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Full Length Research Paper

Analysis of phenanthrene biosorption to the roots of *Cyperus hermaphroditus* by microscopy, spectroscopy and photoacoustic techniques

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Understanding the process of contaminant accumulation by plants is essential to assessing crop contamination; organic chemicals may be adsorbed to roots and then be taken up, translocated, metabolized, or transpired by plants. The biosorption of phenanthrene by the radical system of *Cyperus hermaphroditus,* was studied by plants exposed to different concentrations of this pollutant, during 3 and 12 days and phenanthrene adsorbed was detected by optical and scanning electron microscopy (SEM) techniques and measured by photoacoustic spectroscopy (PAS). The sorption of phenanthrene in this species increased with plant age and the exposition time to the contaminant, due to the root mass with more surface area that enhanced affinity of the roots for the contaminant. The radical system of *C. hermaphroditus* may thus provide a surface for phenanthrene biosorption; that could be an important control in the immobilization of polycyclic aromatic hydrocarbons.

Key words: Phenanthrene, radical system, photoacoustic spectroscopy.

INTRODUCTION

Understanding the process of contaminant accumulation by plants is essential to assessing crop contamination and subsequent human exposure. The accumulation of xenobiotics by roots can be the result of active uptake or surface adsorption mechanisms; both controlled by chemical and physical compound characteristics and plant roots properties. Experiments involving the uptake on no ionized chemicals from hydroponic solution into plant roots have demonstrated that the uptake process consists of two components; first the equilibration of the aqueous phase in the plant root with the concentration in the surrounding solution and second the sorption of the chemical onto lipophilic root solids, include lipids in membranes and cell walls (Briggs et al., 1983; Patterson et al., 1991; Schwab et al., 1998; Li et al., 2005).

The uptake in terrestrial plants has been studied for many plant-contaminant combinations and quantitative models have established to predict uptake rates (Trapp, 2004). The adsorption of chemicals to roots was believed to be an important process causing chemical incorporation into root tissues (Wild and Jones, 1992). The process of Polycyclic Aromatic Hydrocarbons (PAH) being taken up into plant roots consist of three parts: first, contaminants dissolved in the aqueous solution within the apparent free space of the roots (but not adsorbed), second, adsorbed on the root surface and third, penetrating into root tissues (Schwab et al., 1998).

The degree of organic contaminant uptake is influenced by the properties of the contaminant, the plant species and the soil (Li et al., 2005).

Monitoring uptake and location of organic compounds in roots is therefore complicated, because its concentration varies along the roots and with time the root grows

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and elongates.

The growing root represents a linear sequence of cell functional differentiation-from the cap to the branching zone-, with respect to substrate uptake, cell growth, and cell structure and function. Key structural components of root cells, such as cellulose, proteins, hemicellulose lignin, and pectin, differ in proportion/volume along a developing root. It is hypothesised that the progressional change in cell structure from the root tip to the branching zone is likely to affect the uptake and processing of Persistent Organic Pollutants (POP) (Wild et al., 2005).

During the past decades, there has been considerable interest in understanding plant uptake and accumulation of organic contaminants including PAHs to the assessment of crops and produces (Briggs et al., 1982; Wild and Jones, 1992; Simonich and Hites, 1994; Gao and Zhu, 2004; Lin et al., 2006, Jiao et al., 2007; Su and Zhu, 2007; Cofield et al., 2008; Gao et al., 2008). Numerous studies showed that PAHs were accumulated in both vegetable roots and leaves; however, it is still under investigation whether PAHs can be translocated within plants. These compounds are lipophilic and the absorption to the surfaces of roots may be an important sink for these in soils and the first step in phytoremediation (Schwab et al., 1998). Root concentration factor, shoot concentration factor and transpiration stream concentration factor have been frequently been used to model plant uptake and translocation (Su and Zhu, 2008).

