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Benzo[a]pyrene induced lipid changes in the monoxenic arbuscular mycorrhizal chicory roots

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ABSTRACT

Arbuscular mycorrhizal (AM) colonization may be one of the means that protects plants and allows them to thrive on polycyclic aromatic hydrocarbon-polluted soils including the carcinogenic benzo(*a*)pyrene (B[a]P). To understand the mechanisms involved in the AM symbiosis tolerance to B[a]P toxicity, the purpose of this study was to compare the lipid compositions as well as the contents between mycorrhizal and non-mycorrhizal chicory root cultures grown *in vitro* under B[a]P pollution. Firstly, B[a]P induced significant decreases of the Glomalean lipid markers: C16:1ω5 and 24-methyl/methylene sterol amounts in AM roots indicating a reduced AM fungal development inside the roots. Secondly, whereas increases in fatty acid amounts after B[a]P application were measured in non-mycorrhizal roots, no changes were shown in mycorrhizal roots. On the other hand, while, after treatment with B[a]P, the total phospholipid contents were unmodified in non-mycorrhizal roots in comparison with the control, drastic reductions were observed in mycorrhizal roots, mainly owing to decreases in phosphatidylethanolamine and phosphatidylcholine. Moreover, B[a]P affected AM root sterols by reducing stigmasterol. In conclusion, the findings presented in this paper have highlighted, for the first time, significant changes in the AM root lipid metabolism under B[a]P pollution and have culminated on their role in the defense/protection mechanisms.

1. Introduction

The contamination of soil with polycyclic aromatic hydrocarbons (PAHs) is an issue of great environmental concern; many individual PAHs are genotoxic, mutagenic and carcinogenic [1]. The US Environmental Protection Agency (US EPA) has classified some PAHs, including benzo[*a*]pyrene (B[a]P) used in this study, as human carcinogens [2]. These organic compounds mainly arise in incomplete combustion from both anthropogenic and natural activities. The industrial and chemical manufacturing processes such as coal gasification produce effluents containing PAHs which are released into the environment. B[a]P is one of the most prevalent PAHs with five condensed rings. Its persistence is largely due to its structure and extremely low solubility in water. Owing to the acute toxicity of this contaminant, there is an urgent need to develop an effective and affordable technology to remove it from the soil. Unconventional techniques involving biological processes can have strong potentialities. In particular, phytoremediation, the use of green plants to remove environmental pollutants, is recognized as a cost-effective, sustainable, and environmentally friendly approach to the problem resulting in great advantages for the large-scale clean-up of contaminated sites [3,4]. The efficiency of phytoremediation depends, besides the substrate type, on several characteristics of the plant. Among them, we can mention the plant ability to eliminate the pollutant, to tolerate the pollutant toxicity, to grow fast and to develop a deep and extended root system. Mycorrhizal colonization may be one of the means that protects plants and allows them to thrive on polluted sites [5–8]. Enhanced remediation of PAHs-contaminated soils by AM plants has been reported over the past few years. The role of AM fungi concerns two aspects: improved establishment and development of plants on polluted soil as well as the enhancement of PAHs degradation [9–16].

AM symbiosis, a keystone to the productivity and diversity of terrestrial ecosystems, occurs in more than 80% of land plants and represents the most widespread symbiosis on Earth [17]. In the mycorrhizal association, the fungus provides the plant with essential mineral nutrients taken up from the soil by the extraradical mycelium. In turn, the fungus receives carbon supplies from the plant [18,19]. Thereby, the benefits of the AM symbiosis on plant fitness are largely known, including not only a better mineral nutrition but also an increased ability to overcome biotic and abiotic stresses [5–8,20–24].

When plants are subjected to environmental stress conditions, as pollutants, the metabolism of reactive oxygen species (ROS)

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is disturbed. Consequently, and then, the balance between ROS production and elimination is broken. Therefore, enzymatic and non-enzymatic antioxidant systems are needed to decrease the damage to tissues [5,25,26]. Indeed, it is well established that the overproduction of ROS induces oxidative damage to various cellular constituents such as lipids, proteins and nucleic acids [5,26,27]. One of the most damaging oxidative effects is the peroxidation of membrane lipids which results in the concomitant production of malondialdehyde (MDA), a secondary end product of polyunsaturated fatty acid oxidation [28–30]. Several abiotic stresses such as pollution and drought have been shown to induce oxidative stress resulting in MDA production by plant cells [7,8,31–33].

In a previous study, we showed that B[a]P induced oxidative stress in transformed *Cichorium intybus* roots grown *in vitro* and colonized or not by *Glomus intraradices* [8]. The AM fungus development was significantly reduced in response to B[a]P increasing concentrations. The higher length of AM roots compared to non-AM roots following B[a]P exposure pointed out a lower toxicity of B[a]P in mycorrhizal roots, thereby suggesting protection of the roots by mycorrhizal colonization. Accordingly, in B[a]P-exposed AM roots, significant decreases in MDA, a lipid peroxidation marker, were observed. This work suggests an essential role of mycorrhizal fungi in protecting roots subjected to PAHs, notably by reducing PAHs-induced oxidative stress damage. The increase of MDA content in roots indicates an oxidative stress, which can be involved in mediating compositional membrane alterations [8]. Indeed, it was shown that plants respond to several environmental stresses such as pollutants toxicity diversely which in part includes the immediate changes in the membrane lipid composition [34–36]. The regulation of the lipid composition and the adjustment of the unsaturation level of membrane fatty acids (FA) are very important to deal with pollutant toxicity rendering the plant tolerant in unfavourable conditions [32–34]. Previously, several authors reported studies on lipid disruption in plants to cope with abiotic stress negative effects [33,37–48]. However, in spite of the several studies quoted, not only, the trend of lipid modifications was not clear but none has worked on the response of mycorrhizal plants to PAHs. Moreover, due to their hydrophobic property, PAHs are susceptible to disrupt plant and fungal cell membranes and lipid vesicles where PAHs are able to be stored [15,49,50].

