



Impact of phenanthrene on primary metabolite profiling in root exudates and maize mucilage

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Abstract

This study was conducted to assess the impact of polycyclic aromatic hydrocarbon on the composition of rhizodeposits. Maize was submitted to increasing phenanthrene (PHE) concentrations in the substrate (0, 25, 50, and 100 mg PHE.kg⁻¹ of dry sand). After 6 weeks of cultivation, two types of rhizodeposit solution were collected. The first one, called rhizospheric sand extract, resulted from the extraction of root adhering sand in order to collect mucilage and associated compounds. The second one, the diffusate solution, was collected by the diffusion of exudates from roots soaked in water. The impact of phenanthrene on maize morphology and functioning was measured prior to the analysis of the main components of the rhizodeposit solutions, by measuring total carbon, protein, amino acid, and sugars as well as by determining about 40 compounds using GC-MS and LC-MS. As maize exposure to PHE increased, different trends were observed in the two rhizodeposit solutions. In the diffusate solution, we measured a global increase of metabolites exudation like carbohydrates, amino acids, and proteins except for some monoglycerides and organic acids which exudation decreased in the presence of PHE. In the rhizospheric sand extract, we witnessed a decrease in carbohydrates and amino acids secretion as well as in fatty and organic acids when plants were exposed to PHE. Many of the compounds measured, like organic acids, carbohydrates, amino acids, or fatty acids, could directly or indirectly drive PAHs availability in soils with particular consequences for their degradation.

Keywords Rhizodeposition · Organic analysis · Carbohydrate · Polysaccharide · Amino acid · PAH

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous, and toxic to humans. They are of great concern due to their long-term health effect. Sixteen of them have been identified for their mutagenicity, including phenanthrene which is one of the most frequent PAHs (US EPA 1984; EFSA 2008; Lerda 2011). PAHs are generated during the incomplete combustion of organic matter (e.g., coal, oil, wood), mainly due to anthropogenic activities like traffic, petroleum refineries, or coke

production (Conte et al. 2001; Keyte et al. 2013; Abdel-Shafy and Mansour 2016; Fabiańska et al. 2016). The major vector of PAH exposure for human population is air, due to the high volatility of these compounds. However, PAH exposure can also occur through the ingestion of contaminated foods or soil. Indeed, PAHs can be absorbed by crops via roots or leaves, although the uptake mechanisms remain unclear (Zhan et al. 2012; Abdel-Shafy and Mansour 2016; Dupuy et al. 2016). PAHs uptake depends on the nature of the compounds and on their affinity with the root plasma membrane, but also on their concentration in soils, which can vary substantially. Their concentration is generally less than 1 mg.kg⁻¹ in arable, grassland, and forest soils, but can easily reach 4 mg.kg⁻¹ in urban soils especially next to roads and can exceed 700 mg.kg⁻¹ in industrial wasteland soils (Wilcke 2000; Barnier et al. 2014).

PAH dissipation in soil could be improved through a phytoremediation process (Binet et al. 2000; Ouvrard et al. 2014). Indeed, in the presence of plants, soil PAH content declines, although plant uptake is negligible (less than 0.01% for phenanthrene (PHE) and 0.24% for pyrene (PYR)

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(Gao and Zhu 2004). PAH remediation mostly takes place in the rhizosphere, being directly or indirectly, enhanced by rhizodeposition (Binet et al. 2000; Corgié et al. 2003). The rhizodeposition is the emission of compounds, mainly organic, released by roots and called rhizodeposits (Shamoot et al. 1968; Nguyen 2003). Major rhizodeposits can be classified into four groups (Grayston et al. 1997; Nguyen 2003): (1) water-soluble root exudates comprising low molecular weight substances, lost passively by the roots through the plasma membrane according to a concentration gradient (amino acids, organic acids, sugars, phenolics, phytosiderophores, and many secondary metabolites); (2) secretions comprising high molecular weight substances and mucilage, depending on active metabolic processes for their release, which can take place against the electrochemical potential gradient. Mucilage is mainly composed of polysaccharides, phospholipids, and polygalacturonic acids of high molecular weight; (3) excretions emitted actively into the rhizosphere with ATP consumption (ethylene, carbon dioxide, electrons); and (4) lysates released from autolyzed and sloughed-off cells.

The presence of rhizodeposits can modify PAH dynamics in soil/plant systems by modifying the rhizosphere characteristics, physically, chemically, and biologically, leading to reduced PAH concentrations and/or modified its bioavailability. Reilley et al. (1996) showed that PAHs' degradation increases from 30 to 44% on a vegetated soil compared to a bare soil. This phenomenon is mainly attributed to the capacity of rhizodeposits to enhance microbial activity (Joner et al. 2002; Joner and Leyval 2003). Indeed, the massive production of rhizodeposits by plants and especially sugar (e.g., glucose, fructose, sucrose), amino acids, and organic acids (e.g., malate, citrate, oxalate, or aspartate), which are easily degradable, induces a significant increase in bacterial biomass in the rhizosphere because of their chemoattractant characteristics (Heinrich and Hess 1985; Reinhold et al. 1985; Grayston et al. 1997). In addition to increased microbial biomass and activity, rhizodeposition also modifies the diversity of bacterial community in the root's vicinity (Rovira 1959; Grayston et al. 1997; Benizri et al. 2002). This statement is observable in the case of PAH pollution; the total number of cultivable heterotrophic microorganisms able to degrade PHE increases as the distance from the plant root decreases (Muratova et al. 2009). The PAH biodegradation by microorganisms is possible in both aerobic and anaerobic conditions, through the oxidation of PAH aromatic rings (Cerniglia 1993). However, plants can also produce oxidative enzymes including peroxidase in particular, which are directly involved in PAH degradation. These enzymes are located on the root surface and in the rhizospheric soil solution (Adler et al. 1994; Liste and Alexander 1999).

Nevertheless, the main obstacle to phytoremediation of PAH-contaminated soils is the bioavailability of these molecules (Ouvrard et al. 2014). They are easily sorbed on the soil

solid phase especially on organic matter, which make them inaccessible to biodegradation (Wilcke 2000; Ockenden et al. 2003). Some rhizodeposits are able to modify this availability by increasing the soil organic matter content, resulting either in a greater PAH partitioning to soil, or contrarily, by emitting surfactant or chelating compounds (Ouvrard et al. 2006; Wang et al. 2016). Rhizodeposition can modify rhizospheric pH, depending on plant species and soil conditions (Sas et al. 2001; Hinsinger et al. 2003; Paterson 2003). The pH is an important factor in PAH desorption. At low pH levels, comprised between 2 and 3, the release of PAHs is minimal, while it is maximal at pH 7–8 and diminishes after that (Yang et al. 2001). Therefore, an optimal pH combined with an increase in microbial activity leads to a higher and faster PAH degradation (Pawar 2015). Root-emission of organic acids would particularly increase the PAHs availability in the rhizosphere. Injections of sodium citrate into soils significantly increased PAH desorption, as did malic, malonic, citric, and oxalic acid (Yang et al. 2001; Ouvrard et al. 2006; Ling et al. 2009; Sun et al. 2012). Amino acids, like serine and alanine and saccharides, including fructose also gave significant results in PAH desorption, but were less effective than organic acids. The former compounds, especially alanine, are more efficient at lower concentrations (0.42 to 5.03 g.kg⁻¹) (Sun et al. 2012). Effects of rhizodeposits were visible when adding compounds individually, but also as a mixture. Joner et al. (2002) added to a contaminated soil a mixture of saccharides, amino and organic acids, representing the main families of rhizodeposits. The experiment resulted in an increase in the Soxhlet extractable five and six-ring PAHs, attributed to the desorption of initially non-extractable molecules. Gao et al. (2010), as well as Liste and Alexander (1999), also observed an increased desorption of PHE and PYR by adding root exudates. In the previous works, artificial root exudates were added, but high molecular weight rhizodeposits, including mucilage, were not considered. Indeed, those compounds of high molecular weight could affect PAH dynamic as well as root exudates. Additionally, the feedback effect of PAH exposure on the quality and quantity of the rhizodeposits is poorly studied. Indeed, if the general composition of the rhizodeposits is well-known, its composition when plants are affected by the presence of pollutant is very little studied. An altered composition of rhizodeposits in the presence of PHE could result from a strategy from the plant to resist to the presence of pollutants. Rhizodeposits presence seems to modify the dynamics of PAHs in the soil, but the opposite is also true: pollution could induce quantitative and qualitative modifications of the rhizodeposits, as it does for other stresses.

Indeed, some abiotic stress can modify rhizodeposition (Neumann and Röhmheld 2000). This is the case, for example, for nutritional deficiencies. A plant submitted to phosphorus deficiency emits specific amino or organic acids to facilitate phosphorus intake by chelation mechanisms (Hinsinger

2001; Shen et al. 2002, 2005; Wittenmayer and Merbach 2005; Tang et al. 2009). In case of iron deficiency, phenols, malate, and other acids are secreted to facilitate iron bioavailability and absorption (Vranova et al. 2013). Water stress also induces changes in root exudates. Song et al. (2012) showed that maize grown in hydroponic conditions and exposed to water stress exuded more organic acids (malic, lactic, citric, or maleic) than non-exposed plants. Preece and Peñuelas (2016) reported an increase in root exudation of sugar per gram of plant biomass under moderate drought. Plants may upregulate rhizodeposition under moderate drought and release more mucilage in order to ease the movement of roots through dry soil (Ahmed et al. 2014). The presence of toxic compounds like Cd can modify rhizodeposition through the release of larger amounts of acetic, oxalic, glutamic, citric, aconitic, and succinic acid or amino acids like proline (Fan et al. 2001; Lapie et al. 2019). Organic pollutants may also modify rhizodeposition. For instance, *Plantago lanceolata* submitted to a herbicide increased the carbon flow emitted from its roots (Porteous et al. 2000). Despite this quite extensive knowledge, modifications to the rhizodeposition submitted to PAH in both mucilage and root exudates have been very little studied until now.

