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Phytoremediation of polycyclic aromatic hydrocarbons (PAH) by cv. Crioula: A Brazilian alfalfa cultivar

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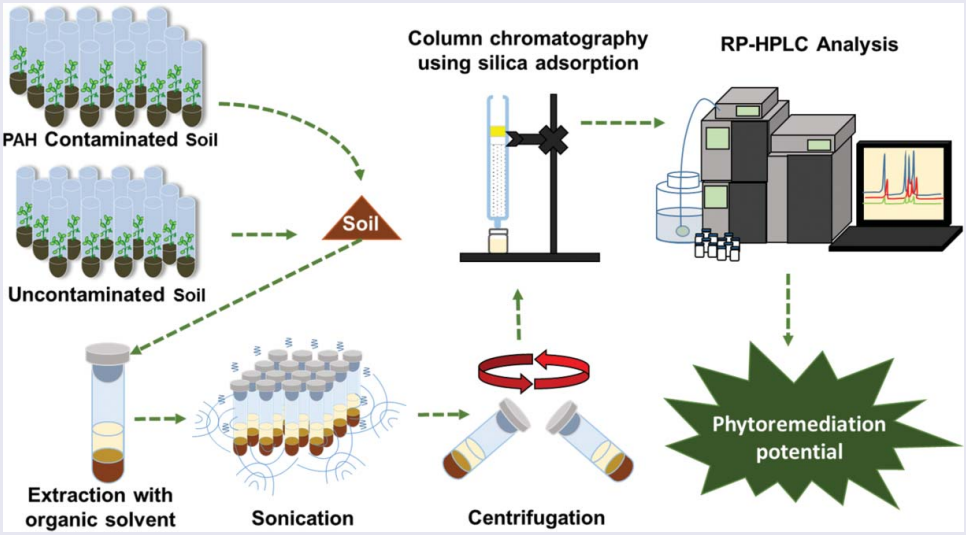
ABSTRACT

This work aimed to evaluate the phytoremediation capacity of the alfalfa cultivar Crioula in soils contaminated with polycyclic aromatic hydrocarbons (PAHs), primary pollutants with mutagenic and carcinogenic potential. Alfalfa was grown from seed for 40 days on soil amended with anthracene, pyrene, and phenanthrene. Soil and plant tissue was collected for biometric assay, dry mass analysis, and PAH analysis by liquid chromatography. Increased total PAH concentration was associated with decreases in plant biomass, height, and internode length. The Crioula cultivar had a satisfactory phytoremediation effect, reducing total PAH concentration (300 ppm) in the experimental soil by 85% in 20 days, and by more than 95% in 40 days. The PAH showed a tendency to be removed in the temporal order: phenanthrene before pyrene before anthracene, and the removal ratio was influenced by the initial soil concentration of each PAH.

GRAPHICAL ABSTRACT

Extracts containing PAH were obtained from soils cultivated with Medicago sativa L. (cv. Crioula) in the presence and absence of 150, 300, and 450 ppm total of PAH (phenanthrene, anthracene, and pyrene). After ultrasonication steps and purification of PAH by silica adsorption chromatography, samples containing PAH were quantified by high-performance liquid chromatography in reverse phase. At the end of this study, it was determined that the cv. Crioula showed a satisfactory phytoremediation potential. It presented the reduction of the total of PAH at around 85% in 20 days.



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Medicago sativa L.; phytoremediation; polycyclic aromatic hydrocarbons

1. Introduction

Since the Industrial Revolution, and with the development of metropolitan areas, natural resources have been exploited without regard for environmental impacts; the increasing emission of industrial wastes and other pollutants resulting from uncontrolled development poses serious issues for the health of our planet. Accidents involving direct spillage of oil derivatives on the ground and in bodies of water have resulted in the

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accumulation of compounds such as polycyclic aromatic hydrocarbons (PAHs), which are toxic to humans and to the environment (Lemieux *et al.* 2015; Martins *et al.* 2011). Once absorbed by animal cells, PAHs from oil derivatives can bind to DNA, forming DNA adducts (Meire *et al.* 2007) and eventually inducing tumors in many human tissues (Chen *et al.* 2013). PAHs accumulate in the soil, and exposing human skin to soils with 50–100 mg kg^{-1} of PAHs or 0.5–5 mg kg^{-1} of benzo(a) pyrene is considered a risk factor for skin cancer (Harvey *et al.* 2002). Countries where economic expansion has been most pronounced, such as China and Brazil, are particularly vulnerable to PAH contamination. In Brazil, the greatest PAH levels are found in large urban and industrial centers such as São Paulo and Rio de Janeiro, which are among the 300 most polluted cities in the world (World Health Organization – WHO 2012). Several techniques aimed at removing pollutants from the environment have been developed to reverse this scenario. Phytoremediation stands out as a green technology because of its low cost, efficiency, and ease of implementation, and it may help reverse this scenario (Cunningham and Ow 1996; Pilon-Smits 2005; Landmeyer 2012).

Because it uses the natural capacity of plants and microorganisms to degrade, sequester, or immobilize pollutants, phytoremediation is a cost-effective approach for PAH removal from a variety of environmental matrices (Pilon-Smits 2005). *Medicago sativa* L. (alfalfa) and similar plants are under study as potential phytoremediation agents, *M. sativa* L. has shown great potential for phytoremediation of environments contaminated with organic pollutants (Kirk *et al.* 2005; Fan *et al.* 2008; Wang *et al.* 2012). This plant grows on soils highly contaminated (30–500 ppm) with PAH, and it is capable of remediating, within a few weeks (60 days on average), up to 80% of the PAH contaminants, including pyrene, anthracene, phenol (Flocco *et al.* 2002), and benzene (Ferro *et al.* 1997). These types of basic studies are expected to help turn phytoremediation into an efficient and economically competitive technology in the future, offering remediation solutions for environments contaminated with recalcitrant organic compounds (Harvey *et al.* 2002).

