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Impact of Phenanthrene on Organic Acids Secretion and Accumulation by Perennial Ryegrass, *Lolium perenne* L., Root

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Abstract A solution culture experiment was performed to investigate the impact of phenanthrene (PHE) on organic acids secretion and accumulation by *Lolium perenne* L. root. Data showed that, oxalic acid was the dominant composition of organic acids in root and root exudates. In root exudates, increased levels of PHE resulted in higher oxalic acid and its secrete proportion; oxalic acid arranged from 3.00 to 4.72 mg/g FW under spiked PHE treatments, in control, it was 2.33 mg/g FW. In root, oxalic acid rose to 25.61 mg/g FW at 1 mg/L PHE treatment, while the PHE concentration was continuously increasing, organic acids in root decreased.

Keywords Ryegrass · Organic acids · Phenanthrene · Root exudates

Polycyclic aromatic hydrocarbons (PAHs) are a series of persistent organic pollutants which are well known for their carcinogenic and mutagenic properties (Menzie et al. 1992) and they are widespread in the environment from several sources including artificial origins, such as incomplete combustion of fossil fuels, pyrolysis of organic materials, exhaust emission of vehicles and natural activities, such as volcano eruption, microbial metabolism, etc. (Thiele and Brümmer 2002). Over the last few decades, the researchers paid a lot of attention to the phytoremediation using different plants both in contaminated soil (Aprill and Sims 1990; Wilson and Jones 1993; Joner and Leyval 2003; Gao and Zhu 2004) and water (Gao et al. 2006). As the high lipophilic nature of PAHs, most of the researches proposed that plant uptake, metabolism and translocation are not the major end-result of the PAHs in contaminated soils (Wild and Jones 1992; Simonich and Hites 1995; Gao and Zhu 2004). However, the dissipation of soil organic pollutant is significantly enhanced by the presence of plants; this conclusion has also been proved by several reports (e.g. Reilley et al. 1996; Binet et al. 2000). This promotion can be explained from the factors below: (1) the root exudates have a large amount of easily degradable organic compounds, which can not only increase the quantity of soil microbe but also enhance the microbial degradation activity by means of co-metabolism (Rentz et al. 2005; Parrish et al. 2005); (2) apart from the nutrients, there are several enzymes in the root exudates, some of them, such as polyphenol oxidase and dehydrogenase can directly participate in the PAHs degradation (Liu et al. 2004); (3) plant root can change the physical chemical properties of soil, consequently improving bioavailability and dissipation of PAHs. The first two effects have already been intensively studied in recent years, while the third one is not so clear yet. Now, in short, the plant root exudates play an important role in PAHs phytoremediation.

Some recent study shows that organic acids affect desorption of organic pollutant in soil and enhance its bioavailability significantly (White 2002; Luo et al. 2006). As an important composition of root exudates, organic acids in root exudates have been researched extensively under the heavy metal stress (Huang et al. 1998; Xu et al. 2007), in contrast, they were poorly understood in PAHs contaminants. To explore organic acids in root exudates in response to PAHs contaminants, phenanthrene (PHE) as typical PAHs was selected. We chose *Lolium perenne* L. (perennial ryegrass) as our plant material because: (1) the

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amount of root exudates was quite considerable (Meharg and Killham 1995); (2) ryegrass was in common use of researches on PAHs phytoremediation. Our present study used a solution culture experiment to test: (1) the effect of spiked PHE solutions on root viability; (2) the changes of low molecular weight organic acids in root exudates under the PHE stress; (3) the changes of low molecular weight organic acids accumulation in plant.

Materials and Methods

Phenanthrene (>97%) was purchased from Aldrich Chemical Co., and methanol was used as co-solvent in culture solution. PHE was first dissolved in methanol to make a stock solution. In culture solution, certain volume of stock solution was added into nutrient solution to make the final concentration of PHE at 1, 2, 4, 8 mg/L. The concentrations of methanol in all solutions were adjusted to 0.1%, and a control, which only has 0.1% methanol in nutrient solution, was designed to avoid the effect of methanol. Considering the solubility of PHE in solution at 25°C is only 1.18 mg/L, the solutions were homogenized by ultrasonication for 2 h, and the bath temperature was kept below 40°C. All distilled water in this experiment was prepared by a Milli-Q Biocel system (USA) and microbes were strictly controlled during the whole procedure.