This is why in this experiment, we aimed at (1) characterizing as exhaustively as possible the compounds in mucilage and root exudates and (2) evaluating qualitatively and quantitatively the impact of plant exposure to PAH on these compounds (mainly carbohydrates, amino acids, organic acids, polysaccharides, and proteins) in order to discuss their role in PAH availability, tolerance, and degradation. Phenanthrene was chosen as a model pollutant and maize (*Zea mays*) as a model plant.

Materials and methods

Plant cultivation

The experiment was conducted in a growth chamber (photoperiod 16 h, 23/18 °C day/night temperatures, relative humidity 70%, photosynthetic photon flux density (PPFD) 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Maize (*Z. mays* L., MB862, INRA) was cultivated in sand (particle size of 0.4–0.8 mm, Sibelco, France) in polyvinyl chloride cylindrical pots (diameter 7.3 cm, height 30 cm, 1725 g dry sand per pot). Maize is a model plant used in many rhizodeposition studies due to its wide root system with an important exudation and a large production of mucilage, visible to the naked eye (Paull et al. 1975; Bacic et al. 1986; Morel et al. 1991; Read et al. 1999; Iijima et al. 2000; Yoshitomi and Shann 2001; Ma et al. 2010; Carvalhais et al. 2011; Lapie et al. 2019). Sand was used to mimic the mechanical impedance of the soil on the roots. Because sand does not strongly interact with organic

compounds, it enabled a better control of the plant exposure to PHE (all PHE is bioavailable) and of the rhizodeposit collection.

The sand was washed with 12% hydrochloric acid to remove any carbonates and contaminants, rinsed with deionized water, and then dried in an oven (70 °C, 48 h, then 200 °C, 2 h). There were four phenanthrene (PHE) levels (0, 25, 50, 100 mg PHE.kg sand⁻¹) in five replicates randomly distributed in the growth chamber. Phenanthrene concentrations were chosen because of their toxic but non-lethal impact on plant (Dupuy et al., 2016). These concentrations were higher than those found in arable or forest (0.3–0.9 mg kg⁻¹) or non-contaminated urban area (4 mg kg⁻¹) and can be found in industrial wasteland soils (up to 770 mg kg⁻¹) (Barnier et al., 2014; Wilcke, 2000).

Sand was contaminated following the Dupuy et al. protocol (2016). A PHE stock solution was prepared in HPLC grade dichloromethane. Then, a sub-sample of dry sand, representing 10% of the required total mass, was spiked at 16% v/w by a PHE solution diluted from the stock solution to achieve concentrations of 250, 500, and 1000 mg.kg⁻¹. The solvent was evaporated under a fume hood and the spiked sand was homogeneously mixed with the remaining fraction (90%) of non-contaminated sand in order to obtain the final concentrations (25, 50, and 100 mg PHE.kg⁻¹). The control treatment was spiked with the same volume of pure dichloromethane and treated similarly. The sand was contaminated just before the beginning of the experiment.

Nutrients were added in a single dose at the very beginning of the cultivation in a 250-mL nutritive solution adapted from Hoagland and Arnon (1938), 5 mM Ca(NO₃)₂·4H₂O, 1 mM KH₂PO₄, 2 mM MgSO₄·7H₂O, 90 μM MnCl₂·4H₂O, 10 μM ZnSO₄·7H₂O, 3 μM CuSO₄·5H₂O, 460 μM H₃BO₃, 1 μM MoO₃, 10 μM Fe-EDTA, and pH 5.4–5.5. The nutrient solution volume corresponded to 80% of the water holding capacity and humidity was maintained at 80% with deionized water (18 M Ω) every 2 days. Maize seeds were sterilized (H₂SO₄ 95% 30 s, ethanol 95% 5 min, H₂O₂ 10% 30 min) before planting and pre-germinated in petri dishes with growth medium (4 g.L⁻¹ tryptic soy agar, 12 g.L⁻¹ agar/agar, Sigma-Aldrich). After 2 days, only non-contaminated germinated seeds were planted in our systems. After 2 weeks, a 10-mL solution of 100 μM Fe-EDTA was added to each pot.

Photosynthesis and respiration measurement

The photosynthesis and respiration rates were measured after 6 weeks with the gas exchange open system Li-Cor 6400 xt (Li-Cor, USA) on the 7th leaf. Each leaf was placed inside the Li-Cor chamber, in the following conditions of air temperature, CO₂ concentration, and PPFD, 23 °C, 400 μmol CO₂.mol⁻¹, and 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Collection of rhizodeposits

After 6 weeks of cultivation, the plants were harvested, and two rhizodeposit solutions were collected. The first resulted from the sand extraction. Sand adhering to roots is known as rhizospheric sand (Pinton et al. 2007). Mucilage was collected by washing the root system with a phosphate-buffered saline (150 mL PBS, 8 g.L⁻¹ NaCl, 1.44 g.L⁻¹ Na₂HPO₄, 0.24 g.L⁻¹ KH₂PO₄, 0.11 g.L⁻¹ KCl). PBS is commonly used for washing procedures because of its isotonicity with cells and its capacity to avoid protein denaturation (Vaughn and Campbell 1988; Sano et al. 1999; Šamaj et al. 1999; Willats et al. 2001; Borg et al. 2019). It prevents or at least reduces the release of root exudates, contrarily to water or other non-isotonic solutes and permits mainly mucilage to be collected. The sand in the buffer solution was agitated for 2 h in an overhead shaker (Reax 20, Heidolph, 15 RPM) and then removed by filtration on Whatman paper. The resulting solution is referred to as rhizospheric sand extract.

The second rhizodeposit solution was collected according to Luo et al., 2014. After sand removal, roots were washed with deionized water and plunged into 150 mL of deionized water for 6 h, to collect the root exudates. This second solution is called diffusate solution.

Both rhizodeposit solutions were kept at -20 °C. A 50-mL volume of each rhizodeposit solution was freeze-dried until all the water was completely sublimated (alpha 1-4 LDplus, Martin Christ, Germany). The freeze-dried residue obtained was re-suspended in 3 mL of deionized water and the concentrated solution was filtered with 0.45-µm filters (Syringe filters, Acrodisc) to remove any dead cells and root parts before chemical analyses. The concentrated solution was used for protein, carbohydrates, and amino acids analysis. For GCMS analysis, 50 mL of non-concentrated rhizodeposit solution were also freeze-dried and re-suspended with 3 mL of acetonitrile (ACN, HPLC-S Gradient Grade) and filtered with 0.45-µm filters.

Two different solvents were used, either water or ACN, because organic acids are more soluble in organic solvents like ACN, while sugars, amino acids, and most proteins are preferably dissolved in water. In order to maximize solubilization and homogenization, concentrated solutions were submitted to ultrasound for 45 min (Bransonic ultrasonic bath, Branson 5210, USA). Even after these steps, there is a possibility that some compounds could remain insoluble and were lost with filtration.

Plant morphological measurements

After both solutions had been collected, foliar symptoms were noted, and root and shoot lengths were measured. Leaves, stems, and roots were separated and weighed. Leaves were scanned using a portable leaf area meter and the transparent

conveyor belt accessory (LICOR LI-3000C and LI-3050C, USA). The roots were scanned, and the images obtained were analyzed using the WinRhizo® software (WhinRhizo, 2005, Regent Instruments Inc., Canada) in order to determine the root surface area of each plant. The fresh material was stored at -20 °C until being manually cryogenically ground, using a pestle and mortar together with liquid nitrogen.

Plant biochemical measurements

Hydro-alcoholic extractions were performed in order to extract carbohydrates and amino acids from plant matter, according to Harrison et al. (2003). One hundred milligrams of fresh material was added to 1 mL ethanol 80% (v/v) and agitated (17 RPM, 1 h, 4 °C). The extract was centrifuged (10 min, 14,000 g) and the supernatant was sampled and kept to one side. The pellet was submitted to a second and a third extraction similar to the first one with 60% ethanol and then with water. The three supernatants were merged to constitute a solution designated as hydro-alcoholic extract.

The anthrone-sulfuric acid assay described by Leyva et al. (2008) was used to quantify the carbohydrate content in the hydro-alcoholic root extract. The anthrone reagent was prepared immediately prior to analysis by dissolving anthrone (0.1%, w/v) in a concentrated sulfuric acid (96%) solution. Standard solutions were prepared by diluting glucose (1.0 g.L⁻¹ in demineralized water). One hundred fifty milliliters of anthrone reagent and 50 µL of the standard or sample were added to each well of a microplate. The latter was kept at 4 °C for 10 min, then incubated at 100 °C for 20 min. Microplates were then cooled at room temperature and the absorbance was read at 620 nm with a microplate spectrophotometer (Synergy HT, Biotek Instruments, USA).

Total soluble amino acids were determined with ninhydrine according to Rosen (1957) and glutamine was used as a standard. One hundred microliters of the standard or sample (hydro-alcoholic extract or rhizodeposit solution) was added to the deep well microplate. Then, 50 µL of KCN (10 mM, in 2.6 M acetate buffer, pH 5.2) and 50 µL of ninhydrine solution (3% w/v in ethyl glycol monomethyl ether) were added before incubating the microplate at 100 °C for 15 min. At the end of the incubation, the reaction was stopped by adding 500 µL of an isopropanol/water mix (50/50, v/v). The deep well microplate was cooled at room temperature and 200 µL were transferred into a classic microplate before reading the absorbance at 570 nm with a microplate spectrophotometer.

Plant proteins were extracted from 100 mg of plant fresh material, using 400 µL of extraction buffer (100 mM tricine, 40 mM CaCl₂, 0.5% (w/v) polyvinylpyrrolidone, 0.5% (v/v) triton 100 X, 1 mM ethylenediaminetetraacetic acid, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl, pH 8). The suspension was agitated for 3 min. The supernatant was

collected after centrifugation (15,000g, 15 min, 4 °C) to constitute the soluble protein extract.