In keeping with the search for novel and improved phytoremediation technologies, and with the need to elucidate the physiological and biochemical phenomena underlying phytoremediation, this work aimed to evaluate the phytoremediation capacity of the *M. sativa* L. cultivar Crioula (EMBRAPA) in soils artificially contaminated with phenanthrene, anthracene, and pyrene. We chose this cultivar because it is adapted to the Brazilian climate, being widely cultivated in the South and Southeast regions of Brazil at high productivity levels (Rassini *et al.* 2008), and thus has the potential to decrease the time necessary for phytoremediation, minimizing its impact on local activities.

2. Materials and methods

2.1. PAHs used in this study

Phenanthrene, pyrene, and anthracene at a concentration of 98% were obtained from Sigma-Aldrich (St. Louis, MO, USA). We chose these PAHs because of the risk they pose to the environment, show similarities with other molecule architectures known to have a mutagenic and carcinogenic effects, such as benzo [a] pyrene and benzo [a] anthracene, and they are considered priority pollutants by the United State Environmental Protection Agency (USEPA - United State Environmental Protection Agency 2000).

2.2. Soil contamination

Experiments were performed as previously described (Brinch *et al.* 2002), with specific modifications: 430 g of soil (pH 5.65, humidity of 51%, 64 mg/kg of N, 128 mg/kg of P_2O_5, and 153,6 mg/kg of K_2O) was sifted through a 2-mm sieve and mixed with 430 g of organic fertilizer and 860 g of vermiculite (previously sifted through 2-mm sieves). This substrate was dried for 24 h at 30°C and divided into four portions of 430 g in 1-L autoclaved Erlenmeyer flasks (autoclaved for 40 min, 1 atm; 121°C). Fractions of each of the four portions (107.5 g, 25%) were transferred to four sterilized 500 mL beakers and amended with 50 mL acetone solutions containing 50, 100, or 150 ppm each of phenanthrene, anthracene, and pyrene (total PAH = 150, 300, and 450 ppm, respectively). Acetone was added to one of the 25% fractions as a control (0 ppm). The fractions and solvent were vigorously homogenized every 2 h using glass rods (previously sterilized for 8 h) and left in a laminar flow cabinet for 8 h until complete evaporation of the organic solvent. Each of the 107.5-g soil fractions containing the contaminants was added to a 75% soil fraction (the remaining 322.5 g, solvent-free) and homogenized by agitation inside the Erlenmeyer flask. Four 430-g substrate portions were thus obtained with the desired PAH concentrations (control, 150, 300, and 450 ppm). The substrates were stored until used for growing alfalfa seedlings.

2.3. Cultivation of cv. Crioula

Germination of alfalfa seeds (cv. Crioula, obtained from EMBRAPA) was induced after surface disinfection using the Silo-Suh *et al.* (2002) methodology. After the seeds started to germinate, seedlings were grown for the biometric, dry biomass, and phytoremediation assays in an aseptic environment. Cultivation was carried out in a biochemical oxygen demand system with a 14/10 h (light/dark) photoperiod at 28°C (LUCADEMA® model B.O.D LUCA-161/02). Each plant was watered with 1.5 mL Hoagland solution (Hoagland and Arnon 1950) every 3 days until the end of the experiment (day 40).

2.4. Biometric and dry biomass assays

Biometric and dry biomass data from cv. Crioula were obtained as previously described by Wang *et al.* (2012). Soil (140 g) was weighed on an analytical balance and transferred to eight sterile, 500-mL screw-cap glass jars, with two jars used for each PAH treatment (control, 150, 300, and 450 ppm of total PAH). For the biometric assays, 10 Crioula seedlings were grown in each jar. Ten plants from each treatment were collected 20 and 40 days after seeding (DAS), and their aerial parts and roots were isolated for the biometric and dry biomass assays.

2.5. Phytoremediation assays

Substrate samples (10 g) were weighed and transferred to sterile test tubes (22 × 200 mm) with cotton caps (nine tubes for each of the control, 150, 300, and 450 ppm total PAH treatments), and one cv. Crioula seedling was added to six tubes of each treatment ("cultivated soil samples"), while the other tubes remained without a seedling ("uncultivated soil samples"). At 0, 20 and 40 DAS, cultivated and uncultivated soil samples (10 g) from each treatment were collected in biological triplicates for the PAH extraction rounds.

2.5.1. Extraction of PAHs from soil samples

The procedure was optimized from Wang *et al.* (2012), Gao and Zhu (2004), Banjoo and Nelson (2005), Souza (2008), Helaleh *et al.* (2005), Sun *et al.* (2008), and Lau *et al.* (2010). Cultivated and uncultivated soil samples (10 g) collected at 0, 20 and 40 DAS were dried with anhydrous sodium sulfate (10 g) at 30°C for 16 h, transferred to screw-cap test tubes, and mixed with a 1:1 (v/v) dichloromethane and n-hexane solution (10 mL), followed by sonication for 30 min (Bransonic® Ultrasonic Cleaner, model 2510R-DHT, puissance HF: 100 W; 42 KHZ ± 6%). The extracts were collected in new test tubes and the extraction/extract collection process was repeated 2 more times, after which the collected extracts were centrifuged for 10 min at 2000 rpm to remove impurities and particulate matter from the solution. 10 mL of the supernatant was collected in 15 mL penicillin flasks and the solvent was allowed to evaporate in a hood, this step was repeated 3 times to complete evaporation of the total extract from each sample (30 mL by extraction). Samples were stored at −20°C for subsequent chromatography using silica adsorption.