Most of these molecules are adsorbed on but not absorbed by roots; only low molecular weight PAHs were able to migrate to shoots when high molecular weight PAHs were strongly adsorbed on the root epidermis (Larson and Sahlberg, 1981; Wild and Jones, 1992b; Kipopoulou et al., 1999). Phenanthrene is a tricycle aromatic hydrocarbon widely present in the environment because of pyrolytic processes. Although phenanthrene is not mutagenic or carcinogenic, it has been shown to be toxic to aquatic organisms and is often used, as a model substrate for studies on the metabolism of carcinogenic PAHs (Narro et al., 1992).

Based upon the conversion of absorbed optical energy into heat, photothermal (PT) science encompasses a wide range of techniques that have been successfully applied in a variety of fields belonging to the life sciences. Current applications in biotechnology, medicine, and the environmental sciences include the thermal and optical characterization of solid, liquid, and gaseous specimens. Among the PT techniques, Photoacoustic spectroscopy (PAS) is well suited for obtaining the optical absorption spectra of highly transparent or opaque materials. In addition, as PAS is insensitive to light scattering, this technique has the capability of analyzing highly light-scattering samples, e.g., biological systems (Cruz et al., 2004); due to the different absorption properties of biological surfaces layers. The photoacustic technique is able to monitor the presence of compounds deposited on the surfaces, by the absorption of the modulated light in biological samples (O'Hara et al., 1983).

Southeastern México has sites with various levels of oil-derived pollution from accidental spillage or disposal of oil wastes. Likewise, the growth of some plant species in these sites has called the attention to identify the physiological mechanisms that have led to the adaptation of these species to oil-polluted conditions. The aim of this work was to investigate the phenanthrene biosorption by the roots of *Cyperus hermaphroditus* (Jacquin Standley) a petroleum hydrocarbon soil polluted abundant species, through the *in situ* phenanthrene detection at roots by the evidence of photoacoustic and microscopy techniques; allowed us to the knowledge of how this species stabilizes an organic contaminant.

MATERIALS AND METHODS

Plant material and growth conditions

For purposes of this study, some specimens of the abundant swamp species that posses high tolerance to contaminants *Cyperus hermaphroditus* (Jacquin Standley) (30 to 45cm height) with mature inflorescences, were collected from the Santa Alejandrina swamp adjacent to the Minatitlan refinery,"Lázaro Cárdenas del Río" in the state of Veracruz, México, from a contaminated area with 81,640 mg/kg dry soil of Total Petroleum Hydrocarbons (TPH) (I.M.P.-U.A.M.-I, 1997). Seeds of *C. hermaphroditus* were obtained from the collected samples, germinated in washed and sterilized sand with sterile distilled water and maintained in a humid chamber at 36 ºC.

Once germinated, the seedlings were allowed to grow in amber glass containers (300 ml) with a plastic cap with two holes on its surface; one hole for the plant and the other for a glass tube, connected to an air pump to supply air to the system; filled with 250 ml of nutrient solution that contains: 200 mM $NH_4H_2PO_4$, 500 mM NH_4NO_3, 1150 mM $Ca(NO_3)_2$, 260 mM $CaCl_2$, 200 mM $MgCl_2 \cdot 6H_2O$, 200 mM $Mg(NO_3)_2 \cdot 6H_2O$, 400 mM $MgSO_4 \cdot 7H_2O$, 200 mM KH_2PO_4, 120 mM KNO_3, 500 mM K_2SO_4, 40 mM $FeCl_3 \cdot 6H_2O$, 12 mM H_3BO_3, 0.12 mM $CuCl_2 \cdot H_2O$, 2.3 mM $ZnCl_2$, 0.44 mM $MnCl_2 \cdot 4H_2O$, 6 x 10^{-3} mM $Na_2MoO_4 \cdot H_2O$, 7.19 mM EDTA and 7.12 mM $FeSO_4 \cdot 7H_2O$ (pH 6), all of these compositions were SIGMA and BAKER Co. analytical grade with a purity of > 98%. The seedlings were maintained during three months in a greenhouse with temperatures of about 35-37°C in the daytime and 15-17°C in the nigh time, and with 56% relative humidity. Previously, all of the materials and solutions employed were sterilized and the volume of the containers during the study was reviewed every week, and if lost of solutions was present, this was replenished with the same amount of distilled water.