Proteins were determined following the Bio-Rad Protein Assay instructions according to Bradford, 1976. The standard was bovine serum albumin (1.37 mg/mL, Bio-Rad). Ten microliters of each standard or protein extract from plants and 200 μ L of five times diluted dye reagent was mixed into each well. The absorbance was read at 595 nm after 5 min of incubation at room temperature.

Analyses of rhizodeposits

Total carbon in non-concentrated rhizodeposit solutions was measured using a total organic carbon analyzer TOC-VSCN (Shimadzu, Japan). Rhizodeposit solutions were previously filtered with 0.45 μ m syringe-filters (Acrodisc). A TOC standard solution (TOC Standard, SCP Science, France) was used both as standard and control sample.

Total carbohydrate, amino acid, and protein contents of concentrated rhizodeposit solutions were measured using the same protocol as for plant material. Thus, (1) carbohydrate content was quantified using the anthrone-sulfuric acid assay used for plant extract (Leyva et al. 2008), (2) total soluble amino acids and ammonium were determined according to Rosen (1957), and (3) protein content was determined following the Bio-Rad Protein Assay protocol for microplates, according to (Bradford 1976).

Molecular analyses were performed on the concentrated solutions. Mono- and disaccharides were quantified following the protocol described by Ricochon et al. (2011). Compounds of interest were quantitatively analyzed on a LC-MS system (Thermo Scientific, USA) including a binary solvent delivery pump connected to a photodiode array detector (PDA) and a LTQ mass spectrometer equipped with an atmospheric pressure ionization interface operating in atmospheric pressure chemical ionization negative mode (APCI⁻). Chromatographic analysis was performed on a Polyamine II HILIC column (250 \times 4.6 mm, 5 μ m, YMC, Germany) fitted with a guard column (7.5 \times 4.6 mm, 5 μ m, YMC, Germany). Mobile phases consisted in a mix of ultrapure water (phase A) and 1% of chloroform in acetonitrile (phase B). Twenty microliters of extracts were injected into the system and sugars were separated at a flow rate of 1 mL.min⁻¹ through the isocratic mode (A/B: 25/75). Sugars were analyzed in SIM mode, thanks to chlorinated adducts [M + 35]⁻ and [M + 37]⁻ which correspond to stable chlorine isotopes. The m/z windows used for the specific screening were set as follows, 167–188 m/z for xylose; 198–202 m/z for rhamnose; 214–232 m/z for fructose, glucose, mannose, and mannitol; and 376–382 m/z for sucrose and cellobiose. Mass spectrometric conditions were as follows: spray voltage was set at 6 kV; source gases were set (in arbitrary units min⁻¹) for sheath gas, auxiliary gas, and sweep gas at 40, 10, and 10, respectively; capillary temperature was set at 225 °C; capillary voltage was set at –

10 V; tube lens, split lens, and front lens voltages at –47, 78, and 5.25 V, respectively. Ion optics parameters were optimized in APCI⁻ mode by automatic tuning, using a standard solution of glucose at 0.1 g.L⁻¹ infused in mobile phase (A/B: 25/75) at a flow rate of 5 μ L.min⁻¹. Furthermore, in order to determine the polysaccharide content of the rhizospheric sand extracts, a volume of 1 mL of concentrated rhizodeposit was hydrolyzed in acidic conditions according to a protocol adapted from Happi Emaga et al. 2012 (15 min, 105 °C, 7.7% HCl, 45 min temperature ramp) before LC-MS analysis. The external calibration curves of sugars were plotted from 0.001 to 0.1 mg.L⁻¹ for diffusate solutions and from 0.001 to 0.5 mg.L⁻¹ for rhizospheric sand extracts.

Native amino acids were quantitatively analyzed using the same LC-MS system, but operating in electrospray positive mode (ESI⁺). A porous graphitic column (Hypercarb, 100 \times 2.1 mm, 5 μ m, Thermo Hypersil-Keystone), fitted with a guard column (7.5 mm \times 4.6 mm cartridge, YMC, Germany), was used to separate 19 amino acids according to the protocol described by Chaimbault et al. (2000), thus, glycine, serine, alanine, threonine, asparagine, cysteine, aspartic acid, proline, histidine, arginine, phenylalanine, tyrosine, tryptophan, glutamine, glutamic acid, valine, lysine, methionine, and leucine/isoleucine isomers (impossible to differentiate due to their identical retention time and MS² fragmentation patterns). Mobile phases consisted in a mix of water modified with nonafluoropentanoic acid (20 mM) (Phase A) and pure acetonitrile (HPLC-S Gradient Grade) (Phase B). Separation of compounds of interest was achieved, with a first linear gradient from 0 to 70% of B for 50 min and then a second linear gradient from 70 to 100% of B for 6 min. The flow rate was set at 200 μ L.min⁻¹ and the injection volume was set at 16 μ L. The retention times of amino acids in complex mixtures were first validated thanks to MS² screening and then the quantitative analyses were routinely performed in MS full scan mode (from 70 to 300 m/z), thereby allowing the screening of parent ions corresponding to mono-protonated form [M + H]⁺. Mass spectrometric conditions were as follows: spray voltage was set at 4.5 kV; source gases were set (in arbitrary units min⁻¹) for sheath gas, auxiliary gas, and sweep gas at 20, 5, and 5, respectively; capillary temperature was set at 250 °C; capillary voltage at –16 V; tube lens, split lens, and front lens voltages at 50, –60, and –5.75 V, respectively. Ion optics parameters were optimized in ESI⁺ mode by automatic tuning using a standard solution of norleucine at 0.1 g.L⁻¹ infused in mobile phase (A/B, 50/50) at a flow rate of 5 μ L.min⁻¹. External calibration curves were plotted from 0.0003 to 0.03 mg.L⁻¹ for both rhizodeposit solutions.

An additional semi-quantitative analysis of rhizodeposit was performed using the GC-MS system following a protocol adapted from Luo et al. (2014). A 1-mL volume of the concentrated solution in ACN was added to a GC-MS-compatible vial, then blown to dryness under a nitrogen flow; after which,

the dry residue was suspended in 1 mL of n-hexane ($\geq 95\%$). Samples were derivatized with 40 mL of methoxyamine hydrochloride ($20 \text{ mg}\cdot\text{mL}^{-1}$ in pyridine $> 99\%$, 2 h, 37°C) and 70 mL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, 30 min, 37°C). The GC-MS system consisted of gas chromatography (7890A, Agilent Technologies, USA) coupled with a mass spectrometer (5975C, Agilent Technologies, USA) equipped with an electron ionization source (EI) and a Gerstel MPS autosampler. A DB-5MS capillary column ($25 \text{ m} \times 0.25 \text{ mm} \times 0.251 \text{ m}$) from Agilent J&W (Agilent Technologies, USA) was used. The injection was performed in splitless mode with $1 \mu\text{L}$ of samples at 230°C . The carrier gas (helium) flow rate was 1 mL min^{-1} . The column temperature was held for 2 min at 50°C , 13 min ramp to 180°C and held for 5 min, 14 min ramp to 250°C and 10 min at 250°C . Interface and ion-source temperatures were respectively adjusted to 250 and 220°C . Mass spectra were recorded at $2.66 \text{ scans}\cdot\text{s}^{-1}$ with a m/z 50–600 scanning range.

Data processing

Raw GCMS signals were processed on the MassHunter Workstation software (Qualitative Analysis, v. B.06.00, Agilent Technologies) after chromatogram deconvolution. Spectra were compared with two search unit mass libraries: NIST 14 (NIST Mass Spectral Library) and Wiley 275 (Wiley Online Library). Compounds were identified when similarities were greater than 70%. Then, chromatograms were integrated using the MassHunter Workstation software (Quantitative Analysis, v. B.07.00, Agilent Technologies). LCMS signals were processed using the XCALIBUR software program (v. 2.1). All biochemical or compound contents were expressed per gram of dry root.

For statistical analyses, data were tested for the normality (Shapiro test) and homogeneity of variances (Bartlett test) for $p < 0.05$. In case of normality and homoscedasticity, a one-way ANOVA and the Tukey’s HSD (Honestly Significant Difference) tests were used to compare means (Test 1). Alternatively, the Kruskal-Wallis’ method followed by the Fisher’s LSD (Least Significant Difference) test were used (Test 2). The principal component analysis (PCA) was performed using the FactomineR package (v.1.34), and the graphic as well as the confidence ellipses with a confidence interval of 95% were edited using the Factoshiny package (v1.0). Data were analyzed using R Studio (v. 3.4.4).

Results and discussion

Plant response to phenanthrene

All the plants survived until the end of the experiment and visually, they did not show any toxic foliar symptoms.

However, dry leaf biomass decreased with PHE exposure as well as dry stem biomass (Fig. 1). Leaf biomass tended to decrease from the first level of PHE, but this reduction became significant at $100 \text{ mg PHE}\cdot\text{kg}^{-1}$ (-32%). Stem biomass followed the same gradual trend with a decrease of -27 , -40 , and -42% , respectively, for the modalities 25, 50, and $100 \text{ mg PHE}\cdot\text{kg}^{-1}$ compared to the control. On the other hand, dry root biomass did not seem impacted by the presence of PHE.

These trends were confirmed by an increasing root to shoot ratio, which was significant from the $50 \text{ mg PHE}\cdot\text{kg}^{-1}$ treatment. This trend has already been witnessed in the case of equivalent PHE exposure (Dupuy et al. 2016). These symptoms appeared as water stress-type response for PAH and/or fuel oil hydrocarbon exposure (Adam and Duncan 2002; Merkl et al. 2005; Langer et al. 2010). Metallic stress could also affect plant biomass allocation (Rellán-Álvarez et al. 2006; Lapie et al. 2019). In the same way, PHE exposure at low concentration ($10 \text{ mg}\cdot\text{kg}^{-1}$) on sorghum, corn, and red beans did not cause any significant effects on plant survival and growth while a higher level of exposure ($100 \text{ mg PHE}\cdot\text{kg}^{-1}$) inhibited accumulation of shoot and root biomass (Baek et al. 2004; Muratova et al. 2009). The biomass of rice submitted to PHE and PYR also showed no significant decrease below $200 \text{ mg}\cdot\text{kg}^{-1}$ (Li et al. 2008).