2.5.2. Column chromatography using silica adsorption (Clean-up)

The column was modified from the "miniaturized chromatographic column" used by Banjoo and Nelson (2005), with the column built in n-hexane using a 5 mL-graduated pipette as column and containing a primary glass wool layer followed by 2 g of 70–230 mesh silica gel, activated for 2 h at 120 °C. A 1-cm anhydrous sodium sulfate layer was added on top, and packing was allowed to occur by gravity. The dried PAH extracts, solubilized in 1 mL of n-hexane, were applied to the top of the column, and the concentrated analytes were eluted with 14 mL of the n-hexane-dichloromethane (8:2) mobile phase into 15-mL penicillin flasks. After solvent evaporation in a hood, samples were stored at −20°C for subsequent analysis and quantification of PAH by reversed phase high performance liquid chromatography (RP-HPLC).

2.5.3. Qualitative analysis of the phytoremediation potential of cv. Crioula

The phytoremediation potential of alfalfa was evaluated by the presence of purified PAH crystals, formed at the bottom of the flasks after evaporation of the solvent. The crystal images were obtained from samples (three biological replicates) of cultivated and uncultivated soil for 40 DAS. By observing the amount of crystals deposited, it is possible determine the influence of the cultivation of an individual of *M. sativa* L. in 10 g contaminated soil, where less deposition of crystals in the bottom of the containers indicates that there was phytoremediation of PAH. These data were confirmed by analytical and quantitative methods in RP-HPLC-UV (Supplemental data S1).

2.5.4. Preparation of purified PAH samples for reversed phase high performance liquid chromatography (RP-HPLC-UV)

Biological triplicates from PAH samples already purified in the *Clean-up* stage were solubilized in 1 mL of a 60% acetonitrile/water mobile phase and immediately filtered through a poly (1,1,2,2-tetrafluoroethylene) (PTFE) membrane with 0.22-μm pores. The concentrated samples were diluted within the detection range of the standard curves, and analytical triplicates (with a 20-μL injection volume) were used to obtain the chromatographic profiles. PAH concentrations were determined by integrating the area under each PAH peak and comparing the values with those obtained from integration of the areas under the peaks in the analytical curves for each PAH standard (Souza 2008) (Supplemental data S2).

The analysis of PAH concentration in cultivated and uncultivated soil samples was optimized in an HPLC system (Prominence, Shimadzu, Kyoto, Japan) with a specific program for data acquisition and peak integration (Shimadzu LCsolution). PAHs were separated with a reversed phase column packed with octadecylsilane, Kromasil-C18 (AkzoNobel, Bohus, Sweden) using a Shimadzu LC-20AT bomb, an acetonitrile/water (6:4 v/v) mobile phase with a 1.4 mL min^{-1} flux velocity, and a column thermostatically controlled at 35°C with an oven (CTO-20A). Phenanthrene and anthracene were detected at 254 nm and pyrene was detected at 235 nm using a variable wavelength UV/visible detector (SPD-M20A, Shimadzu). Retention time (rt) was 10.331 min for phenanthrene, 11.299 min for anthracene, and 15.248 min for pyrene, determined from a 20-min chromatography run for a standard mixture containing 100 μg/mL of each of the PAHs (Souza 2008).

2.6. Statistical analyses

Statistical analyses were performed with Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA). For biometric and dry mass data, we used two-way ANOVA, and averages were compared with Tukey's multiple comparison test ($p < 0.05$) to evaluate plant response to PAH, or with the Holm–Sidak test ($p < 0.05$) to evaluate growth and mass increase in the interval of 20–40 DAS. Regarding PAH phytoremediation data, PAH removal calculations were performed according to Teng *et al.* (2011) using two-way ANOVA for the averages, followed by Tukey's test ($p < 0.05$).

3. Results

3.1. Development of cv. Crioula in PAH-contaminated soils

3.1.1. Phenotype of cv. Crioula grown at different PAH concentrations

Plants of the Crioula cultivar were able to develop and survive until the end of the experiment (DAS 40) at all PAH

concentrations tested (Holm–Sidak $p < 0.05$). Nevertheless, compared to the plants grown in control soil, we observed morphological changes such as reduced internode length and shorter leaves, which became more pronounced as PAH concentrations increased. In soil contaminated with 450 ppm total PAH, developmental differences in the aerial part were obvious from DAS 20 onward (Figure 1A), becoming exacerbated by DAS 40, with retarded growth, smaller leaves, and internode shortening observed (Figure 1B and C). Compared to controls, average height of the aerial parts was approximately 2 times shorter for plants grown in soil amended with 150 ppm total PAH and 2– 3 times shorter in soils contaminated with 300 and 450 ppm total PAH (Figure 2A). This tendency for reduction in height in the presence of contaminants was significant (Tukey $p < 0.05$) throughout the development of the plants, although there was no significant difference between the heights of the aerial parts of plants grown on amended soils at 20 and 40 DAS (Tukey $p < 0.05$). Shortening of the internode regions contributed to the observed growth profile (Figure 2A).