Exposition of *Cyperus hermaphroditus* plants to phenanthrene

The plants were transferred to clean amber glass containers with 900 ml of the nutrient solution, which posses the same characteristics as the used for the culture of seedlings and the containers were cover with aluminium foil to keep the roots in darkness.

Phenanthrene (from concentrated ethanol stocks pure grade HPLC, SIGMA Chemical, Co.) was added to the mineral solution of each container at test concentrations of 40, 80 and 120 mg/ L, with constant aeration to ensure the distribution of the contaminant in

the system. Controls without phenanthrene were considerate. The hydroponic cultures were maintained in a greenhouse at 37-17°C and 56% relative humidity. The hydroponic systems have a constant aeration to ensure that roots were supplied with nutrients and O_2 and homogeneous distribution of the contaminant in the system. The plants were exposed for 3 and 12 days to phenanthrene, and the experiments were performed by triplicate.

After the exposition time of 3 and 12 days, the root and shoot biomass was weighted and the roots were equal divided in three fractions for the quantify of phenanthrene sorbed at radical surface by photoacoustic spectroscopy analysis and root microscopy.

In situ photoacustic detection and quantify of phenanthrene immobilized at the radical system

10 mg root samples were selected from all of the experiments, dried at 40°C for two hours, placed in the photoacoustic (PA) cell and their optical absorption spectra were obtained in the range of 270 to 535 nm with a homemade photoacoustic spectrometer.

The photoacoustic spectra were recorded by varying the wavelength of the excited radiation and keeping the modulation frequency constant. This is called wavelength scanning and it is expected to obtain characteristic peaks (bands) due to the presence of phenanthrene detected along the root samples, therefore, it is possible to measure the quantity of the compound by means of photoacoustic analysis, to detect distribution of phenanthrene in the radical samples at various concentrations. The experimental setup consists of a 1000 W xenon lamp (Oriel), a variable frequency mechanical chopper set at 17 Hz, a monochromator, and an air-filled brass cell equipped with a condenser microphone. The PA signal from the brass cell provides the input to the signal channel lock-in amplifier (SR-850), which is interfaced to a personal computer that simultaneously displays the wavelength-dependent signal amplitude and phase. The data were normalized to the root dry weight and expressed as mg of phenanthrene per mg of root dry weight to quantify the phenanthrene detected at the root surface.

Microscopy analysis

In order to detect any perceivable changes in root tissues and the adhesion of phenanthrene to the radical surface, a microscopy analysis was done after the period of exposition to the contaminant; samples of the radical systems from 40, 80 and 120 mg/L experiments, were analyzed by optical and scanning electron microscope analysis.

For optical microscopy, 1 cm^2 hand root sections were taken at 10 mm below the root-shoot junction from fourth plants, these were previously rinsed with sterile water and finally mounted using non-colored glycerine gelatine. The root sections were observed and photographed using a Nikon Labophot-2 Microscope employing the DCI technique (Differential Contrast of Interferences); the areas of the root sections and the external phenanthrene adhered to the root surfaces were measured and quantified using the Motic Images 2000, Ver.1.3 Program.

For scanning electron microscopy (SEM), other root samples were dried at 40 ºC for two hours, gold coated with sputtering and observed in a JEOL 554-6300 Scanning Electron Microscope at an accelerated Voltage of 10 kV.

Statistical analyses

Results are the means from three replicates of the experiments and the significance of differences between mean values of root dry and fresh weight and phenanthrene photoacoustic technique was determinate by a two-way Analysis of Variance (ANOVA) and comparison among means was performed using a Tukey´s Test. These analyses were done applying the FAUANL ver. 1.4 software for Experimental Data Statistics (Olivares, 1989) and the GraphPad Instat Ver. 2.03 Statis Software for experimental designs (Aceves, 1993).