Hence, the purpose of this work, was to compare the total lipid [FA, sterols, phospholipids (PL), and FA associated to PL (PLFA)] compositions and contents between mycorrhizal and non-mycorrhizal chicory roots grown in monoxenic conditions under B[a]P pollution. The findings of the current study will contribute to the understanding of the mechanisms involved in the protection against PAHs toxicity by the AM symbiosis.

2. Materials and methods

2.1. Culture of mycorrhizal and non-mycorrhizal chicory roots in the absence and in the presence of B[a]P

All the experiments were carried out with monoxenic cultures of chicory roots (*Cichorium intybus* L.), transformed by *Agrobacterium rhizogenes* [51] and colonized or not by the AM fungus: *Glomus irregulare* Blaszk., Wubet, Renker and Buscot (DAOM 197198) [52,53].

Cultures were inoculated with standardized 2-month-old monoxenic cultures of Ri T-DNA transformed chicory roots, colonized or not by *G. irregulare*, and using a 10 mm cork borer. A disk of culture medium containing roots from monoxenic cultures (non-mycorrhizal or mycorrhizal chicory roots) was placed in the middle of Petri dishes. Root colonization of the inoculum was about 60%.

Transformed chicory roots, colonized or not by *G. irregulare*, were grown on a modified M medium [54] [solidified with 0.25% (w/v) gellan gum (phytagel, Sigma, St. Louis, MO, USA)] supplemented or not with B[a]P (Sigma–Aldrich) at different concentrations (140 and 280 μM) and incubated for 9 weeks at 27 °C in the dark until the transition from a mainly vegetative state (absorptive) to a reproductive phase (sporulative) was initiated [55].

To prepare PAH-enriched medium, B[a]P was dispersed in gelatin (0.5%) and NaCl (0.9%) and sonicated for 10 min at room temperature. 6 ml of diluted B[a]P solution was added to each flask containing 600 ml of M medium (sterilized at 121 °C, 30 min), to obtain final concentrations of 140 and 280 μM. The culture medium was distributed in sterile Petri dishes of 90 mm diameter (25 ml/Petri dish). The control consisted in 600 ml of M medium plus 6 ml of the solution previously used to disperse B[a]P [gelatin (0.5%) and NaCl (0.9%)], without B[a]P.

2.2. Quantification of root colonization

One part of the root samples was cleared in KOH (10%) and stained with Chlorazol black E [56] to quantify arbuscular mycorrhizal colonization using the magnified intersect method [57].

2.3. Sterol and fatty acid extraction and separation

Lipid extractions were performed on a second part (about 25 mg dry weight) of 9 weeks previously freeze-dried root material. The biological material was saponified with 3 ml of 6% (w:v) in methanolic KOH at 70 °C for 2 h. Two successive extractions were carried out with cold hexane. The first extraction allowed to recover the unsaponifiable fraction containing sterols and the second the saponifiable fraction containing fatty acids.

2.4. Sterol extraction, analysis and identification

Sterol extraction, analysis and identification were performed according to the method described in Campagnac et al. [58] by GC–FID (PerkinElmer, Autosystem, Norwalk, CT, USA). Steryl acetates were identified by gas chromatography-mass spectrometry (GC–MS Varian, Walnut Creek, CA, USA) by using specific fragmentation pattern.

2.5. FA (total FA and PLFA) extraction, analysis and identification

Total FA and PLFA extraction, analysis and identification were carried out following the method reported in Campagnac et al. [33]. After addition of 1 vol of distilled water, the saponifiable fraction was extracted three times with 5 vol of hexane and evaporated under N_2. FA were methylated using 3 ml of BF_3:methanol (14%) at 70 °C for 3 min, and reaction was stopped in ice. FA methyl esters were extracted three times with 5 vol of hexane after the addition of 1 ml of distilled water. These extracts were evaporated under N_2 and transferred to chromatography vials. FA methyl esters were analyzed using a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionisation detector (Norwalk, CT, USA) and a ECTM-1000 (Alltech Associates Inc., Deerfield, IL, USA) capillary column (30 m × 0.53 mm i.d.) with hydrogen as carrier gas (3.6 ml min^{-1}). The temperature programme included a rapid increase from 50 °C to 150 °C at 15 °C min^{-1} and then a rise from 150 °C to 220 °C at 5 °C min^{-1}. FA were quantified using heptadecanoic acid methyl ester (C17:0) as an internal standard. Their identification relied on the retention times of a wide range of standards (Sigma–Aldrich).

2.6. PL extraction and analysis

PL extraction was performed on the third fraction of 9 weeks freeze-dried chicory roots (25 mg dry weight). PL extraction was

carried out as described by Avalli and Contarini [59]. The root samples were dissolved in 20 ml of dichloromethane:methanol (2:1, v:v) at 75 °C during 2 h. After filtration and concentration under N_2, samples were collected in 2 ml of chloroform and applied to solid phase extraction (SPE) cartridges. A silica gel bonded column (GracePure 3 ml volume, 500 mg sorbents, Grace Davidson Discovery Sciences, Alltech, Deerfield, USA) was used. After conditioning with 6 ml of hexane and 3 ml of Chloroform: 2-propanol (2:1, v:v), samples were added to the column. Total lipids were eluted with 6 ml of the precedent solvent. FA were eluted with 6 ml of 2% acetic acid in diethyl ether. PL were eluted using two different conditions: (1) with 6 ml of methanol and (2) with 6 ml of chloroform:methanol:H_2O (3:5:2, v:v:v). The recovered fraction was dried under N_2 and was re-dissolved in 0.2 ml of chloroform. A first part (0.1 ml) was injected into HPLC system; the second part (0.1 ml) was separately collected to extract PLFA.