We detected significant differences in average root length of plants grown in amended soil at 40 DAS but not at 20 DAS. Root length of plants grown in contaminated soil at 40 DAS was approximately half as that of plants grown in the control soil, with no difference in root lengths between the 150, 300, and 450 ppm PAH treatments (Tukey $p < 0.05$). Therefore, root growth was impaired in contaminated soils between 20 and 40 DAS, compared to controls (Holm–Sidak $p < 0.05$). Other than the control plants, only those grown on soil with 150 ppm of total PAH showed significant root growth (Figure 2B).

3.1.2. Analysis of dry biomass data for cv. Crioula at different PAH concentrations

Plants grown on amended soil had significantly lower dry biomass of the aerial parts compared to controls at 40 DAS (Tukey $p < 0.05$) but not at 20 DAS. Dry biomass decreased as contaminant concentration increased, with only plants grown on soil with 150 ppm total PAH (and plants grown in control soil) showing a significant increase in biomass of the aerial parts between 20 and 40 DAS (Holm–Sidak $p < 0.05$, Figure 2C). Root biomass was 3, 4, and 5 times smaller than that of the control for plants grown on soil amended with 150, 300, and 450 ppm total PAH, respectively. Only plants grown on soil containing 300 ppm total PAH (and control plants) showed a significant increase in root biomass between 20 and 40 DAS

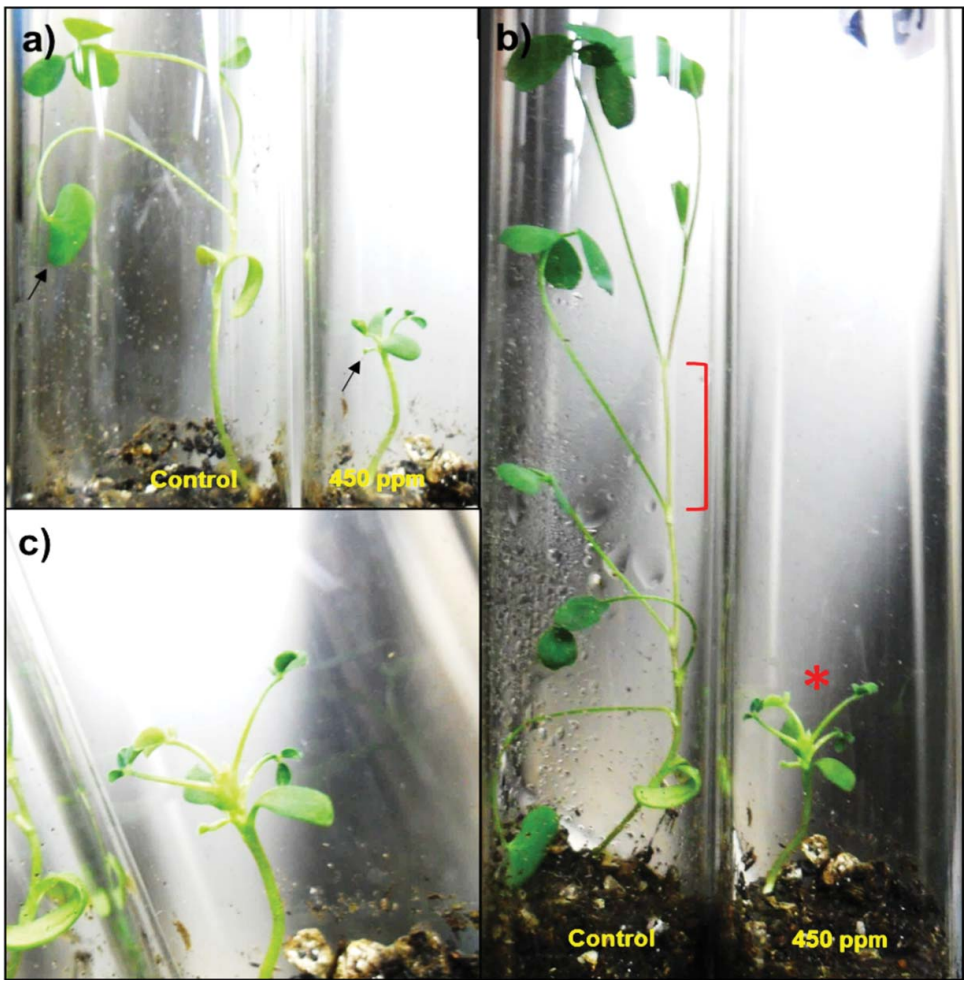

Figure 1. Differences in the development of plants grown in the presence and absence of polycyclic aromatic hydrocarbons. (a) Cultivated plants for 20 DAS. Arrows indicate the size difference between the control initial unifoliolate leaf and 450 ppm initial unifoliolate leaf. (b) Cultivated plants for 40 DAS. In red is shown the size difference between cultivated plants in contaminated soil and control (*). An internode region is represented by the red key in the control plant. (c) Highlight for cultivated plants in the concentration of 450 ppm PAH, with tiny leaves and low development of internodes.

