aromatic compound dissipation were observed among PAH types with regard to AM symbiosis functionality. No clear relationship between the dissipation of aromatic compounds and PAH characteristics was found as the aromatic heterocycle dibenzothiophene showed a dissipation pattern similar to that for anthracene. We can therefore conclude that the dissipation of PAHs by *R. custos* in root organ cultures depends on the nature of the compound and also on the concentrations of these compounds in the medium.

*R. custos* has been shown to be endowed with a special resistance to the presence of anthracene; it enhanced root growth, extraradical mycelium and consequently this hydrophobic compound's adsorption surface, and had a marked tendency to attach itself to cell walls. Spores could be used as storage for this compound as anthracene has been detected in these structures. The inhibition of anthraquinone formation in the M root could also be a protective strategy, although further analyses of oxidative stress enzymes may be necessary in order to clarify the resistance of AM

fungi to oxidated PAHs. In experiments with phenanthrene and dibenzothiophene, we detected some damage to M fungal structures and a decrease in root growth as well as in hyphal length. However, in the case of dibenzothiophene, *R. custos* appears to be involved in the removal of this compound from the medium and in impeding its migration to roots.

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