PLFA analysis was carried out, as described by Calonne et al. [60], using an HPLC Waters 600 Controller (Meadows Instrumentation Inc., Bristol, UK) instrument with an automatic injector.

2.7. Statistical analysis

The data from roots exposed to various B[a]P concentrations (0, 140 and 280 μM) was compared to each other. All the experiments were done in 5 replicates. A two-way ANOVA was carried out. The method used to discriminate between the means was the LSD test ($p \leq 0.05$) using Statgraphics release 5.1. Variance homogeneity was checked using Levene's test, before the use of multiple comparison procedure.

We chose to use the Mann–Whitney *U* non parametric test instead of a parametric one to compare non-mycorrhizal and mycorrhizal roots, since normality tests were invalid and *F* test results suggested that variances were heterogeneous.

The data of sterol, FA, PL and PLFA percentages were converted to arcsine values before the analysis of two-way ANOVA and LSD test.

3. Results

3.1. B[a]P effect on root colonization

The percentage of mycorrhizal colonization in control treatment (*i.e.* without B[a]P) reached 61% (Fig. 1). The hyphal colonization of the chicory roots was significantly reduced by B[a]P treatments as compared to the control. Only 21 and 22% of colonization were obtained with 140 and 280 μM of B[a]P, respectively.

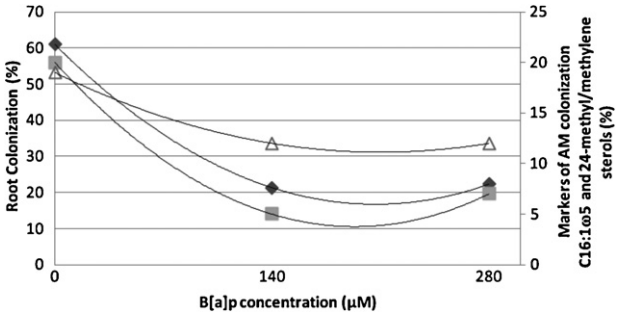

Fig. 1. Correlation between root colonization (♦) and the mycorrhizal lipid markers: 24-methyl/methylene sterol proportions (Δ) and C16:1ω5 FA proportions (■) in the absence and in the presence of B[a]P.

3.2. B[a]P effect on the chicory root FA

The FA composition of non-mycorrhizal roots grown in the absence of B[a]P (Table 1) was ranged from C16:0 to C18:3. The main FA that characterized the roots were C18:2 (linoleic acid), C16:0 (palmitic acid) and C18:3 (linolenic acid). They constituted more than 96% of the total FA. C18:2 was the major compound (60%). The FA profile as well as the total FA contents remained unchanged after treatment with B[a]P compared to the control, except in the presence of B[a]P at 140 μM where the total FA content was significantly increased in the comparison with the control (Fig. 2). In fact, some FA concentrations were affected by the presence of B[a]P at 140 μM: C18:0, C18:2 and C18:3 significantly increased compared to the control. In the presence of B[a]P at 280 μM, only the concentration of C18:0 per g of dry weight was significantly increased in the comparison with the control.

The FA composition of mycorrhizal roots grown in the absence (control) and in the presence of different B[a]P concentrations (Table 1) differed from the non-mycorrhizal ones by the presence of C16:1ω5, which is a major FA for most of *Glomus* species [61–65]. It accounts for 20% of the total FA. The total FA contents were not changed in the presence of the PAH. Conversely, the quantities of some FA were affected by the presence of B[a]P. In fact, the AM fungal marker C16:1ω5 [66] significantly decreased in the presence of B[a]P as compared to the untreated roots. Whereas C18:0 presented a moderate increase both in the presence of 140 and

Table 1
Total fatty acid (FA) compositions and contents (mg g^{-1} of dry weight) of non-mycorrhizal (NMR) and mycorrhizal chicory roots (MR) by *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different B[a]P concentrations. Data are presented as means ± SD. Different letters indicate significant differences at $p \leq 0.05$ as determined by a two-way ANOVA followed by a multiple range test (LSD), between increasing B[a]P concentrations (a, b for each component and A, B for total content in NMR; a′, b′ for each component and A′, B′ for total content in MR). The absence of a′, b′ in MR indicates an interaction between mycorrhizal colonization and treatments, and all values were statistically compared together.