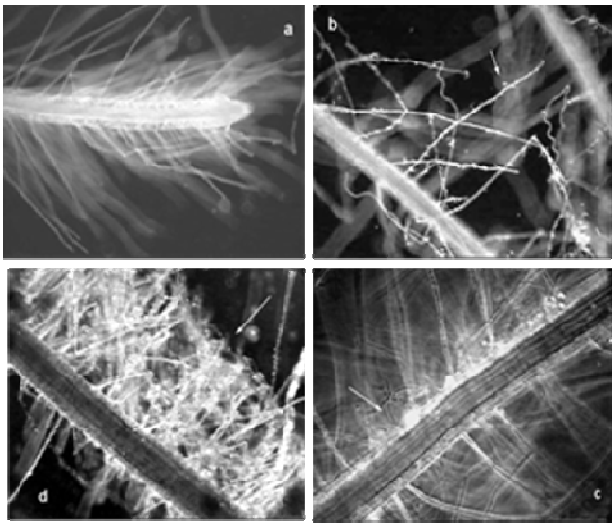

Figure 1. Microscopy analysis of root fragments of *Cyperus hermaphroditus* by Differential Contrast Interferences Technique: a) without phenanthrene, b) 40 mg/L, c) 80 mg/L and d) 120 mgl experiments (200x). The arrows indicate the phenanthrene adhered at root hairs and epidermis.

RESULTS

In order to evidence and relate the sorption of phenanthrene, *C. hermaphroditus*, roots were directly analyzed after their exposition to phenanthrene employing photoacoustic spectroscopy and optical and scanning electron microscopy.

Microscopy analysis of the roots was performed in order to demonstrate the phenanthrene sorption. Figure 1 (a, b, c and d) shows the image of a *C. hermaphroditus* radical fragments exposed to phenanthrene; the contaminant (white mass) is clearly observed adhered to the radical surfaces as the experimental concentrations increase and the percentage of phenanthrene adhered at the root surface was 6%, 14% and 40% for 40, 80 and 120 mg/L phenanthrene concentrations, respectively, at 12 days of exposition.

The Figure 2 shows the SEM image of a radical fragment exposed during 12 days to the highest phenanthrene concentration (120 mg/L), where is clearly observed that the root epidermis is covered with phenanthrene adsorbed at the radical surface.

This work considerate the phenanthrene detection by PAS technique, applied in a previous assay with this

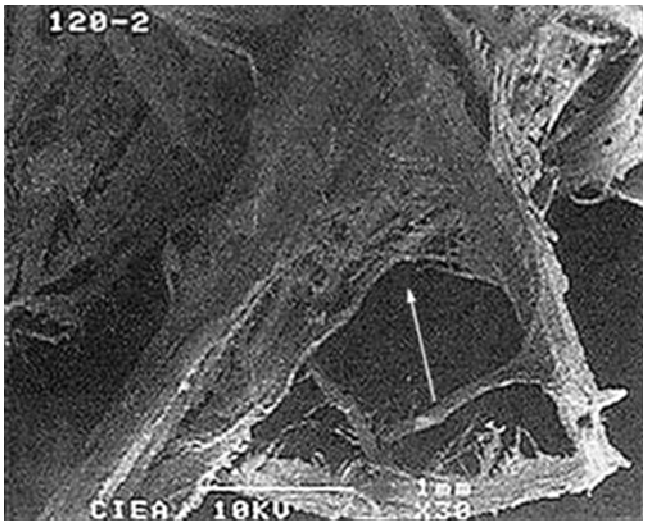

Figure 2. Scanning electron micrograph (30x) showing root epidermis of *C. hermaphroditus* cover with phenanthrene from a plant exposed during 12 days to 120 mg/L.