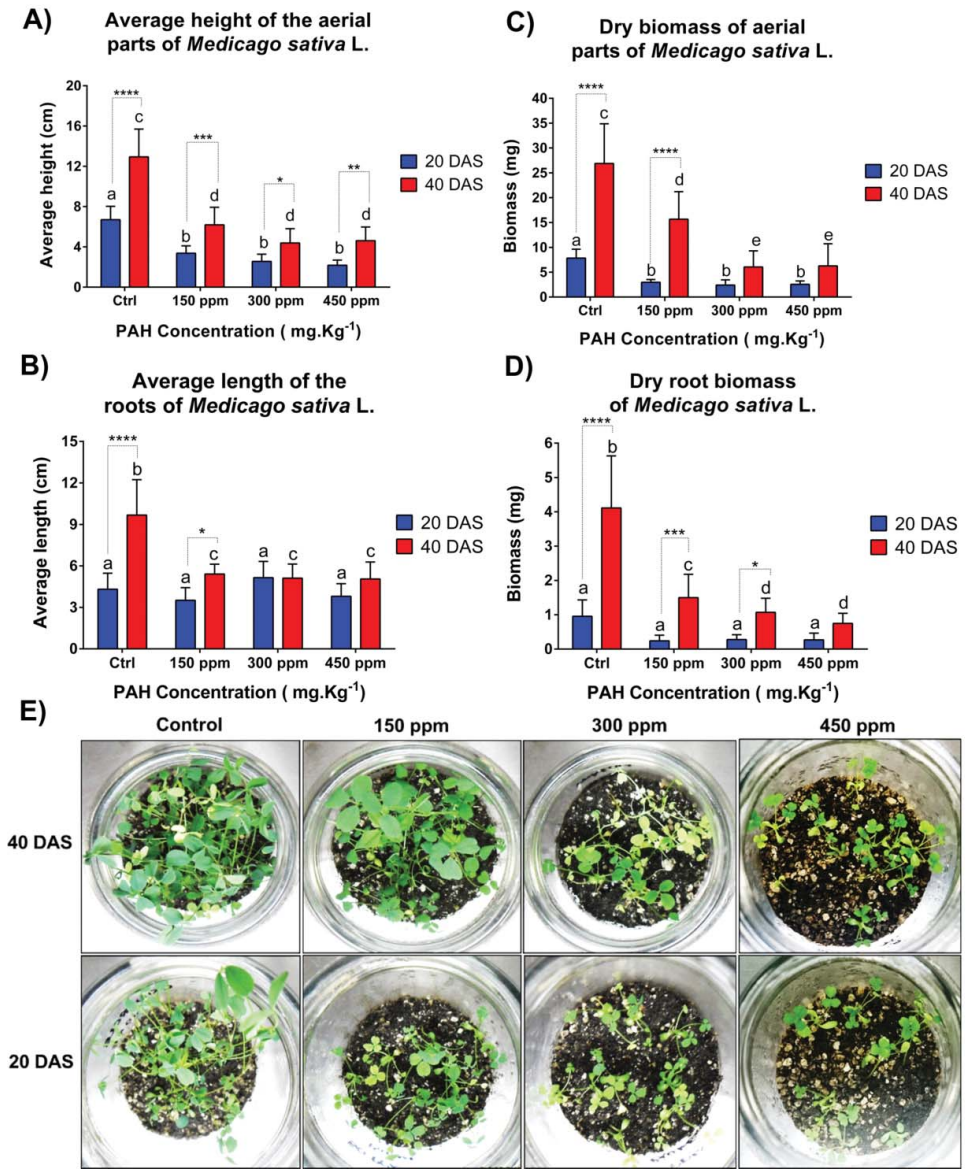

Figure 2. Biometric data from shoots and roots of *Medicago sativa* L. The graphs show a reduction of the average length (A and B) and dry biomass (C and D) of the aerial parts and roots of plants grown in the presence of PAH (phenanthrene, anthracene and pyrene) for 20 and 40 days. Each column represents the mean ± standard deviation, and 10 plants, different letters indicate significant differences between the conditions for two-way ANOVA, followed by Tukey's multiple comparisons test ($p < 0.05$). Evaluation of the increase of the biomass and growth of plants was obtained by two-way ANOVA followed by the Holm-Sidak ($p < 0,05$), where stars indicate significant difference for the same conditions between times 20 and 40 DAS (Confidence intervals: **** = 99.9%/= 99% ***/** = 95%, and * = 90%). In E, the appearance of *Medicago sativa* L. with various concentrations of total PAH after 20 and 40 DAS is observed.

(Holm–Sidak $p < 0.05$, Figure 2D). Figure 2E shows the growth profile of cv. Crioula for different PAH concentrations throughout the 40-day experiment.

3.2. Phytoremediation potential of cv. Crioula

PAHs extracted from both cultivated and uncultivated soils were purified by silica adsorption chromatography, after which the eluates were collected and concentrated by complete evaporation of the solvent, yielding purified crystals at the bottom of the flasks. For all PAH concentrations tested, cultivated soils in which cv. Crioula had been grown for 40 days yielded a smaller quantity of crystals (Figure 3A) than the uncultivated soils (Figure 3B). The observed reduction in crystal quantity per flask resulted from growing a single cv. Crioula plant in the amended soil. Thus, the presence of cv. Crioula in the contaminated soil clearly contributed to the 85% reduction in soil PAH concentration at 20 DAS, which yielded a PAH level significantly lower than its value at the beginning of the experiment and that of uncultivated soil (Tukey $p < 0.05$, Figure 4A–D). The presence of cv. Crioula plants in contaminated soil was correlated with a reduction in the concentrations of the studied PAH as early as 20 DAS. For phenanthrene, the decrease was 98% at all levels of contamination, which was significantly different from the removal level in the absence of plants. At 40 DAS, however, phenanthrene removal, even in the absence of cv. Crioula plants, exceeded 98% for all tested concentrations except the highest (150 ppm phenanthrene), at which level the presence of cv. Crioula plants resulted in significantly higher removal compared to the control (Tukey $p < 0.05$, Figure 5A).