	Control				B[a]P (μM)							
	0				140				280			
	NMR		MR		NMR		MR		NMR		MR	
Fatty acids (FA)	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%
C16:0 (palmitic acid)	1.19 ± 0.2^{a}	24^{a}	1.08 ± 0.1$^{a'}$	21$^{a'}$	1.41 ± 0.3^{a}	21^{a}	1.06 ± 0.4$^{a'}$	20$^{a'}$	1.25 ± 0.3^{a}	21^{a}	1.15 ± 0.4$^{a'}$	20$^{a'}$
C16:1ω5 (palmitoleic acid)			1.04 ± 0.6$^{a'}$	20$^{a'}$			0.29 ± 0.03$^{b'}$	5$^{b'}$			0.37 ± 0.1$^{b'}$	7$^{b'}$
C18:0 (stearic acid)	0.06 ± 0.01^{a}	1^{a}	0.08 ± 0.03$^{a'}$	2$^{a'}$	0.19 ± 0.03^{b}	3^{a}	0.15 ± 0.1$^{b'}$	3$^{a'}$	0.16 ± 0.03^{b}	3^{a}	0.15 ± 0.04$^{b'}$	3$^{a'}$
C18:1 (oleic acid)	0.16 ± 0.03^{a}	3^{a}	0.09 ± 0.1$^{a'}$	2^{b}	0.19 ± 0.04^{a}	3^{a}	0.17 ± 0.1$^{ab'}$	3^{a}	0.17 ± 0.1^{a}	3^{a}	0.19 ± 0.1$^{b'}$	3^{a}
C18:2 (linoleic acid)	3.00 ± 0.5^{a}	60^{a}	2.40 ± 0.6$^{a'}$	47^{b}	4.03 ± 0.6^{b}	61^{a}	3.08 ± 1.2$^{a'*}$	57^{a}	3.60 ± 0.7^{ab}	61^{a}	3.20 ± 0.8$^{a'}$	56^{a}
C18:3 (linolenic acid)	0.60 ± 0.1^{a}	12^{bc}	0.40 ± 0.1$^{a'}$	8^{a}	0.81 ± 0.1^{b}	12^{bc}	0.61 ± 0.2$^{a'}$	11^{bc}	0.75 ± 0.2^{ab}	13^{c}	0.63 ± 0.2$^{a'}$	11^{b}
Total FA (mg g^{-1} of dry wt)	5.01 ± 0.8^{A}		5.09 ± 0.4$^{A'}$		6.63 ± 1.2^{B}		5.36 ± 1.7$^{A'}$		5.93 ± 1.2^{AB}		5.69 ± 1.4$^{A'}$	
Saturated/unsaturated FA	0.3		0.3		0.3		0.3		0.3		0.3	

* Significant differences between medians of MR and NMR according to the Mann–Whitney *U*-test ($p \leq 0.05$)

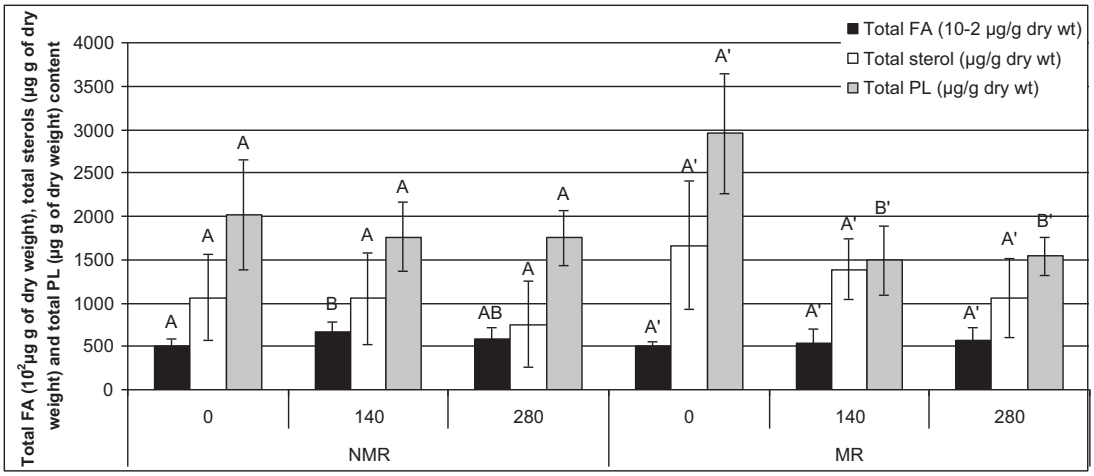

Fig. 2. Total fatty acids (FA) (10^{-2} µg g^{-1} of dry weight), total sterols (µg g^{-1} of dry weight) and total phospholipids (PL) (µg g^{-1} of dry weight) content of non-mycorrhizal (NMR) and mycorrhizal chicory roots (MR) by *Glomus irregulare* after 9 weeks of growth in the absence and in the presence of B[a]P at different concentrations. Data are presented as means ± SD. Each type of lipid was statistically evaluated separately from others. Different letters indicate significant differences at $p \leq 0.05$ as determined by a two-way ANOVA followed by a multiple range test (LSD), between increasing concentrations of B[a]P (A, B for total content in NMR and A′, B′ for total content in MR). The absence of * indicates no significant difference between MR and NMR according to the Mann–Whitney *U*-test ($p \leq 0.05$).

280 µM of B[a]P, C18:1 amount showed a moderate increase only at the highest B[a]P concentration compared to the control.

The total FA contents as well as the ratio saturated/unsaturated FA were quite similar in non-mycorrhizal and mycorrhizal roots.

3.3. B[a]P effect on the chicory root sterols

In control treatment (without PAH), sterols of non-mycorrhizal roots (Table 2) were identified as stigmasterol, sitosterol, 24-methylcholesterol and cholesterol. A pentacyclic triterpen, α-amyrine, was also detected. The sterol profile as well as the total sterol contents remained unchanged after treatment with PAH in comparison with the control. A significant reduction of cholesterol content with B[a]P was observed at 280 µM.

Similarly, in mycorrhizal roots, the total sterol contents did not change in the presence of different B[a]P concentrations (Fig. 2). In contrast, a reduction of particular sterol contents was observed in the presence of B[a]P. 24-methylcholesterol amounts significantly decreased by 48% and by 59% respectively at 140 and 280 µM of B[a]P, whereas the stigmasterol content was significantly decreased by 39% only at the highest B[a]P concentration as compared to the control. The ratio 24-ethylsterols/24-methylsterol [(sitosterol + stigmasterol)/24-methylcholesterol)] specifically decreased in mycorrhizal roots non treated by B[a]P.

After the colonization of the root by *G. irregulare*, the appearance of 24-methyl/methylene cholesterol and 24-methyldesmosterol was observed in the mycorrhizal roots in comparison with the non-mycorrhizal roots. No significant difference in total sterol content was shown in mycorrhizal roots treated or not by different concentrations of B[a]P when compared to the non-mycorrhizal roots. Interestingly, 24-methylcholesterol content of mycorrhizal roots differed from non-mycorrhizal ones. It represents a drastic increase of 126%. In contrast, this 24-methylcholesterol content remained unchanged after B[a]P treatment of mycorrhizal and non-mycorrhizal roots. The ratio 24-ethylsterols/24-methylsterol clearly decreased in mycorrhizal roots compared to the non-mycorrhizal roots when grown in the absence of PAH (Table 2).