Root surface area seemed to slightly and non-significantly decrease in contrast to leaf area. The latter decreased gradually with PHE exposure and the reduction was significant from $50 \text{ mg PHE}\cdot\text{kg}^{-1}$. The decrease in root surface was more visible in other species like Sorghum bicolor, whose root surface decreased significantly at $100 \text{ mg PHE}\cdot\text{kg}^{-1}$ (Muratova et al. 2009).

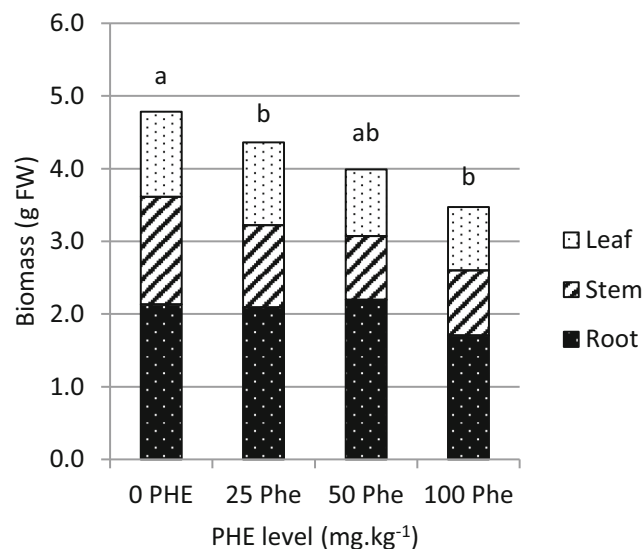


Fig. 1 Mean root, stem, and leaf biomass of maize submitted to PHE (0, 25, 50, and $100 \text{ mg PHE}\cdot\text{kg}^{-1}$), $n = 5$. Mean values with different letters are significantly different ($n = 5$, $p = 0.05$)

Photosynthesis, measured by CO₂ exchange on the 7th leaf, increased from the first PHE exposure concentration and this increase reached + 155% of the control for the highest exposure. Generally, PHE exposure induces a decrease in photosynthesis (Clijsters and Van Assche 1985; Küpper et al. 2002, 2007; Oguntimehin et al. 2008; Liu et al. 2009), but at low PAH concentrations, chlorophyll content could be enhanced. Li et al. (2008) already observed this phenomenon on rice submitted to PHE and PYR at concentrations below 200 mg.kg⁻¹. This could be explained by an increasing SOD activity, which has a role in protecting photosynthetic apparatus, such as chloroplast. Chloroplast are usually exposed to various reactions producing oxygen free radicals (Halliwell 1984; Monk et al. 1989; Li et al. 2008).

Total biochemical analysis measured on roots gave the concentration in amino acids, proteins, and carbohydrates, expressed per gram of dried tissues (Table 1). Amino acid content in roots increased with PHE exposure, and this increase was significant for the 100 mg PHE.kg⁻¹ modality, whose concentration in amino acids was twice that of the control. Proline is a well-known example of amino acid increase. Indeed, in the presence of abiotic stress like drought, salinity, heavy metal, or PAH presence, proline as well as amino acid contents generally increase in plant tissue, resulting in a better tolerance of these stresses (Costa and Spitz 1997; Ashraf and Foolad 2007; Gargallo-Garriga et al. 2018). Exposure to PYR induced a significant increase of proline content in leaves and roots of *Kandelia candel* from 13 mg PYR.kg⁻¹ (Song et al. 2011). Concerning proteins, their content in our samples was stable except for the 50-mg PHE.kg⁻¹ modality, where their presence decreased significantly. Carbohydrate content in roots was also stable in

spite of PHE exposure. Li et al. (2008) observed the same trend on rice, for which no significant impact of PHE and PYR was visible, even at high concentrations (400 mg.kg⁻¹).

In this experiment, even if no foliar symptoms were visible, plants appeared to be affected by PHE exposure at a biochemical and physiological level, thereby confirming that plant function modifications always appear before morphological alterations (Radetski et al. 2000; Alkio et al. 2005; Muratova et al. 2009).

Influence of PHE on total carbon, carbohydrate, amino acid, and protein in rhizodeposit solutions

Total carbon in rhizodeposit solutions corresponded to organic carbon because inorganic carbon was negligible in our samples. Carbon content was not significantly affected by PHE in either rhizodeposit solution, even if its content seemed to increase in the diffusate solution (Fig. 2a) and to decrease in the rhizospheric sand extract (Fig. 3a). The average concentration of organic carbon was, respectively, 1.99 and 2.76 mg.g⁻¹ for the diffusate solution and the rhizospheric sand extract (values are expressed per gram of dry root). In *Festuca ovina* and *Plantago lanceolata*, carbon exudation is around 0.2 mg.g⁻¹.h⁻¹ (Hodge et al. 1996).

In the same way, carbohydrate emission in both diffusate solution (Fig. 2b, 0.64 mg.g⁻¹ dry root weight.h⁻¹ on average) and rhizospheric sand extract (Fig. 3b, 6.79 mg.g⁻¹ dry weight) was not significantly modified by PHE exposure. Krafczyk et al. (1984) also measured carbohydrates in 36-day old maize root exudate. They obtained a concentration of 8.74 mg.g⁻¹ dry weight). This concentration is higher than in our experiment because it resulted from 36 days of

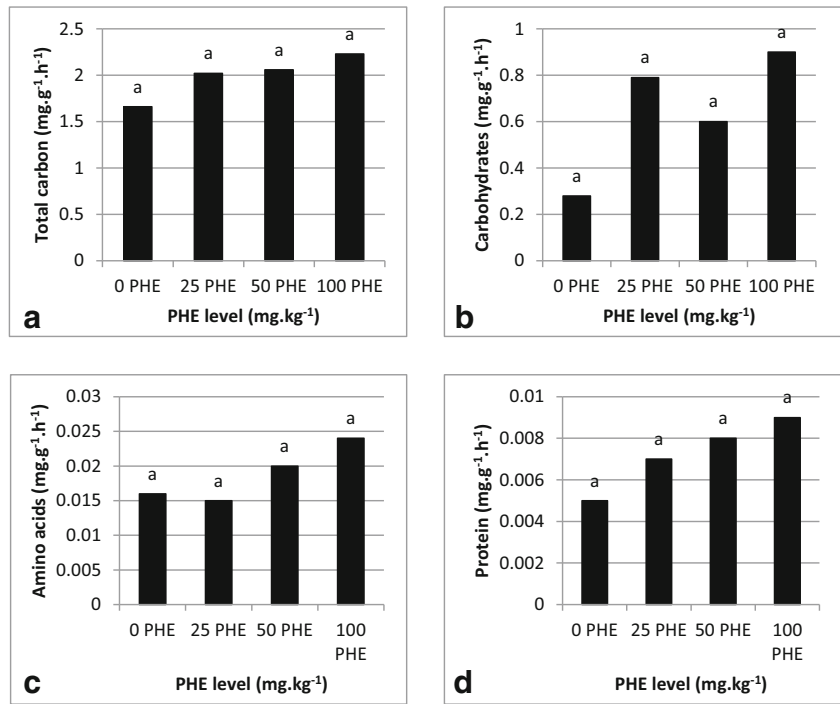
Table 1 Maize plant characteristics (mean values ± standard deviation) according to the different levels of phenanthrene (PHE) exposure

PHE level (mg.kg ⁻¹)	Organ	Test	0	25	50	100
R/S ratio		2	0.40 ± 0.05 c	0.51 ± 0.06 bc	0.78 ± 0.30 a	0.62 ± 0.25 ab
	Leaf	2	718 ± 27 a	698 ± 48 a	549 ± 155 b	518 ± 83 b
Surface area (cm ²)	Root	1	1109 ± 143 a	1090 ± 222 a	1160 ± 274 a	840 ± 151 a
	7 th leaf	1	3.71 ± 0.45 b	5.74 ± 1.86 ab	5.44 ± 1.95 ab	7.06 ± 1.58 a
Amino acids (mg.g ⁻¹ DW)	Root	1	2.66 ± 1.02 b	2.79 ± 1.49 ab	4.65 ± 2.55 ab	5.78 ± 1.33 a
		1	3.85 ± 1.26 a	4.28 ± 0.63 a	1.94 ± 0.67 b	3.99 ± 1.38 a
Proteins (mg.g ⁻¹ DW)		1	161.72 ± 45.08 a	149.84 ± 23.87 a	130.73 ± 42.53 a	144.61 ± 53.81 a
	Carbohydrates (mg.g ⁻¹ DW)	1				

For a given parameter, mean values with different letters are significantly different (n = 5, p = 0.05)

R/S root to shoot biomass ratio

Fig. 2 Total carbon (a), carbohydrate (b), amino acid (c), and protein (d) mean content in the diffusate solution of maize according to the phenanthrene (PHE) exposure level (\pm standard deviation). Expressed in milligrams of metabolite per gram of dry root. For a given parameter, mean values with different letters are significantly different ($n = 5$, $p = 0.05$)



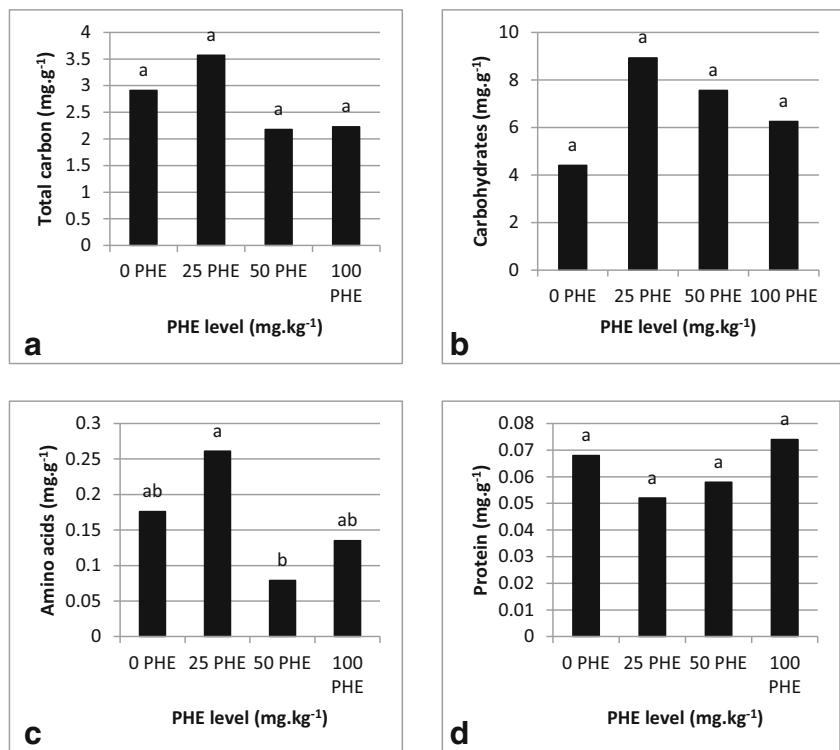
cultivation while in our experiment; the diffusate solution was obtain after 6 h of diffusion and is expressed per hour.