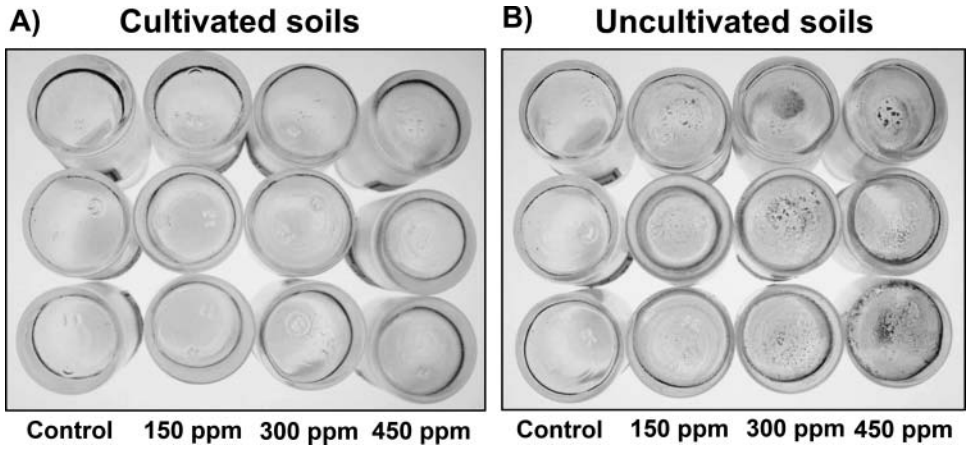

Figure 3. Crystals of polycyclic aromatic hydrocarbons (PAH) extracted and purified from cultivated and uncultivated soils with *Medicago sativa* L. for 40 days. Each vial contains PAH extracted by ultrasonication and purified by adsorption chromatography on silica, from 10 g of uncultivated (A) or uncultivated soil B) for 40 days. The experiment was performed in biological triplicates. (A) PAH crystals extracted and purified from soil cultivated with Medicago sativa L. (B) crystals PAH extracted and purified from uncultivated soils for 40 DAS. Detail for noticeable decrease of PAH crystals in the presence of alfalfa plants in comparison with uncultivated soil.

Anthracene removal was significantly greater from cultivated soil than that from uncultivated soil (Tukey $p < 0.05$), with a removal ratio for the 50 and 100 ppm PAH treatments of 60–80% at 20 DAS and reaching 90–100% at 40 DAS. For soil contaminated with 150 ppm anthracene, the removal ratio was 50% at both 20 DAS and 40 DAS (Tukey $p < 0.05$, Figure 5B). Finally, soil in which cv. Crioula was grown also had significantly reduced pyrene for all tested concentrations (Tukey $p < 0.05$), with removal ratios varying between 60% and 85% at 20 DAS and reaching an average of 95% at 40 DAS (Tukey $p < 0.05$, Figure 5C).

4. Discussion

This study evaluated the ability of cv. Crioula to tolerate and to phytoremediate the PAH phenanthrene, anthracene, and pyrene. We monitored morphological changes in plants grown with different concentrations of these contaminants for 40 days using biometric and dry biomass assays, and we determined the *in vitro* phytoremediation potential of this cultivar. Our results showed that cv. Crioula plants grown in a PAH-amended soil suffered a reduction in size and biomass proportional to the increase in contaminant concentration. Baek *et al.* (2004), describing the effects of crude oil on growth of bean (*Phaseolus nipponensis* Ohwi) and corn (*Zea mays* L.), reported reduction of plant size in soils contaminated with organic pollutants. Phytotoxicity in these species increased with the number of aromatic rings, and *Z. mays* showed more sensitivity to soil contaminated with PAH than did *P. nipponesis*. Ekundayo *et al.* (2001) also reported a reduction in *Z. mays* plant growth in polluted soils (oil spillage), using stalk height and width as well as leaf area index as criteria. Xu and Johnson (1995), Hester and Mendelssohn (2000), and Pezeshki *et al.* (2000) proposed an explanation for the reduction in size of plants grown on soils contaminated with organic compounds, reporting that organic pollutants such as oil and oil-derivatives can form an impermeable layer over the surface of the roots, hindering their uptake of water and mineral salts from the soil. As a result, plant height and leaf area become smaller and the number of stomata increases. As lipophilic compounds, the PAHs studied in our work could potentially adhere to roots of *M. sativa*, compromising water intake and therefore causing a reduction in size. Plants in the 150, 300, and 450 ppm total PAH treatments were much smaller than the controls, with a proportional reduction in the length of the internode region. This reduction in internode length is drastic, generating "dwarf plants" that can be easily distinguished from plants grown in non-contaminated soils, suggesting that alfalfa cv. Crioula is a good candidate as a phytoindicator of soil pollution with PAH.