species (Cruz et al., 2004) by the analysis of the optical absorption spectra obtained from the radical systems of plants exposed to different phenanthrene concentrations for 3 and 12 days. By using PAS technique, the characteristic optical absorption peaks of phenanthrene were obtained. These peaks, at 295, 343 and 378 nm, agree with those reported in the literature (Cruz et al., 2004) and are related with those obtained for solid phenanthrene. Figure 3 (b, c and d), shows the quantity of phenanthrene present (adsorbed) in the roots, for each one of the tested phenanthrene concentrations. The phenanthrene quantified at the root surface by this spectroscopy analysis, showed that the method detected the highest quantity at the 80 mg/L experimental concentration, for 3 and 12 days (Figure 4). It is important to note here that this quantity of phenanthrene corresponds to the compound strongly adhered at the root surface, these analysis were done considerate only the roots that were dried after the exposition to the contaminant, so there was the solely phenanthrene that established the interaction with the root epidermis components.

DISCUSSION

Organic chemicals may be adsorbed to the roots and then be taken up, transported, metabolized, or transpired by plants; the first step is the sorption of chemicals to roots (Dietz and Schnoor, 2001). The increment of phenanthrene adsorption in 12 days could be attributed to the partitioning of the chemical between the lipophilic root solids and the aqueous phases of the roots (Briggs et al., 1982; Bell 1992; Chang and Corapcioglu, 1998). The sorption behaviour of exposition was similar to the results obtained by Zhu and Zhang (2008) with ryegrass (*Lolium multiflorum* Lam) to phenanthrene and pyrene; showed a linear increase against uptake time, where the amount of phenanthrene accumulated in ryegrass root based on the total mass of roots; showed that the amount of phenanthrene in roots increased rapidly.

The photoacoustic spectroscopic method allowed the detection of phenanthrene at the root surface of the samples, and thus this method could be used to monitor other biochemical or biophysical processes of plants *in situ.*

The photoacoustic signal corresponds mainly to the phenanthrene strongly adhered at the root surface; these analysis were done by considerate only the roots that were dried after the exposition to the contaminant, so there was the solely phenanthrene that established the interaction with the root epidermis components. Plant roots have two physiological sheaths: the endodermis and exodermis that play important roles in basic root function and protection against stresses like drought, pathogens, organic contaminants and heavy metals (Enstone et al., 2003).

As the amplitude of the photoacoustic signal is a function of the optical absorption coefficient, which depends on the concentration of the absorbing compound (Schmid et al., 2002), the influence of absorption by substances other than phenathrene detected in the root epidermis becomes evident by strong increase of the photoacoustic signal, detecting peaks at 375 and 400nm in roots without phenanthrene (Figure 3a); it suggests that were present root pigments that can absorb in these wavelengths, that may have very similar spectroscopic behaviour as PAHs (Kobayashi et al., 2008). Most carotenoids absorb maximally at these wavelengths (Rodríguez and Kimura, 2004). The results clearly demonstrate the strong influence of root structure and its optical properties on the photoacoustic signal.

This sorptive event on the root epidermis of *C. hermaphroditus* obey to the interaction that was present between the contaminant and the radical surface. Where phenanthrene possibly has no active H atoms to form H bonds and therefore can interact only nonspecifically with the sorbent, suggesting that partitioning is the main sorption mechanism for this contaminant, and the hydrophobic domains of the radical surface are the major sorptive sites (Chefetz, 2003). This also could be related to the Van der Waals or covalent bond that keeps strongly PAH's at the root surfaces (Fismes et al., 2002).