Even if results were not significant, carbohydrates tended to increase in the diffusate solution as well as in the rhizospheric sand extract. These results could be correlated with the increase in photosynthesis activity. In fact, about

17% of net carbon fixed by photosynthesis is emitted by the roots as rhizodeposits (Nguyen 2003). Therefore, it is likely that the amount of carbon lost by rhizodeposition is dependent on photosynthesis.

Reflecting the nitrogen metabolism, amino acids (Fig. 2c) and proteins (Fig. 2d) in the diffusate solution also increased

Fig. 3 Total carbon (a), carbohydrate (b), amino acid (c), and protein (d) mean content in the rhizospheric sand extract of maize according to the phenanthrene (PHE) exposure level (\pm standard deviation). Expressed in milligrams of metabolite per gram of dry root. For a given parameter, mean values with different letters are significantly different ($n = 5$, $p = 0.05$)



(respectively + 60% and + 50% for the 100 mg PHE.kg⁻¹ exposure) with PHE exposure in a non-significant way. The proportion of total metabolites in the diffusate solution is close to those of other studies. In our experiment, the proportion of total carbohydrates and amino acid is about 95 and 5%, respectively. In a study from Krafczyk et al. (1984), the proportion of carbohydrates emitted by maize roots after 36 days of cultivation is about 97% while that of total amino acids is about 3%. In both cases, the proportion of carbohydrate predominates. In the rhizospheric sand extract, amino acid decreased significantly from the 50-mg PHE.kg⁻¹ modality (Fig. 3c) and proteins did not seem to be impacted by PHE exposure (Fig. 3d). Proteins are mostly composed of enzymes like peroxidase or oxidoreductase (Uren 2007). Mucilage proteomes of maize have already been identified, being involved in cell wall metabolism, nutrient acquisition, scavenging of reactive oxygen species, or stress response (Ma et al. 2010). Even when the plant is not submitted to abiotic stress, it emits heat shock proteins in the rhizosphere, and their quantity can be modified in the presence of PHE, thereby affecting the total protein emission.

The composition of the rhizodeposit solutions match those cited in previous works. Indeed, the main families of compounds found in exudates are sugars, amino and organic acids, but also lower quantities of proteins and alcohols, which correspond to compounds determined in our diffusate solution (Grayston et al. 1997; Neumann and Röhmheld 2000; Nguyen 2003; Uren 2007). Indeed, Boeuf-Tremblay et al. (1995) measured total carbohydrates from root exudates of maize and obtained an average concentration of 0.20 mg.g⁻¹.h⁻¹ while in our experiment, these values were comprised between 0.28 and 0.90 mg.g⁻¹.h⁻¹. They also measured a 0.070-mg.g⁻¹.h⁻¹ average concentration of amino acids, while in our study, these values lay between 0.015 and 0.024 mg.g⁻¹.h⁻¹. Concerning proteins, Muratova et al. (2009) measured total proteins in root exudates from plants exposed to 10 and 100 mg PHE.kg⁻¹. However, contrarily to our experiment, they observed a decrease in protein secretion, even if enzymatic activities like oxidoreductases tended to increase. In their experiment, they used sorghum and it is well-known that plant exudation can vary from one species or a variety to another (Aulakh et al. 2001; Fan et al. 2001). Regarding the mucilage matrix, this is composed of 95% (w/w) polysaccharides and 5% (w/w) protein (Harris and Northcote 1970; Bacic et al. 1986). Accordingly, in our rhizospheric sand extracts, the main compound family was carbohydrates, which includes mono- and polysaccharides, representing almost 95% (w/w) of total biochemical compounds measured. Proteins and amino acids represented, respectively, 1.5 and 3.8% (w/w) of the measured compounds.

Influence of PHE on diffusate solution composition

In the diffusate solution, we were able to determine three saccharides: fructose, glucose, and cellobiose (Table 2).

Xylose and sucrose were detected below the limit of quantification (LOQ) and rhamnose, mannose, and mannitol were below the limit of detection (LOD). The most abundant saccharide in the diffusate solution was fructose, with concentrations from 44 to 129 mg.g⁻¹ dry root.h⁻¹, followed by glucose (from 5 to 10 mg.g⁻¹.h⁻¹) and by cellobiose, whose concentrations were below 1 mg.g⁻¹.h⁻¹. Values were expressed per hour and per gram of dry root. However, in a similar experiment, glucose was the most abundant saccharide (3.90 mg.g⁻¹ root dry weight), followed by arabinose, sucrose, and fructose (Krafczyk et al. 1984). Chaboud and Rougier (1984) also measured glucose as the predominant sugar in their soluble fraction of rhizodeposits collected after a sterile cultivation of maize. The comparison of these studies should be taken with caution due to the different conditions of cultivation and rhizodeposit collection. In our experiment, glucose was not significantly impacted by the PHE presence, while fructose and cellobiose content increased significantly even at the lowest PHE exposure level. Fructose was almost three times more concentrated in the highest exposure level than in the control. Cellobiose also gradually increased from 0.171 for the control to 0.564 mg.g⁻¹.h⁻¹ for the highest PHE exposure. To our knowledge, only one other study was dedicated to the impact of PAHs on rhizodeposit composition and especially on root exudates (Muratova et al. 2009). They witnessed a rise in sugar content (fructose, glucose, maltose, and galactose) on sorghum, significant for maltose, but only at low PHE exposure (10 mg PHE.kg⁻¹).

Twelve amino acids were above the LOQ: glycine, serine, alanine, threonine, histidine, arginine, phenylalanine, tyrosine, glutamic acid, valine, lysine, and leucine (Table 2). Cysteine was not detected and asparagine, aspartic acid, proline, tryptophan, glutamine, and methionine were below the LOQ. Lysine and leucine were the most abundant amino acids in the diffusate solution with an average concentration of, respectively, 1.05 and 1.25 µg.g⁻¹ dry root.h⁻¹. The same proportion of amino acids in exudate is not found in other studies. Carvalhais et al. (2011) measured asparagine and glutamic acid as the most abundant amino acids in root exudates collected after 15 days on maize; in this study, their concentration is, respectively, 0.92 and 0.88 µg.g⁻¹ dry root.h⁻¹. Asparagine and glutamic acid were also the dominant amino acid in the publication of Krafczyk et al. (1984).

Most amino acids, including alanine, threonine, arginine, phenylalanine, tyrosine, glycine, serine, and leucine, gradually and significantly increased with PHE exposure. For example, threonine was six times more concentrated for the highest PHE exposure (0.67 µg.g⁻¹ dry root.h⁻¹) than for the control (0.11 µg.g⁻¹.h⁻¹). Histidine content remained stable over PHE exposure, with a mean concentration of 0.11 µg.g⁻¹.h⁻¹, while glutamic acid and lysine decreased with increasing PHE exposure. This is not in line with the study of Muratova et al. (2009), in which amino acid contents were not significantly affected by PHE exposure.

Table 2 Metabolite profiling (mean values, related to root dry weight ± standard deviation) in diffusate solution of maize according to the phenanthrene (PHE) exposure level