Even though PAH soil contamination reduced the average height and length of the aerial and root parts of the plant, as well as the dry biomass of those plant parts, cv. Crioula plants were capable of surviving to the end of the experiment (40 days), indicating high tolerance of this cultivar to high PAH soil concentrations. At 150 ppm total PAH, cv. Crioula plants had significant growth of root and aerial parts, as well as significant biomass increase between 20 and 40 DAS with few visual signs of stress, indicating that cv. Crioula is capable of developing in soils under these conditions. According to the Environmental Protection Agency of the state of São Paulo, Brazil (CETESB), the PAH concentrations that pose a risk to human health and that require the implementation of remediation and monitoring techniques are 15 ppm for agricultural land, 40 ppm for residential soil, and 90 ppm for industrial soil (Companhia Ambiental do Estado de São Paulo - CETESB 2014). Thus, our results suggest that the alfalfa cultivar Crioula is viable as a phytoremediation tool for agricultural, residential, and industrial soils in Brazil. Furthermore, cv. Crioula plants were efficient phytoremediators at all PAH contamination levels tested. Our qualitative analysis revealed a significant reduction in the quantity of PAH extracted and purified from contaminated soil in which cv. Crioula had been grown for 40 DAS, demonstrating that a single cv. Crioula plant grown in 10 g of amended soil was capable of removing the majority of contaminants during the experiment. Quantitative analysis of the purified PAH extracts determined that, on average, the plant was capable of remediating more than 85% of the PAH in 20 days, particularly at lower concentrations (150 ppm total PAH). Removal efficiency was highest for phenanthrene (98% in just 20 days). However, not all of the removal of

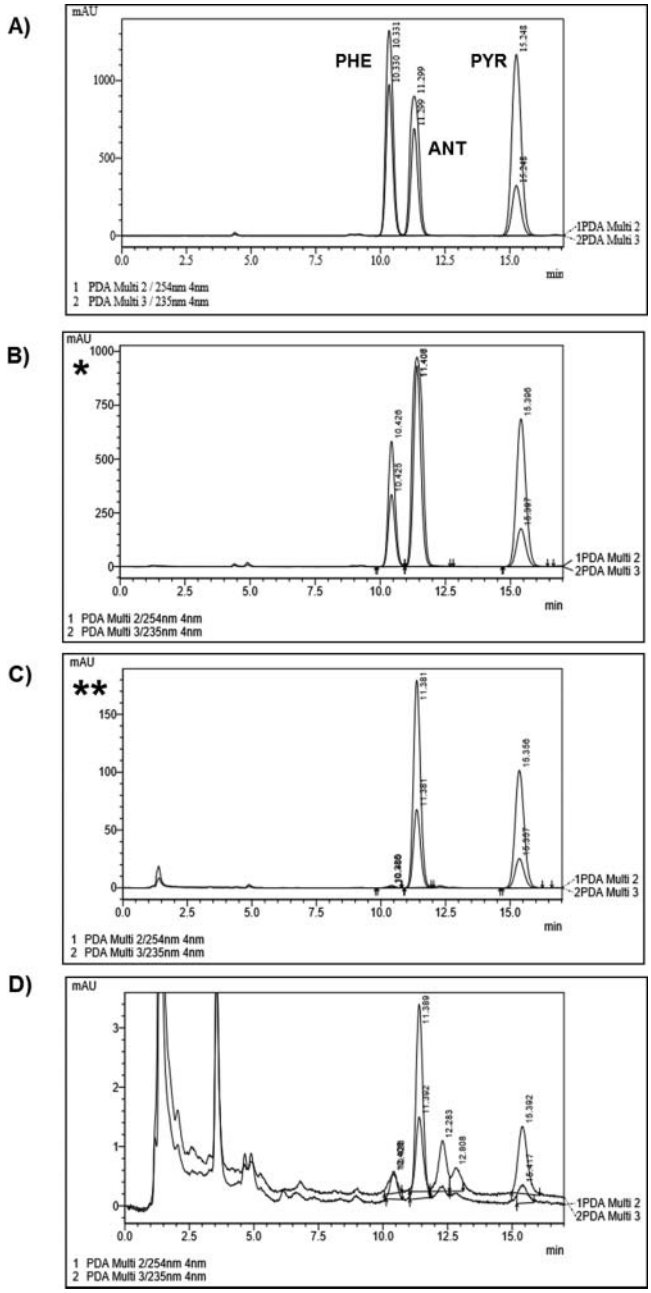

Figure 4. Chromatographic profile of polycyclic aromatic hydrocarbons (PAH) samples eluted by reversed phase high performance liquid chromatography (RP-HPLC). The retention times obtained for the phenanthrene (PHE), anthracene (ANT), and pyrene (PYR) is 10 min, 11 min and 15 min respectively. (A) Chromatogram of the standard mixture of PAH in 60% acetonitrile. (B) Chromatogram of soil samples contaminated with 300 ppm of total PAH not cultivated for 20 days. (C) Chromatogram of soil samples contaminated with 300 ppm of total PAH cultivated with *Medicago sativa* L. for 20 days. (D) Chromatogram of samples of control soil. (*) Reduction of 85% concentration of contaminants in soil (difference in scale in mAU). (**) Remarkable decrease of phenanthrene peak.