Table 2
Sterol compositions and contents (µg g^{-1} of dry weight) of non-mycorrhizal (NMR) and mycorrhizal chicory root (MR) by *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of B[a]P at different concentrations. Data are presented as means ± SD. Different letters indicate significant differences at $p \leq 0.05$ as determined by a two-way ANOVA followed by a multiple range test (LSD), between increasing concentrations of B[a]P (a, b for each component and A, B for total content in NMR; a′, b′ for each component and A′, B′ for total content in MR). The absence of a′, b′ in MR indicates an interaction between mycorhizal colonization and treatments, and all values were statistically compared together. Absence of asterisk indicates no significant differences between MR and NMR according to the Mann–Whitney *U*-test ($p \leq 0.05$).

	Control				B[a]P (µM)							
	0				140				280			
	NMR		MR		NMR		MR		NMR		MR	
Sterols	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%	µg g^{-1}	%
Cholesterol	25.9 ± 10.0^{a}	2^{a}	32.0 ± 9.8$^{a'}$	2$^{a'}$	20.9 ± 11.0^{ab}	2^{a}	25.6 ± 4.8$^{a'}$	2$^{a'}$	13.1 ± 5.2^{b}	2^{a}	23.1 ± 8.5$^{a'}$	2$^{a'}$
24-Methylcholesterol§	139.1 ± 67.7^{a}	13^{a}	315.2 ± 56.8$^{a'}$	19^{b}	127.7 ± 56.0^{a}	12^{ac}	165 ± 43.8$^{b'}$	12^{c}	89.4 ± 55.5^{a}	12^{ac}	130.3 ± 46.4$^{b'}$	12^{ac}
Stigmasterol	335.5 ± 162.5^{a}	32^{a}	547.5 ± 192.5$^{a'}$	33$^{a'}$	333.5 ± 162.8^{a}	32^{a}	425.5 ± 107.9$^{a'b'}$	31$^{a'}$	244.3 ± 150.7^{a}	32^{a}	333.1 ± 135.2$^{b'}$	31$^{a'}$
Sitosterol	403.5 ± 200.5^{a}	38^{a}	567.9 ± 268.2$^{a'}$	34$^{a'}$	408.3 ± 215.0^{a}	39^{a}	551.3 ± 135.3$^{a'}$	40$^{a'}$	288.4 ± 196.8^{a}	38^{a}	414.5 ± 183.4$^{a'}$	39$^{a'}$
α-Amyrine	159.6 ± 72.4^{a}	15^{a}	201.6 ± 73.0$^{a'}$	12$^{a'}$	160.1 ± 215.0^{a}	15^{a}	221.2 ± 86.7$^{a'}$	16$^{a'}$	119.0 ± 90.0^{a}	15^{a}	158.2 ± 90.5$^{a'}$	15$^{a'}$
Total sterols (µg g^{-1} of dry wt)	1063.6 ± 502.3^{A}		1664.2 ± 740.3$^{A'}$		1050.5 ± 530.5^{A}		1388.6 ± 354.3$^{A'}$		754.2 ± 493.3^{A}		1059.2 ± 459$^{A'}$	
24-ethylsterols/24-methylsterol	5.3		3.5		5.8		5.9		6.0		5.7	

§ In MR, Methylcholesterol corresponds to a mixture of 24-methyl/methylenecholesterol and 24-methyldesmosterol not separated in our GC-conditions.

Table 3
Phospholipid (PL) compositions and contents ($\mu g\,g^{-1}$ of dry weight) of non-mycorrhizal (NMR) and mycorrhizal chicory roots (MR) by *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different B[a]P concentrations. Data are presented as means ± SD. Different letters indicate significant differences at $p \leq 0.05$ as determined by a two-way ANOVA followed by a multiple range test (LSD), between increasing concentrations of B[a]P (a, b for each component and A, B for total content in NMR; a′, b′ for each component and A′, B′ for total content in MR). The absence of a′, b′ in MR indicates an interaction between mycorrhizal colonization and treatments, and all values were statistically compared together. Absence of asterisk indicates no significant differences between MR and NMR according to the Mann–Whitney *U*-test ($p \leq 0.05$).

	Control				B[a]P (μM)							
	0				140				280			
	NMR		MR		NMR		MR		NMR		MR	
Phospholipids (PL)	$\mu g\,g^{-1}$	%	$\mu g\,g^{-1}$	%	$\mu g\,g^{-1}$	%	$\mu g\,g^{-1}$	%	$\mu g\,g^{-1}$	%	$\mu g\,g^{-1}$	%
Phosphatidylcholine (PC)	130.8 ± 22^{a}	6^{a}	199.2 ± 34^{b}	$7^{a'}$	135 ± 14^{a}	8^{a}	153.6 ± 28^{a}	$10^{a'}$	160.08 ± 14^{a}	9^{a}	147.2 ± 16^{a}	$10^{a'}$
Phosphatidylethanolamine (PE)	682.4 ± 524^{ab}	34^{a}	1676.8 ± 384^{a}	$57^{a'}$	370.8 ± 140^{b}	21^{a}	303.2 ± 76^{ab}	$20^{b'}$	583.4 ± 328^{ab}	33^{a}	266 ± 10^{b}	$17^{b'}$
Phosphatidylserine (PS)	1205.4 ± 206^{a}	60^{a}	$1079 \pm 136^{a'}$	$37^{a'}$	1251 ± 298^{a}	71^{a}	$1032.8 \pm 366^{a'}$	$69^{b'}$	1003.8 ± 186^{a}	57^{a}	$1125 \pm 184^{a'}$	$73^{b'}$
Total PL ($\mu g\,g^{-1}$ of dry wt)	2018.6 ± 634^{A}		$2955 \pm 692^{A'}$		1756.8 ± 398^{A}		$1489.6 \pm 396^{B'}$		1748 ± 310^{A}		$1538.2 \pm 222^{B'}$	
PE/PC	5.2		8.4		2.7		2.0		3.6		1.8	