Compounds	Unit	Test	PHE per kg of dry sand (mg.kg ⁻¹)					
			0	25	50	100		
Monosaccharide	Fructose	μg.g ⁻¹ (per hour)	2	43.75 ± 12.67 b	115.42 ± 50.56 a	92.74 ± 12.95 a	128.97 ± 79.60 a	
	Glucose		2	7.06 ± 1.14 a	10.25 ± 7.64 a	4.96 ± 2.86 a	8.92 ± 6.13 a	
Disaccharide	Cellobiose		2	0.17 ± 0.07 b	0.33 ± 0.18 ab	0.29 ± 0.06 ab	0.56 ± 0.40 a	
Amino acid	Glycine		2	0.33 ± 0.12 b	0.56 ± 0.23 ab	0.40 ± 0.11 b	0.99 ± 0.50 a	
	Serine		2	0.23 ± 0.09 b	0.41 ± 0.13 ab	0.39 ± 0.15 ab	1.07 ± 0.96 a	
	Alanine		2	0.29 ± 0.11 b	0.48 ± 0.10 ab	0.45 ± 0.19 ab	1.15 ± 0.93 a	
	Threonine		2	0.11 ± 0.04 c	0.30 ± 0.15 b	0.21 ± 0.08 b	0.67 ± 0.33 a	
	Histidine		2	0.11 ± 0.02 a	0.08 ± 0.01 a	0.11 ± 0.08 a	0.12 ± 0.09 a	
	Arginine		2	0.26 ± 0.09 b	0.35 ± 0.10 ab	0.40 ± 0.10 ab	0.98 ± 0.76 a	
	Phenylalanine		2	0.30 ± 0.09 b	0.41 ± 0.26 ab	0.41 ± 0.33 ab	0.83 ± 0.49 a	
	Tyrosine		2	0.19 ± 0.06 b	0.27 ± 0.17 ab	0.26 ± 0.20 ab	0.49 ± 0.29 a	
	Glutamic acid		2	1.30 ± 0.39 a	0.24 ± 0.07 c	0.41 ± 0.15 b	0.58 ± 0.41 b	
	Valine		2	0.69 ± 0.21 a	0.29 ± 0.10 b	0.43 ± 0.32 ab	0.87 ± 0.80 ab	
	Lysine		1	1.29 ± 0.22 ab	1.42 ± 0.46 a	0.75 ± 0.17 bc	0.65 ± 0.48 c	
	Leucine		2	0.69 ± 0.17 b	0.86 ± 0.15 ab	0.84 ± 0.27 ab	2.33 ± 1.89 a	
	Monoglyceride	1-Monopalmitin	Log(pic area.g ⁻¹)	2	5.86 ± 5.48 a	5.79 ± 5.58 a	5.05 ± 4.80 b	5.38 ± 5.12 b
		1-Monostearin		1	5.49 ± 4.77 a	5.45 ± 4.89 a	5.39 ± 5.31 a	5.46 ± 5.15 a
	Fatty acid	Palmitic Acid		1	5.53 ± 5.13 a	5.58 ± 5.01 a	5.42 ± 5.12 a	5.48 ± 5.11 a
Stearic acid			1	5.64 ± 5.03 a	5.75 ± 5.04 a	5.59 ± 5.36 a	5.71 ± 5.32 a	
Organic acid	Pyruvic acid		2	5.44 ± 4.41 b	5.72 ± 5.40 a	4.28 ± 4.17 c	4.82 ± 4.64 c	
	Lactic acid		2	5.72 ± 5.73 ab	5.71 ± 5.48 a	4.95 ± 4.47 b	5.44 ± 5.07 ab	
	Caproic acid		1	5.67 ± 5.50 a	5.67 ± 5.56 a	5.19 ± 5.23 a	5.68 ± 5.60 a	
	Oxalic acid		2	6.50 ± 6.18 a	6.64 ± 6.34 a	4.48 ± 3.71 c	4.79 ± 4.28 b	
	3-hydroxybutanoic acid		2	4.98 ± 4.83 a	4.88 ± 4.42 a	4.25 ± 4.11 b	4.33 ± 3.93 b	
	Malonic acid		2	5.63 ± 5.32 ab	5.51 ± 4.89 b	5.36 ± 4.64 c	5.73 ± 4.48 a	
	Succinic acid		2	6.52 ± 5.81 a	6.44 ± 6.10 a	4.88 ± 4.11 c	5.22 ± 4.37 b	
	Nonanoic acid		1	4.69 ± 4.33 ab	4.69 ± 4.28 ab	4.44 ± 4.26 b	4.85 ± 4.57 a	
	Alcohol	Heptanol		2	5.42 ± 5.33 a	5.48 ± 5.33 a	4.84 ± 4.15 a	5.32 ± 4.98 a
		Glycerol		1	6.52 ± 5.81 a	6.44 ± 6.10 a	6.30 ± 5.87 a	6.47 ± 6.00 a
1-Dodecanol			2	4.82 ± 4.71 a	4.68 ± 4.53 a	4.49 ± 4.08 a	4.79 ± 4.28 a	
Alcohol amine	Ethanolamine		2	5.58 ± 5.50 ab	5.15 ± 5.08 b	5.36 ± 4.63 ab	5.73 ± 4.48 a	

For a given parameter, mean values with different letters are significantly different (n = 5, p = 0.05)

In the diffusate solution, 16 compounds were analyzed by GCMS. Their contents are shown, in Table 2, as a logarithm of the peak area measured in arbitrary unit per gram of dry root. Half of these were organic acids like pyruvic, lactic, caproic, oxalic, 3-hydroxybutanoic, malonic, succinic, and nonanoic

acid (Table 2). Four were alcoholic compounds: heptanol, glycerol, dodecanol, and ethanolamine, which is an alcohol amine. Two fatty acids were also found, namely palmitic and stearic acid, as well as two monoglycerides, i.e., 1-monopalmitin and 1-monostearin. The emission of almost half

of these compounds was not significantly affected by the presence of PHE. On the contrary, the exudation of oxalic, pyruvic, lactic, 3-hydroxybutanoic, succinic acid, and 1-monopalmitin decreased significantly as PHE exposure increased, from the 50-mg PHE.kg⁻¹ treatment, while the 25-mg PHE.kg⁻¹ treatment did not seem to differ from the control. Ethanolamine, malonic, and nonanoic acid emission decreased significantly until 50 mg PHE.kg⁻¹ exposure but increased in the highest PHE treatment. In their experiment, Muratova et al. (2009) did not observe any significant differences in organic acid exudation of sorghum (expressed per unit of root surface) between PHE treatments. Nevertheless, increasing sugar and organic acids exudations as a response to abiotic stress was observed for other stresses. In the hydroponic experimentation, Song et al. (2012) showed that maize exuded more organic acids (malic, lactic, citric, or maleic acid) under water-stress conditions. Likewise, Preece and Peñuelas (2016) showed an increased sugar root exudation per gram of plant biomass under moderate drought. The increase in these soluble compounds could be related to osmotic regulation, triggered to support survival in such water-stress conditions. Indeed, as with hydrophobic compounds, PAHs could alter the water dynamics and transportation due to their hydrophobic characteristics and therefore act as a drought agent.

Several compounds found in our diffusate solutions have already been studied for their involvement in PAH dynamic, whether directly or indirectly. Root exudates and particularly organic acids including acetic, oxalic, tartaric, and citric acids measured in our samples improve desorption of phenanthrene and pyrene in soils (Gao et al. 2010). And when they are associated to surfactants, organic acids were found to enhance the desorption of PHE from soil into aqueous phase. Combining a surfactant with organic acids enabled a higher desorption of this PAH than that of the surfactant alone (An et al. 2011). Moreover, some microorganisms could produce biosurfactants like glycolipids or rhamnolipids, which are able to improve the bioavailability of PAHs and consequently, render them more easily degraded (Deziel et al. 1996). We did not measure the number of microorganisms present in the rhizosphere and in the rest of the sand, but in many studies, the number of microorganisms in the rhizosphere was higher than that of the bulk soil (Gray and Williams 1971; Osorio Vega 2007). Indeed, root exudates are known to be chemoattractant. Moreover, most of them, like sugars (glucose, fructose, sucrose, trehalose), amino acids (arginine), and organic acids (malate, citrate, oxalate, aspartate), are easily degradable compounds which are used as nutritive substrate for microflora (Heinrich and Hess 1985; Reinhold et al. 1985; Grayston et al. 1997; Tian et al. 2017). In the diffusate solution, glucose and fructose are respectively present at concentrations of approximately 8 and 31 µg.g⁻¹.h⁻¹ and could have strong chemoattractive abilities. Indeed, *Escherichia coli* is able to respond to a chemotactic signal < 10 nM of glucose (Lux et al. 1999).

Influence of PHE on composition of rhizospheric sand extracts

The rhizospheric sand extract contained no mono or disaccharides but only polysaccharides (Table 3). They were analyzed after acid hydrolysis and only glucose monomers were found. In another experiment, glucose was found predominant in the polysaccharide composition (38%) of mucilage but was not the only saccharide measured. In another experiment (Chaboud and Rougier, 1984), glucose was found predominant in the polysaccharide composition (38%) of mucilage but was not the only saccharide measured. Indeed, they also measured galactose (20%), xylose (18%), arabinose (14%), fucose (5%) and mannose (5%). In our method, galactose could not be separated from glucose due to their epimeric form and xylose, arabinose and fucose were not measured. Hydrolyzed glucose content decreased significantly with increasing PHE exposure, from 0.043 mg.g⁻¹ for the control to 0.012 mg.g⁻¹ for the highest PHE exposure.

In addition, 11 amino acids were measured in quantities superior to the LOQ: histidine, arginine, phenylalanine, tyrosine, tryptophan, glutamine, glutamic acid, valine, lysine, leucine and methionine (Table 3). Cysteine, glycine, serine, alanine, asparagine, aspartic acid, proline and glutamine were below the LOD and threonine below the LOQ. Arginine and leucine were the most concentrated compounds with a respective average concentration of 0.97 µg.g⁻¹ and 0.94 µg.g⁻¹. Amino acids content in rhizospheric sand extracts decreased significantly with increasing PHE even at the first level of exposure. For example, the tryptophan content decreased by 58% between the control and the 100 mg PHE.kg⁻¹ treatment.

Using GCMS, 20 different molecules were determined in rhizospheric sand extracts. Most of them, precisely seven, were organic acids: sebacic, nonanoic, benzoic, 3- and 4-hydroxybutanoic, lactic and glycolic acid (Table 3). Five alcohols were found (dodecanol, glycerol, 2-ethylhexanol, propanediol and xylitol) as well as five fatty acids, including palmitic, stearic, octanoic, palmitoleic and pentadecanoic acids. Other molecules like monoglyceride (1-monostearin and monopalmitin) and catecholamine were also found. Most of these compounds did not seem significantly impacted by PHE. However, compound contents like 1-monopalmitin decreased significantly in the modalities 25 mg PHE.kg⁻¹ and 50 mg PHE.kg⁻¹, while increasing to the control level in the 100 mg PHE.kg⁻¹ modality. Stearic acid content in rhizospheric sand extract increased gradually and significantly with PHE exposure as did as 4-hydroxybutanoic acid and dopamine. Pentadecanoic acid concentration increased significantly in the 25 mg PHE.kg⁻¹, but was similar to the control for the higher PHE exposures. In contrast, 3-hydroxybutanoic acid content decreased significantly for the two highest PHE exposures.