Perennial ryegrass (polim) seeds were surface sterilized for 20 min with 3% hydrogen peroxide, and were washed with distilled water for several times. The sterilized seeds germinated for about 2 weeks on germination discs, nutrient solutions were changed every 3 days. When the seedlings were about 15 cm tall, they were transferred to the beakers, with each beaker containing ten seedlings, and the seedlings were fastened loosely on a plastic board, which had been perforated to ensure that the roots were all submerged and the stems were just out of the solution. Each beaker contained 0.3 L half strength Hogland's nutrients solution, and the pH was adjusted to 5.5 before use. After being pre-cultured for 3 days, the solutions were changed by nutrient solutions, which contained spiked PHE; the beakers were packed with silver paper to avoid the photolysis of PHE. The whole incubation experiment was conducted in a phytotron where the day and night cycle was 16:8 h and room temperature kept at 22:15°C and the culture solutions were changed every 3 days.

After being treated by PHE for 6 days, the seedlings were harvested, at the same time the culture solutions were tested for pH by an ORION mode 868 digital acidometer (USA).

Root exudates were also collected after 6 days' treatment. Endosperms of the seedlings were wiped off before collection, the roots were washed carefully by distilled water, and then submerged into amber cuvettes containing quantificational distilled water. The collection procedure was done in the phytotron under illumination for 2 h. Afterwards, the plant roots were harvested immediately for inner organic acids extraction. The root exudates were kept in a −20°C refrigerator and analyzed as soon as possible.

Organic acids in root were extracted by water. Roots were cut down just after the forenamed collection and weighing. Then, they were homogenized in a glass mortar with distilled water, the homogenate was transferred into glass tube and kept in 80°C water bath for 20 min to extract organic acids, and then, distilled water was added to make the final volume at 5 mL. The extracts were finally centrifuged under 20,000*g* for 20 min; the supernatants were used for HPLC analysis. These samples were also stored in refrigerator and analyzed as soon as possible.

Organic acids were analyzed with an Angilent 1100 HPLC series consisting of G1311A quart pump, G1379A degasser, G1316A column thermostat, G1315B DAD detector, G1328B manual injector and a XB-18 column (4.6 × 250 mm, 5 μm particle size). 25 mmol H_3PO_4–KH_2PO_4 buffer was used as mobile phase at a flow rate of 0.7 mL/min; the chromatography was performed at 30°C. All the organic acids were detected at 210 nm, the samples and solutions used in HPLC analysis were filtered by 0.45 μm cellulose nitrate membranes beforehand. We used multilevel calibration and peak area calculation method with a series of fresh standard solutions to qualitatively and quantitatively analyze organic acids.

2,3,5-Triphenyltetrazolium chloride (TTC) reduction activity assay performed following the method of Nayyar et al. (2005) with some modification. After harvest, the root was carefully washed and blotted; each replicate had two same weight samples: a heated sample (which was incubated in 95°C water at the very start) and a treated sample, all the samples were put in the tubes which contained 10 mL 50 mM phosphate buffer (pH 7.0) with 0.4% (w/v) TTC. These tubes were incubated in a water bath of 37°C for 2 h in darkness, and then 2 mL of 1 mol/L sulfuric acid was added to cease the reaction, after the solutions were drained, the root was homogenized with 10 mL ethyl acetate. The absorbance of extraction was recorded at 485 nm after filtration. The TTC reduction was measured by a standard curve and TTC reduction activity was calculated by the following formula:

2,3,5-Triphenyltetrazolium chloride reduction activity $= (T_T - T_H)/W \times T$, where T_T is the TTC reduction of treated sample, T_H is of heated sample, W is the fresh weight of sample and T for 2 h.

Each treatment has three replicates. All the statistical analyses were conducted in SPSS v.10.0. Treatment effects were tested by an analysis of variance (ANOVA), and the

Table 1 pH of culture solutions after ryegrass incubation

Phenanthrene concentration (mg/L)	0	1	2	4	8
pH	5.08 ± 0.04b	4.87 ± 0.15a	4.84 ± 0.07a	4.77 ± 0.07a	4.93 ± 0.11a

Each value is mean ± SD (n = 3)

Values marked by different letter means significant difference between them under the least significant difference (LSD) test ($p < 0.05$)

significance of differences between treatments was determined by the least significant difference (LSD) test.