3.4. B[a]P effect on the chicory root phospholipid

In the control treatment (without PAH), phospholipids of non-mycorrhizal roots (Table 3) were identified as phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The phospholipid profiles as well as the total phospholipid contents in non-AM roots remained unchanged after treatment with B[a]P in comparison with the control. However, the PC quantity was significantly increased in the presence of B[a]P 280 μM compared to the control.

After the colonization of the root by *G. irregulare*, the same phospholipid compounds were identified (Table 3). The total phospholipid contents were significantly decreased by 50% and by 48%, in the presence of 140 and 280 μM of B[a]P respectively. Whereas, important decreases of PE (by 82% and by 84%) were observed in the presence of 140 and 280 μM B[a]P respectively, PC was slightly reduced by 26% at the highest B[a]P concentration (280 μM) in comparison with the control.

In mycorrhizal roots, the ratio PE/PC significantly decreased by 76% and by 79% in the presence of 140 and 280 μM B[a]P respectively in comparison with the control.

3.5. B[a]P effect on the chicory root PLFA

The main PLFA identified in the non-mycorrhizal roots grown in the absence (control) or in the presence of different B[a]P concentrations (Table 4) were C18:2 and C16:0. They represented 82% of the total PLFA. C18:2 was the major compound (50%). Minor PLFA were also identified: C18:0, C18:1 and C18:3. The PLFA composition and the total PLFA contents were not modified in the presence of the lower B[a]P concentration used compared to the control. On the contrary, in the roots treated with B[a]P at 280 μM, a very important increase of the total PLFA content (165%) was observed when compared to the control. Indeed, PLFA amounts were affected by the presence of 280 μM of B[a]P; C16:0, C18:2 significantly increased by 164% and by 201% respectively compared to the control (Table 4).

The PLFA relative proportions of mycorrhizal roots were not different from the non-mycorrhizal ones when roots were grown without PAH (Table 4). C16:1ω5 was detected in very low amounts (trace) under our analysis conditions. In the presence of B[a]P at 140 μM, the total PLFA content significantly increased by 175% compared to the control but no effect was observed at 280 μM. In fact, the quantities of some PLFA were affected by the presence of B[a]P at 140 μM: C16:0, C18:2, C18:3 significantly increased by 153, 218 and 146% respectively in comparison to the control.

At 140 and 280 μM of B[a]P, a significant difference was observed between the total PLFA content in mycorrhizal roots compared to the PLFA content in non-mycorrhizal roots.

4. Discussion

In previous study, we showed that B[a]P was toxic to both mycorrhizal and non-mycorrhizal chicory roots. Nevertheless, the B[a]P adverse impacts were less marked on mycorrhizal root growth than on non-mycorrhizal ones, thereby suggesting a protective effect of mycorrhizal colonization. MDA, a product of lipid peroxidation indicating ROS production as well as membrane unsaturated phospholipid oxidative alteration, was increased by B[a]P addition in non-mycorrhizal roots while it was significantly lower in mycorrhizal roots [8]. In the present study, several changes in the lipid contents have been pointed out in the chicory roots grown under B[a]P pollution.

4.1. Fatty acids

FA compositions of non-mycorrhizal chicory roots treated with B[a]P ranged from C16:0 to C18:3 with three major FA (C16:0, C18:2 and C18:3) as published earlier [33]. However, B[a]P treatment induced significant increases of C18:0 at both concentrations and an increase of unsaturated FA (C18:2 and C18:3) amounts at 140 μM. These results concord with the study of Zuninio and Zygaldo [48] which reported an increase of total unsaturated FA of maize root in the presence of the phytotoxic lipophilic monoterpene. But as far as we determine, no data has been reported on the impact of PAHs on plant FA metabolism.

On the other hand and interestingly, in mycorrhizal roots grown in the presence of B[a]P, no significant changes were observed in the polyunsaturated FA (C18:2 and C18:3) quantities. These results can be correlated with our previous study where MDA content of mycorrhizal roots was not increased under B[a]P treatments, indicating less lipid peroxidation in arbuscular mycorrhizal roots compared to non-mycorrhizal roots [8]. These findings suggested that mycorrhizal colonization may have an interesting impact on maintaining a balance in the presence of B[a]P and by reducing the lipid peroxidation.

4.2. Sterols

Sterols are primary components of cellular membranes where they regulate fluidity and permeability. As constituents of the plasma membrane, sterols may influence the function of membrane

Table 4
Phospholipid fatty acid (PLFA) compositions and contents (μg g^{-1} of dry weight) of non-mycorrhizal (NMR) and mycorrhizal chicory roots (MR) by *Glomus irregulare* after 9 weeks of growth in the absence (control) and in the presence of different B[a]P concentrations. Data are presented as means ± SD. Different letters indicate significant differences at $p \leq 0.05$ as determined by a two-way ANOVA followed by a multiple range test (LSD), between increasing B[a]P concentrations (a, b for each component and A, B for total content in NMR; a′, b′ for each component and A′, B′ for total content in MR). The absence of a′, b′ in MR indicates an interaction between mycorrhizal colonization and treatments, and all values were statistically compared together.