Presumably more uptake takes place over time, and the compound can also move further radially and/or longitudinally in the root with increasing time. Like the work of Wild et al. (2005), with anthrancene and phenanthrene in root divisional zones of maize and wheat; the phenanthrene in *C. hermaphroditus* roots was observed at the outer surface of the epidermal cells along the length of the root, mainly at the root hairs. The phenanthrene's

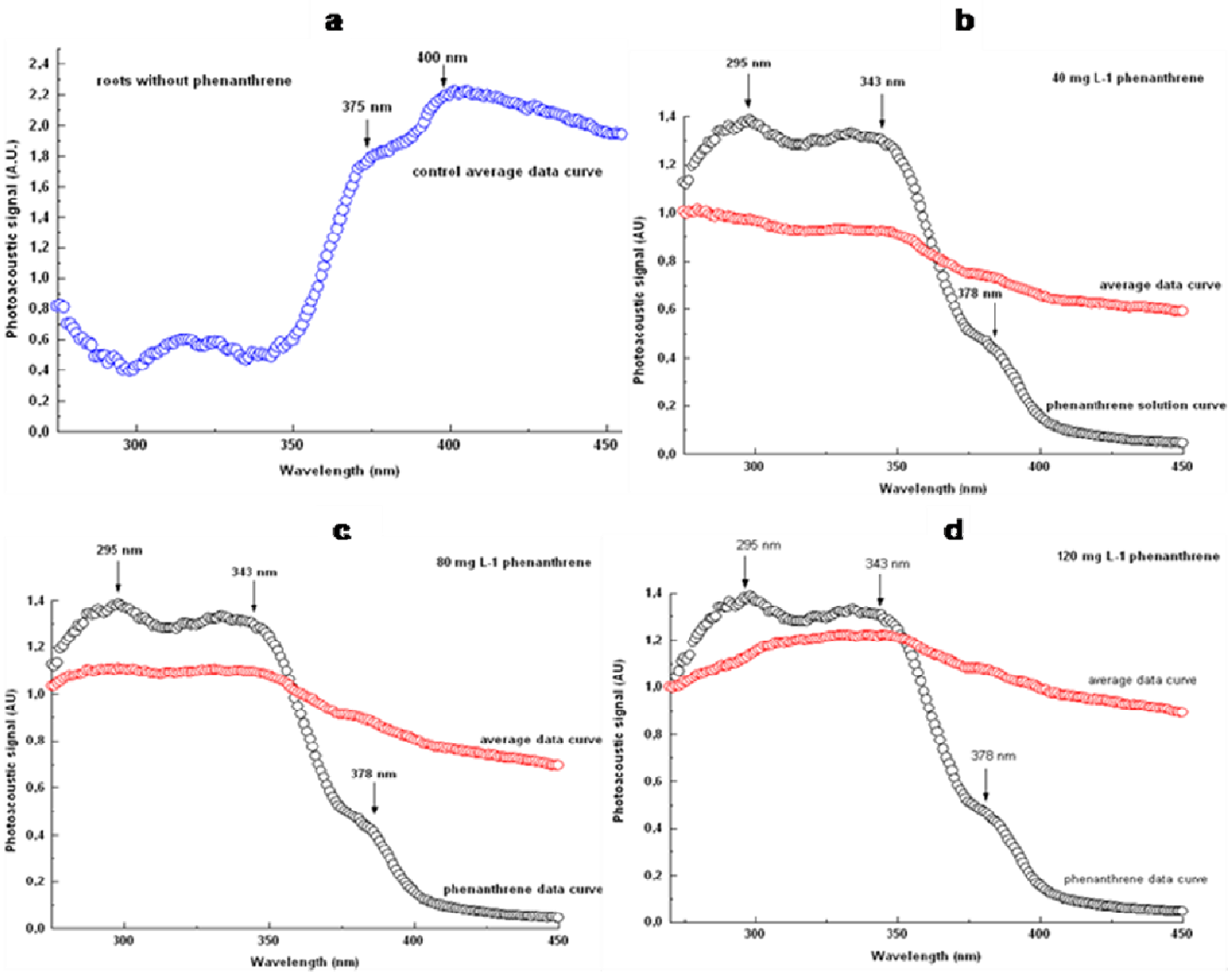

Fig. 3. Photoacoustic optical absorption spectra of *Cyperus hermaphroditus* roots exposed to phenathrene during 12 days (n = 4): a) without phenanthrene, b) 40 mg/L, c) 80 mg/L and d) 120 mg/L.