To our knowledge, there is no other publication relating the influence of PAHs on root mucilage. However, the composition of mucilage is well-known and principally composed of polysaccharides, phospholipids and proteins (Chaboud 1983; Bacic et al. 1987; Read et al. 2003). Chaboud (1983) found that polysaccharides from maize were mostly composed of galactose, fucose, and glucose, while the rhizospheric sand extract analyzed here contained high amounts of polysaccharides mainly composed of glucose chains. The difference might be due to our method, which did not differentiate the glucose from its epimer, galactose. Moreover, the conditions of Chaboud's experiment were very different from ours, as they grew the plant and collected the mucilage in sterile conditions on seedlings aged less than a week, while our collection was conducted on non-sterile 6-week old plants. Both age and sterility are factors which can modify rhizodeposition (Hodge et al. 1996; Aulakh et al. 2001).

The lipidic composition of the rhizospheric sand extract in our experiment is in agreement with fatty acids measured by Read et al. (2003) in maize, lupin, and wheat. In their experiment, saturated fatty acids were predominant, especially C16 and C18, while in our study, four out of five fatty acids were saturated (palmitic, stearic, octanoic, and pentadecanoic acids) and also three were C16 or C18: palmitic acid (C18), stearic acid (C18), and palmitoleic acid (C16). Fatty acids and glycerol are components of phospholipids, major constituents of mucilage. Phospholipids are also surfactant, i.e., organic compounds consisting of both hydrophobic and hydrophilic components. Those amphipathic molecules are known to affect the behaviors and dynamics of organic pollutants such as PAHs by enhancing their solubilization in water and consequently improving their bioavailability for both plant and microorganisms (Deziel et al. 1996; Tiehm et al. 1997; Gao et al. 2006). However, if small amount of surfactant increases absorption and accumulation of PAH by a plant, higher amounts seem to reduce them (Gao et al. 2006). In soils, the presence of those surfactants could improve PAH desorption from the solid phase.

Multivariate approach to the impact of PHE on rhizodeposition

A principal component analysis (PCA, Fig. 4) was used to synoptically evaluate the impact of PHE on both rhizodeposit solutions, including the determination of individual compounds performed with LC-MS and GC-MS. When performed with 31 compounds of the diffusate solution (Fig. 4a, b), the PCA first plane explained 53% of the variability (31.8% for the first component and 21.5% for the second one). The variables which most contributed to the first component were amino acids, fructose, and cellobiose, while the second component mostly resulted from the organic and fatty acids (palmitic, caproic, nonanoic, lactic acid) (Fig. 4a).

Control replicates and replicates exposed to 25 mg PHE.kg⁻¹ seemed to exude in the same way, with their confidence ellipses being almost superimposed. Replicates exposed to the two highest PHE exposures formed two groups apart from the previous one. Diffusate solutions obtained from plants exposed to 100 mg PHE.kg⁻¹ were positively correlated with fructose, cellobiose, and amino acid contents, except glutamic acid and lysine (Fig. 4b). Gargallo-Garriga et al. (2018) observed the same trends with drought stress for amino acids and fructose. They also have shown an increase in the abscisic acid (ABA), a phytohormon which mediates an upregulation of the biosynthesis of amino and organic acids (Ollas et al. 2015; Li et al. 2017). PHE or its downstream products may induce ABA production, or interact with ABA receptors in maize, leading to water-stress responses (Váňová et al. 2009). Conversely, the control or 25 mg PHE.kg⁻¹ treatment was positively correlated with the diffusion of glucose, many organic acids like oxalic or succinic acid, monopalmitin, and some alcohols (dodecanol and heptanol), which could be regarded as exudates of an unperturbed plant. In opposition, the 50-mg PHE.kg⁻¹ exposure reduced the content of organic acid, fatty acid, and alcohol, testifying to a perturbation of the plant functioning, which might be different to that at 100 mg PHE.kg⁻¹ exposure.

The second PCA (Fig. 4c, d) was performed with 31 compounds of the rhizospheric sand extract. The first plane explained about 56% of the variability, with 34.9% for the first component and 21.2% for the second. The variables most contributing to the first component were amino acids (positively), while some organic acids (lactic, sebacic, and 3-hydroxybutanoic acid), fatty acids (pentadecanoic and palmitoleic acid), and xylitol were negatively correlated to this. Other organic acids (benzoic, nonanoic, and glycolic acid), fatty acids (octanoic, palmitic, and stearic acid), dopamine, and glycerol contributed positively to the second component. The pattern in the rhizospheric sand extract compared to the diffusate solution was slightly different. The intermediate exposure concentrations (25 and 50 mg PHE.kg⁻¹) had superimposed ellipses, indicating a similar composition and rhizosphere functioning, while compounds emitted from the control and the 100-mg PHE.kg⁻¹ treatments were distinct both from each other and from those of the two other treatments. This suggests that the highest exposure level favored the production of monoglycerides and most of the fatty acids, while reducing that of amino acids and hydrolyzed glucose. On the other hand, a small group of compounds like sebacic, lactic, 3-hydroxybutanoic, palmitoleic, and pentadecanoic acid and xylitol were more specific to samples exposed to intermediate PHE exposure concentrations.

This PCA highlights two levels of response of the rhizodeposition towards PHE. From 0 to 50 mg PHE.kg⁻¹, rhizodeposition seems to be slightly and progressively modified with increasing PHE exposure, but between 50 and

Table 3 Metabolite profiling (mean values, related to root dry weight \pm standard deviation) in rhizospheric sand extract of maize according to the phenanthrene (PHE) exposure level

Compounds	Unit	Test	PHE per kg of dry sand ($\text{mg}\cdot\text{kg}^{-1}$)					
			0	25	50	100		
Hydrolyzed polysaccharide	Glucose	$\mu\text{g}\cdot\text{g}^{-1}$	2	43 \pm 28 a	22 \pm 9 ab	19 \pm 8 ab	12 \pm 6 b	
Amino acid	Threonine		1	0.26 \pm 0.07 a	0.03 \pm 0.03 b	0.03 \pm 0.04 b	0.06 \pm 0.10 b	
	Histidine		1	0.48 \pm 0.17 a	0.19 \pm 0.10 b	0.12 \pm 0.06 b	0.18 \pm 0.11 b	
	Arginine		1	1.89 \pm 0.52 a	0.68 \pm 0.32 b	0.43 \pm 0.25 b	0.89 \pm 0.65 b	
	Phenylalanine		1	1.39 \pm 0.31 a	0.35 \pm 0.15 b	0.25 \pm 0.16 b	0.69 \pm 0.48 b	
	Tyrosine		1	0.86 \pm 0.20 a	0.31 \pm 0.17 b	0.18 \pm 0.08 b	0.66 \pm 0.23 a	
	Tryptophane		1	0.25 \pm 0.06 a	0.07 \pm 0.04 b	0.04 \pm 0.03 b	0.10 \pm 0.08 b	
	Glutamic acid		1	0.14 \pm 0.05 a	0.04 \pm 0.03 b	0.07 \pm 0.04 ab	0.08 \pm 0.07 ab	
	Valine		1	0.09 \pm 0.02 a	0.06 \pm 0.01 b	0.03 \pm 0.01 b	0.05 \pm 0.02 b	
	Lysine		2	0.44 \pm 0.22 a	0.11 \pm 0.04 b	0.08 \pm 0.05 b	0.07 \pm 0.06 b	
	Leucine		2	1.69 \pm 0.94 a	0.41 \pm 0.11 b	0.40 \pm 0.23 b	1.21 \pm 0.53 a	
	Methionine		2	0.22 \pm 0.04 a	0.06 \pm 0.03 b	0.05 \pm 0.02 b	0.09 \pm 0.07 b	
	Monoglyceride	1-Monostearin	$\text{Log}(\text{pic area}\cdot\text{g}^{-1})$	1	5.81 \pm 5.64 a	4.87 \pm 4.51 a	5.38 \pm 5.22 a	5.89 \pm 4.95 a
		1-Monopalmitin		2	5.72 \pm 5.26 ab	4.36 \pm 3.99 c	4.78 \pm 4.42 bc	5.84 \pm 5.63 a
	Fatty acid	Palmitic acid		1	6.19 \pm 5.61 a	6.25 \pm 5.59 a	6.28 \pm 6.04 a	6.44 \pm 5.61 a
Stearic acid			1	5.86 \pm 5.40 b	5.98 \pm 5.13 ab	6.06 \pm 5.90 ab	6.30 \pm 5.61 a	
Octanoic acid			1	6.01 \pm 5.22 a	5.99 \pm 5.27 a	5.81 \pm 5.26 a	6.07 \pm 5.86 a	
Palmitoleic acid			1	5.05 \pm 4.88 a	5.36 \pm 4.66 a	5.12 \pm 5.07 a	4.84 \pm 4.38 a	
Pentadecanoic acid			1	4.60 \pm 3.98 b	5.09 \pm 4.65 a	4.54 \pm 4.06 b	4.69 \pm 4.36 b	
Alcohol		Dodecanol		1	5.35 \pm 4.90 a	5.17 \pm 4.89 a	5.00 \pm 4.81 a	5.21 \pm 4.57 a
	Glycerol		1	6.39 \pm 5.77 a	6.37 \pm 6.21 a	6.50 \pm 6.41 a	6.62 \pm 6.10 a	
	Ethylhexanol		2	5.45 \pm 5.43 a	5.20 \pm 4.45 a	5.20 \pm 5.01 a	5.56 \pm 5.05 a	
	Propanediol		2	5.37 \pm 5.27 a	5.03 \pm 3.81 a	4.87 \pm 4.17 a	5.10 \pm 5.06 a	
	Xylitol		1	4.01 \pm 3.64b a	5.09 \pm 4.95 a	4.85 \pm 4.83 a	4.55 \pm 4.49 a	
	Organic acid	Sebacic acid		1	5.19 \pm 4.89 a	5.65 \pm 5.47 a	5.61 \pm 5.49 a	5.31 \pm 5.40 a
Nonanoic acid			2	5.25 \pm 4.73 a	5.34 \pm 4.69 a	5.32 \pm 4.72 a	5.51 \pm 5.52 a	
Benzoic acid			2	5.34 \pm 4.63 a	5.34 \pm 4.89 a	5.26 \pm 5.04 a	6.25 \pm 6.19 a	
4-Hydroxybutanoic acid			2	5.36 \pm 4.93 b	5.54 \pm 4.89 ab	5.44 \pm 5.12 ab	6.78 \pm 6.53 a	
3-Hydroxybutanoic acid			2	6.65 \pm 6.45 ab	6.93 \pm 6.37 a	6.58 \pm 6.17 b	6.45 \pm 6.27 b	
Lactic acid			1	5.99 \pm 5.40 a	6.08 \pm 5.56 a	6.08 \pm 5.94 a	5.97 \pm 5.78 a	
Glycolic acid			2	5.74 \pm 5.31 a	5.45 \pm 4.96 a	5.63 \pm 5.38 a	6.11 \pm 6.11 a	
Catecholamine	Dopamine		2	5.75 \pm 5.42 b	5.88 \pm 5.20 ab	5.68 \pm 5.44 b	6.82 \pm 6.55 a	

For a given parameter, mean values with different letters are significantly different ($n = 5$, $p = 0.05$)

100 $\text{mgetPHE}\cdot\text{kg}^{-1}$, plants passed a threshold concentration beyond which the plant deeply and significantly modifies its rhizodeposition. Indeed, in the diffusate solution, control plants

and plants submitted to concentrations below 50 $\text{mgetPHE}\cdot\text{kg}^{-1}$ are in the same axis of the PCA, while plants exposed to the highest PHE concentrations are positioned orthogonally to them.