	Control				B[a]P (μM)							
	0				140				280			
	NMR		MR		NMR		MR		NMR		MR	
Phospholipid fatty acids (PLFA)	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%	μg g^{-1}	%
C16:0 (palmitic acid)	144.0 ± 49.1^{a}	32^{a}	219.7 ± 110.5^{ab}	33$^{a'}$	147.1 ± 17.7^{a}	31^{a}	556.3 ± 104.9^{c}	30$^{a'}$	380.7 ± 14.8^{bc}	32^{a}	367.4 ± 292.1^{c}	41$^{a'}$
C16:1ω5 (palmitoleic acid)	–		Tr	Tr	–		Tr	Tr	–		Tr	Tr
C18:0 (stearic acid)	29.0 ± 9.8^{a}	6^{ab}	36.2 ± 13.9$^{a'}$	5^{ab}	36.1 ± 8.9^{a}	8^{ab}	43.2 ± 10.1$^{a'b'}$	2^{a}	48.2 ± 24.3^{a}	4^{a}	62.8 ± 15.8$^{b'}$	7^{b}
C18:1 (oleic acid)	17.6 ± 4.1^{a}	4^{a}	12.0 ± 4.7$^{a'}$	2^{a}	9.5 ± 2.9^{a}	2^{a}	nd	nd	nd	nd	29.4 ± 4.0$^{b'}$	3^{a}
C18:2 (linoleic acid)	228.0 ± 87.7^{a}	50^{a}	354.2 ± 172.1^{a}	53$^{a'}$	246.7 ± 25.7^{a}	52^{a}	1127.6 ± 341.3^{b}	61$^{a'}$	687.2 ± 275.2^{ab}	57^{a}	378.3 ± 185.4^{b}	42$^{a'}$
C18:3 (linolenic acid)	35.8 ± 15.3^{a}	8^{a}	50.2 ± 24.9^{ab}	7$^{a'}$	34.8 ± 5.4^{a}	7^{a}	123.5 ± 23.8^{c}	7$^{a'}$	86.0 ± 28.6^{bc}	7^{a}	53.8 ± 28.5^{c}	6$^{a'}$
Total PLFA (μg g^{-1} of dry wt)	454.4 ± 168.3^{A}		672.3 ± 314.5$^{A'}$		474.2 ± 52.9^{A}		1850.6 ± 460.7^{B**}		1202.1 ± 290.2^{B}		891.7 ± 284.8$^{A'*}$	
Saturated/unsaturated PLFA	0.6		0.6		0.6		0.5		0.6		0.9	

Tr: traces; nd: not detected.
* Significant differences between medians of MR and NMR roots according to the Mann–Whitney *U*-test ($p \leq 0.05$).

bound enzymes involved in transport activity (H^+-ATPase) [67] or in cellulose synthesis in the building of the cell wall [68]. Furthermore, there is strong evidence today that sterols are essential for normal plant growth [69] and mycorrhizal symbiosis development [70]. The establishment of functional symbiosis implies complex molecular signaling between mycorrhizal partners and recent studies have indicated the strong implication of sterols in the process [71]. To our knowledge, B[a]P impact of plant sterol's metabolism has never been studied. Based on our results the main Δ^5-sterols (4-demethylsterols, the major end-products in higher plant) identified in chicory roots were sitosterol, stigmasterol and 24-methylcholesterol. This data is consistent with the total sterol chicory and carrot root sterol profiles described earlier by Fontaine et al. [51] and Campagnac et al. [70]. The present study demonstrated, for the first time, that B[a]P, under the highest concentration tested, reduced significantly stigmasterol and 24-methyl/methylenesterol (mixture of 24-methyl/methylene cholesterol and 24-methyldesmosterol) content in mycorrhizal roots. It is clear that the B[a]P treatment modify ethyl/methylsterols ratio with an increase of the 24-ethylsterols. It has been reported that several environmental stresses alter the free sterol composition [72] and that during senescence the stigmasterol to other sterols ratio increases [73,74]. Zunino and Zygaldo [48] showed that the phytotoxic monoterpenes quantitatively affected free sterols and PLFA composition, thus producing an increase in the percentage of unsaturated PLFA, stigmasterol of the free sterol fraction, and saturated steryl ester FA. We can suppose that the sterol composition adaptation in the presence of B[a]P could be also a form of protection against PAHs toxicity. We have previously demonstrated that PAHs, lipophilic compounds, tend to accumulate in AM-fungal tissue [15]. A modification of the root sterol composition could help avoiding translocation of PAHs in root tissue and consequently protect the host against PAHs toxicity.

4.3. Lipid signature C16:1ω5 and 24-methyl/methylenesterols

As signature lipids of Glomalean fungi, C16:1ω5 and 24-methyl/methylenesterols have been only detected in mycorrhizal roots. These results are in agreement with those of Olsson [66] and Fontaine et al. [51]. *G. intraradices* has been shown to be particularly rich in C16:1ω5 and a good correlation between the total amount of C16:1ω5 and root colonization by *G. intraradices* has been pointed out by Fontaine et al. [51]. In the present work, significant decreases of both C16:1ω5 found in the total FA and 24-methyl/methylenesterol in AM chicory roots grown in the presence of B[a]P indicate a reduced development inside the roots (Fig. 1). These results corroborate those of Debiane et al. [8], who reported that B[a]P caused a drastic decrease in chicory root colonization and AM fungal development. Indeed the mycorrhizal colonization of chicory roots as well as *G. intraradices* hyphal length and sporulation were significantly inhibited by B[a]P.