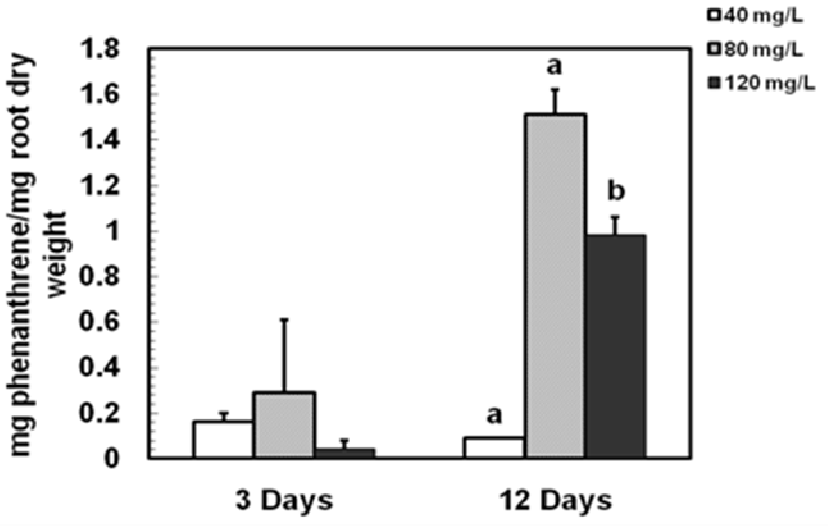

Fig. 4. Phenanthrene quantified at root surface of *C. hermaphroditus* by PAS, for the three experiments. Mean values of four replicates and lower-case letters represent significant differences between experiments ($p < 0.05$, Tukey's test). Bars indicate standard deviation.

movement behavior in this species, was located in the root epidermis, this structure constitutes a barrier to diffusion and slows the radial movement of phenanthrene into the roots, compared to that of water. Thus, having the screening occur in the exodermis near the root surface prevents the concentration of potentially toxic materials in the cortex.

It is possible that the detected and quantified phenanthrene in C. *hermaphroditus* roots was due to its strong adsorption on the root epidermis; in fact, this can occur because roots could be made of suberin, a polymer which is able to adsorb strongly PAHs (Kolattuduky, 1980; Briggs et al., 1982; Schwab et al., 1998; Fismes et al., 2002).

It is important to note here that during the performance of experiments, macroscopically acute effects such as expanded apex of the roots in the presence of phenanthrene were observed; although the general aspect of the plants were healthy and there were no yellowing and lost of leaf turgescence as in Flocco's experiments with alfalfa plants (Flocco et al., 2002). Very few studies have been undertaken to evaluate the toxic effects of PAHs on plants, especially terrestrial dicotyledons (Ren et al., 1996) and findings from limited studies show that different plant species had different responses to PAH contamination. Qiu et al. (1997) pointed out that no significant detrimental effects were caused by the presence of PAHs on seed germination and root radicle elongation of five prairie grasses even at high concentrations of PAHs (Ke et al., 2003).

In summary, the adsorption of phenanthrene in the roots of *C. hermaproditus* was increased with the time of exposition to the contaminant; this fact was due to the greater total root mass with a more surface area as well as the enhanced affinity of phenanthrene to the roots. This observation was confirmed from the evidence obtained by the photoacoustic spectroscopy and optical and scanning electron microscopy.

The photoacoustic spectroscopic method allowed the detection of phenanthrene at the root surface of the samples, and thus this method could be used to monitor other biochemical or biophysical processes of plants *in situ.*

Although the magnitude of the phenanthrene detected by the photoacoustic technique, it allows the demonstration, that phenanthrene was adhered or adsorbed to the radical surface of the exposed plants.

The radical system of *C. hermaphroditus* may thus provide a surface for phenanthrene adsorption, consequently the retention of contaminants on the surface of plant roots is an important control in stabilizing otherwise immobile organic compounds.

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