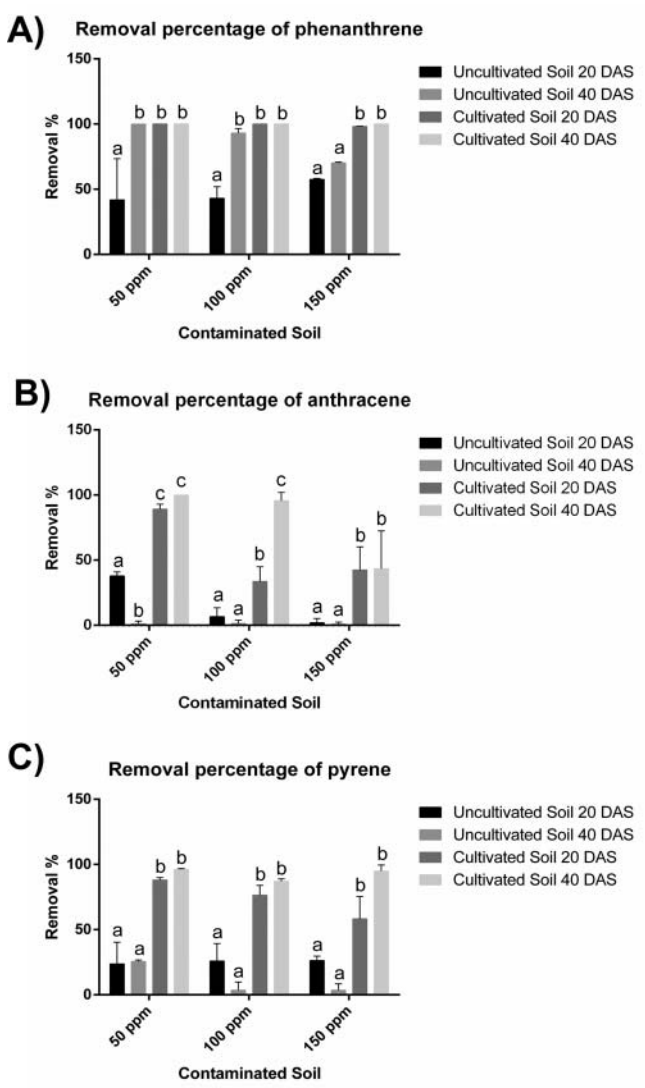

Figure 5. Removal percentage of PAH after 20 and 40 days. Each column represents the mean and standard deviation of the percentage of removal of phenanthrene, (A) anthracene, (B) and pyrene (C) in three soil samples (biological triplicate). Different letters indicate significant difference between the series for each soil contaminated with 50, 100, and 150 ppm phenanthrene, anthracene, and pyrene. Statistical analysis was performed by two-way ANOVA ,followed by Tukey's multiple comparison ($p < 0.05$).

phenanthrene resulted from the cv. Crioula plants; phenanthrene removal reached 40–60% at 20 DAS even in uncultivated soils. This was partly due to the ease with which phenanthrene is volatilized and degraded in the environment, compared with higher molecular weight PAH (Wilcke *et al.* 2000). On the other hand, soils uncultivated for 20 days had low percentages of removal of pyrene and anthracene, whereas cultivation of cv. Crioula for the same period of time resulted in an 85% reduction in those PAH. These results support data from previous studies showing that there is more removal of organic pollutants from cultivated soils than from uncultivated soils (Merkl *et al.* 2005, Lee *et al.* 2008, Su and Zhu 2008, Gao *et al.* 2010, Ma *et al.* 2012). Our results with cv. Crioula also reinforce previous studies that indicated that *M. sativa* L. can be used for phytoremediation of PAH in contaminated soils, suggesting that these plants possess the biochemical machinery necessary for PAH phytoremediation (Kirk *et al.* 2005; Fan *et al.* 2008; Wang *et al.* 2012).

An interesting observation from our work concerns the dynamics of contaminant removal over time. PAH were removed in the following temporal order: phenanthrene > pyrene > anthracene. Phenanthrene was removed almost completely (98%) within a few days, whereas pyrene had over 95% removal by day 40. The removal ratio of anthracene was dependent on its initial soil concentration: it was approximately 90% both at 20 DAS for a concentration of 50 ppm and at 40

DAS for a concentration of 100 ppm, but it never exceeded 50% for soil contaminated with 150 ppm. This pattern may be explained by the presence of bay-regions and K-regions in the molecular structure of phenanthrene and pyrene, which would be absent in anthracene. These regions are more likely to suffer oxidation and possible degradation by enzymes present in *M. sativa* L., including peroxidases released from the roots (Muratova *et al.* 2015). This phenomenon can also be explained by observing the absorption and migration capacity of these PAH in root and aerial tissues of *M. sativa* L. cv. Crioula, through fluorecence microscopy techniques. A recent study by Alves *et al.* (2017) detected, for the first time, the presence of PAH in tissues of *M. sativa* L. cv. Crioula by fluorescence microscopy, and showed differences in the relative intensity of PAH fluorescence in roots and leaves of cv. Crioula, observing high anthracene signals still in the roots, high signs of pyrene in the leaves, and low phenanthrene signs in both tissues, in plants cultivated for 40 days in PAH-amended soils, suggesting that these PAHs have different mobilities in cv. Crioula created by water solubility, where transport via xylem would favor the more efficient absorption of phenanthrene (1.15 $mg.L^{-1}$), followed by pyrene (0.135 $mg.L^{-1}$) and finally anthracene ($4.34e^{-2}mg.L^{-1}$) (Alves *et al.* 2017).

5. Conclusions

M. sativa L. cv. Crioula suffered developmental changes when grown in soil with high levels of PAH contamination. In the 450 ppm total PAH treatment, plants exhibited drastic reductions in internode length, leaf size, and biomass. However, cv. Crioula showed great potential as a phytoremediation tool for agricultural, residential, and industrial Brazilian soils contaminated with pyrene, anthracene, and phenanthrene, growing even in the presence of high levels of these PAH and enhancing their removal. Our work also suggests that the tested PAHs were removed in the temporal order: phenanthrene before pyrene before anthracene, a sequence possibly influenced by the presence, in phenanthrene and in pyrene, of molecular regions more prone to oxidation as well as differences in the mobility of these PAHs within the plant, by differences in water solubility. Nevertheless, how these chemical mechanisms are linked to PAH phytoremediation is a question that requires further investigation. Defining PAH phytoremediation-related proteins in *M. sativa* L. via proteomic analyses may be the key to identifying these biochemical mechanisms.

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