4.4. PL and PLFA

Among the main membrane constituents, PL are more than just structural components of membranes since they can be cofactors for membrane enzymes, signal precursors, or signaling molecules themselves [75]. Three types of PL were detected in the chicory root extracts: PC, PE and PS. This data is consistent with that of Cooper and Lösel [76] who reported a qualitatively similar composition in PL with PE and PC as the major phospholipids in mycorrhizal and non-mycorrhizal clover and onion roots. While total PL contents remained unchanged in non-mycorrhizal roots after treatment with B[a]P in comparison with the control, drastic reductions were observed in mycorrhizal roots, due mainly to decreases in PE and PC. Moreover, the PE/PC ratio was significantly reduced in

mycorrhizal roots grown in the presence of B[a]P. Similarly, the ratio was disturbed in pepper roots under cadmium stress and in *Beta vulgaris* in response to Al [77]. This decrease could indicate that the conversion of PE to PC may have been disrupted. The important drop of PC and PE observed in mycorrhizal roots under B[a]P treatment could be due to B[a]P negative effect on the AM fungal root colonization as previously shown by Debiane et al. [8]. Indeed, PC and PE have been described to be the major PL in *Glomus mosseae* [76], in *Glomus caledonium* [78] and in *G. irregulare* [79].

The second explanation could be to consider the PE and PC decreases as a means of root stress response against the pollutant so as to deal with B[a]P toxicity. PC is known to play an important role in the regulation of cellular functions and intracellular signaling [80]. They may alter the composition of cellular membranes; keep cells at a higher level of hydration, and by that affect metabolite transport, ion selectivity and the activity membrane-bound enzymes [81,82]. PC can also be hydrolysed to choline which can later be oxidized to glycine betaine [83], and then play a role in the osmotic adaptation of plants. We can suppose that PL composition disruption under PAHs treatment could modify some membrane functions and must be determinant for the development of the roots. In contrast, in non-mycorrhizal roots no important changes in PL contents were highlighted in the presence of increasing concentrations of B[a]P in comparison to the control, except a rise in PC at the highest concentration of B[a]P.

Additionally, whereas in mycorrhizal roots a significant increase in the total PLFA content was observed in the presence of B[a]P at 140 μM (due principally to C16:0, C18:2 and C18:3 rises), in non-mycorrhizal roots PLFA total quantity increased only at the highest concentration (280 μM), due mainly to C18:2 amount increase. We can suppose that variations in PLFA unsaturation can produce drastic effects on both physical and functional membrane properties, and membrane fluidity may increase as demonstrated by Stubbs and Smith [84], Karp [85] and Borst et al. [86].

It is noteworthy that in our experimental conditions, C16:1ω5 was surprisingly detected in very low amounts in PLFA extracted from chicory roots colonized by *G. irregulare*. This C16:1ω5 has been shown to be mainly associated in storage lipids (representing 34–78% of the neutral FA) but found in a minority proportion in PLFA (13–23%) of *G. intraradices* profile [65,87,88].

Taken together, our results conform to the study reported by Ouariti et al. [43] who pointed out lipid loss in tomato plants treated by cadmium and copper suggesting a disturbance of the membrane lipid turnover by the two heavy metals. These authors proposed that the loss of membrane lipids may be related to an enhanced rate of catabolism and/or to the suppression of lipid biosynthesis [43]. Both metals enhanced lipoxygenase activity [89,90], which is responsible for catalyzing lipid peroxidation by using membrane lipid components as substrates. Likewise, the products of the lipoxygenase reaction, mainly peroxy, alkoxy and hydroxyl radicals, are themselves reactive and can result in further membrane lipid deterioration [91], and also affect other macromolecules in the cells [92]. Several pollutants such as PAHs and heavy metals are also involved in many ways in the production of activated oxygen species that actively induce peroxidation of membrane lipids [7,8,93]. Therefore, it is conceivable to suppose that a decrease of enzymatic free radical scavengers [91] caused by pollutant-stress may also contribute to the shift in the balance of free radical metabolism towards accumulation, leading further to more breakdown of membrane lipids.

As far as the literature is dedicated, relatively few reports have been devoted to the protective role of mycorrhizal inoculation against PAHs toxicity by adjusting the lipid metabolism and by reducing lipid peroxidation in plants exposed to PAHs [7,8]. It seems that cellular damage by free radicals is alleviated partially by mycorrhizal colonization, which presumably contribute in B[a]P tolerance in chicory roots. ROS-induced oxidative damage could be alleviated by unknown direct and indirect mycorrhizal mechanisms, such as enzymatic pathways and/or presuming detoxifying action of carotenoids, thereby suggesting mycorrhizal colonization played an important role in B[a]P tolerance in chicory roots. This protection of root cells against B[a]P toxicity could be partially explained by the higher superoxide dismutase activity, an antioxidant enzyme, detected in mycorrhizal chicory roots [7,8] and/or the accumulation of apocarotenoids in the roots which is supposed to protect root cells against oxidative damage of membranes by ROS [94].

Summing up, the present work highlights that B[a]P, a high molecular weight PAH frequently found in polluted soils, affects chicory root lipid metabolism. The changes observed in total FA, PL, PLFA and sterols levels implies that membrane structure and function might be disturbed by B[a]P stress. However, the question whether changes in lipids, play a role in the defense/protection mechanisms, or rather reflect damage remains poorly understood and controversial. This question has been addressed by Zhang et al. [95] by exploiting mutants deficient in lipid FA desaturation and in transgenic plants over expressing FA desaturases, in response to various abiotic stresses, including heat, salt and drought [95]. In our study, regarding the comparisons between mycorrhizal and non-mycorrhizal root responses, it is difficult to give a clear significance to some lipid changes observed in the presence of B[a]P. Are they attributed to defense responses or to the toxicity effects of B[a]P on membrane lipids of the mycorrhizal roots? In order to validate these hypotheses, it will be interesting in the future to assess the cell membrane permeabilities of both mycorrhizal and non-mycorrhizal roots grown under B[a]P pollutant.

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