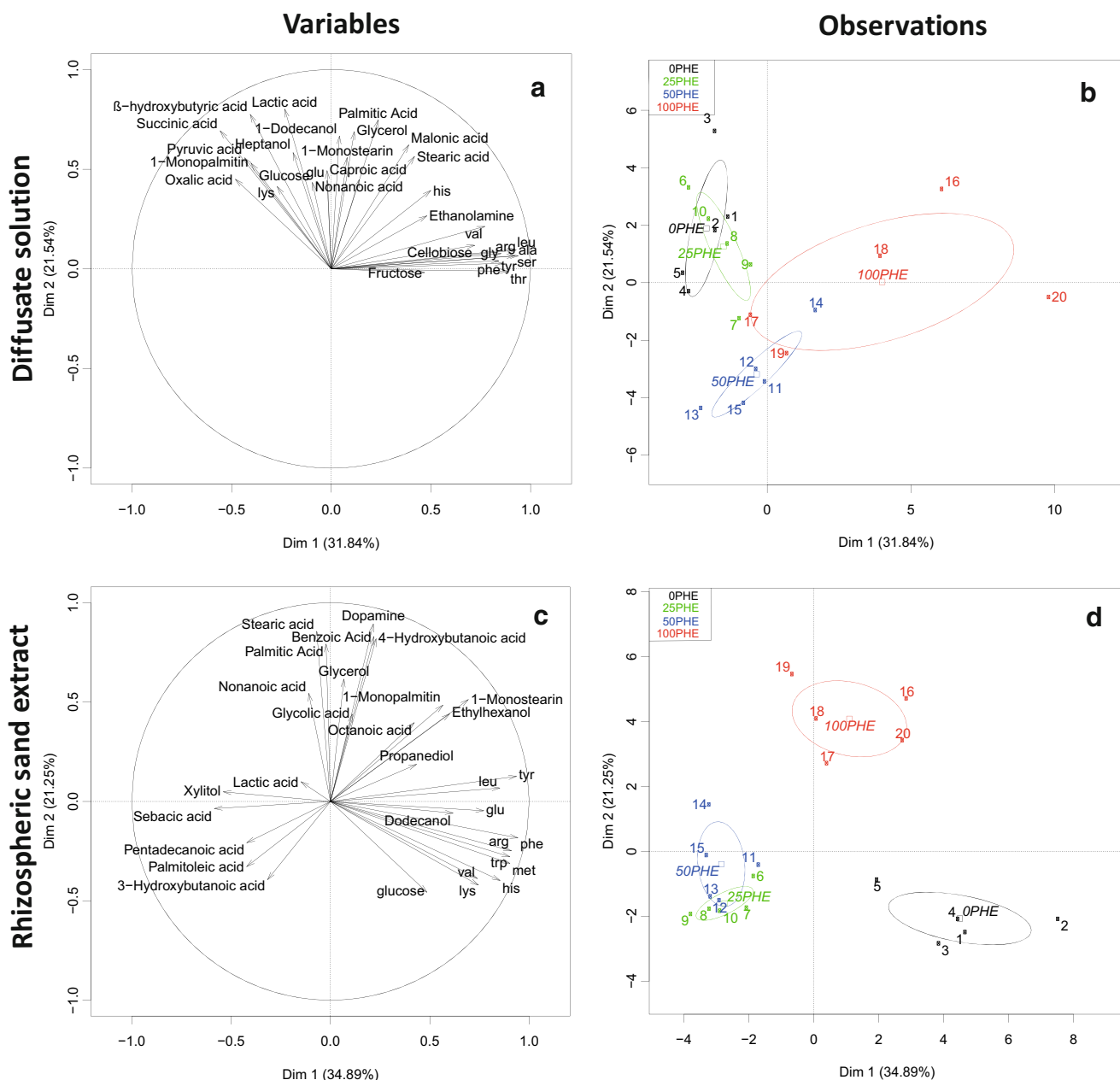


Fig. 4 Principal Component Analysis (PCA) generated from all measured compounds in diffusate solution (a, b) and in rhizospheric sand extract (c, d). Points represent the different replicates according to PHE treatments: 0PHE (0 mg PHE.kg⁻¹) correspond to replicates 1, 2, 3, 4, and

5; 25PHE (25 mg PHE.kg⁻¹) correspond to 6, 7, 8, 9, and 10; 50PHE (50 mg PHE.kg⁻¹) correspond to replicates 11, 12, 13, 14, and 15; 100PHE (100 mg PHE.kg⁻¹) correspond to replicates 16, 17, 18, 19, and 20. Amino acids are designated according to their three-letter code

In the same way in the rhizospheric sand extract, the 100-mgPHE.kg⁻¹ modality is placed on an opposite axis compared to the other treatments. The existence of a threshold concentration has already been witnessed in *S. bicolor* submitted to 100 mg PAH.kg⁻¹ with a substantial decrease in the plant biomass and a modification of the rhizodeposits composition. Additionally, this response could also be variable between the diffusate solution and the rhizospheric sand extract, which are emitted and collected differently. The variability between the two rhizodeposit solutions could be explained by the differences in

compound transportation in rhizospheres and by the differences between the two collection methods. Indeed, compounds of low molecular weight composing the diffusate solution are generally exuded in the rhizosphere via the passive diffusion process through the plasma membrane while compounds of high molecular weight generally resulting from the rhizospheric sand extract, result in active transportation via Golgi vesicles or ATP transporters (Badri and Vivanco 2009). Moreover, the diffusate solution and the rhizospheric sand extract are collected differently; the first one resulting from a diffusion of 6 hours, while the

second one resulting from 5 weeks of cultivation. In the first case, the microorganisms did not have the time to consume root exudates while the second solution reflects an equilibrium between root secretion and microorganism consumption during all the cultivation period.

The rhizodeposition mechanism has been classified according to the mode of transport from the plant to the rhizosphere, whether active or passive. Root exudates are known to be emitted passively across the plasma membrane, and consequently the concentration in the rhizosphere depends on the plant functioning (Lynch and Whipps, 1990; Nguyen, 2003; Rovira, 1969). Indeed, in our experiment, the photosynthetic activity increases in presence of PHE while the diffusion of total carbon and total carbohydrates in the rhizosphere increases too. In this case, the rhizodeposition would reflect the plant functioning (photosynthesis). Amino acids also followed the same trend in the rhizosphere and in the root tissue, they both increased in the presence of PHE. Indeed, amino acids are usually described in the literature as passively emitted by roots through the diffusion process. However, some recent studies suggest that amino acids could be actively emitted into the rhizosphere (Bobille et al., 2019). Protein contents in root tissue, also measured in our experiment, do not seem affected by the presence of PHE except for the 50-mg.kg⁻¹ modality while protein content in the diffusate solution increased. Indeed, proteins are known to be actively emitted in the rhizosphere, suggesting this emission would benefit the plant”.

Conclusion

Plant functioning was impacted by PHE presence in the substrate, with a decrease in plant biomass and a modification of its allocation, preferably to the root. Molecular composition in root tissues was modified as well, essentially with an increase in amino acid content. Rhizodeposition was also affected by PHE exposure. This study brings new information on the metabolic profiling of root exudates and especially on mucilage, which previously had rarely been studied, in the context of organic pollution. The diffusate solution was mainly composed of low molecular weight compounds, such as sugars, amino acids, and organic acids, while the rhizospheric sand extract principally contained higher molecular weight compounds like polysaccharides or components of phospholipids, but also amino acids. Most compounds of the diffusate solution tended to increase in the presence of PHE, and this rise was statistically significant for sugars and amino acids. In the rhizospheric sand extract, most compounds also seemed to be impacted by PHE exposure, except proteins. Amino acid contents decreased significantly in the total and individual analyses, as did the hydrolyzed glucose, while most organic and fatty acids increased. Plant functioning under PAH stress has already been compared to its functioning under drought stress, as water stress induces the production of organic acid and amino acid in root exudates

as in our diffusate solutions submitted to PHE. These similarities could be explained by the similarity between ABA which accumulates in case of drought stress and PHE downstream products which can act as an ABA-like hormone disruptor. In this study, we also measured numerous compounds known to have, directly or indirectly, an impact on PAH dynamic in soil. Those compounds could be involved in plant tolerance against PAH. Indeed, the plant physiology is often studied in contrast to rhizodeposition while this process could be the first chemical barrier to protect plants against the toxicity of pollutants. Rhizodeposits and especially root exudates are often highlighted for their role in phytoremediation while very little information is available on their composition in the presence of pollutants. This study provides new data for understanding of the phytoremediation process, with the measurement of numerous compounds able to desorb PAH from the soil matrix. It also documents the role of mucilage, which is usually less studied than root exudates.

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