Results and Discussion

The pH of culture solutions after incubation was shown in Table 1. All the solutions had a decrease in pH. This decrease was caused by the integrated effect of both root absorbability and root exudation. In this experiment, the differences of pH between PHE treatments and control were significant under the least significant difference test ($p < 0.05$).

In all root exudates samples, oxalic, lactic, malic and acetic acids were detected, of which oxalic acid exceeded 98% of the total acids, it was the dominant composition of the organic acids in ryegrass root exudates and the composition and proportion showed no significant change with the increasing of PHE concentration.

The detected limitations of oxalic acid in this experiment was 1.43 ng, and its recovery averaged 103.2% (n = 10, RSD <2.78%).

As showed in Table 2, the concentrations of oxalic acid in root exudates were higher than control, and oxalic acid rose with the increasing of PHE concentration. The differences between all treatments and control were significant ($p < 0.05$).

The organic acids composition in root had the same character with root exudates of ryegrass; oxalic acid was also the dominant part as a proportion exceeded 97%. Its concentration had a peak value at 1 mg/L PHE treatment. As PHE continuously increasing, oxalic acid in root had a decrease trend, these decreases were significant compared to 1 mg/L PHE treatment, but not significant compared to control except for the highest treatment.

Table 2 Oxalic acid secreted and accumulated by root

Phenanthrene concentration (mg/L)	Oxalic acid in root exudates (mg/g FW)	Oxalic acid in root (mg/g FW)
0	2.33 ± 0.23a	22.78 ± 2.35B
1	3.00 ± 0.15b	25.61 ± 1.97C
2	3.28 ± 0.30b	20.40 ± 1.79AB
4	4.14 ± 0.44bc	20.49 ± 2.15AB
8	4.72 ± 0.32c	18.85 ± 0.46A

Values marked by different letter in a column means significant difference between them under the least significant difference (LSD) test ($p < 0.05$)

Each value is mean ± SD (n = 3)

To research changes of oxalic acid metabolism and secretion under spiked PHE, we used two indexes: total oxalic acid (TA) and secrete proportion (SP).

TA = oxalic acid accumulated by root + oxalic acid secreted by root;

SP = oxalic acid secreted by root/oxalic acid accumulated in root.

Figure 1 showed their changes under spiked PHE. TA had a significant increase ($p < 0.05$) at 1 mg/L PHE treatment, and under other concentrations TA showed no significant difference to control. However, SP rose continuously as the PHE concentrations increasing, from 11.7% at 1 mg/L to 25.2% at 8 mg/L PHE treatment, and oxalic acid secrete proportion under all PHE concentrations were higher then control (10.5%).

Organic acids are of special importance in plant not only at the cellular level but also at the whole plant level from multi-aspects. They are related to several biochemical pathways, such as photosynthesis, formation of precursors for amino-acid biosynthesis and modulating adaptation in

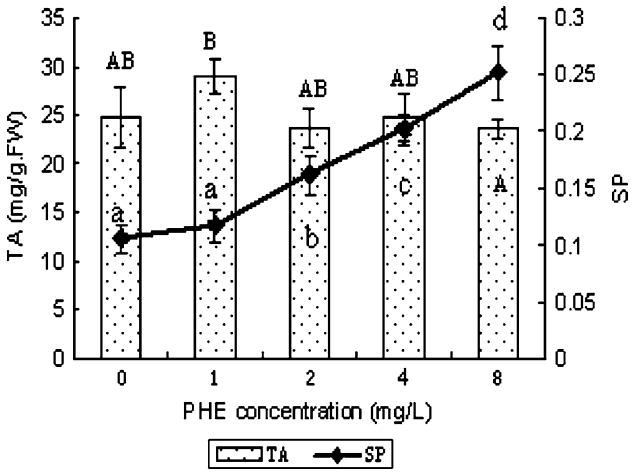

Fig. 1 Total oxalic acid of root and its secrete proportion under different PHE treatments. TA = oxalic acid accumulated by root + oxalic acid secreted by root; SP = oxalic acid secreted by root/oxalic acid accumulated in root. Capital letter showed the least significant difference (*LSD*) test result of TA and lowercase letter showed the result of SP; values marked by *different letter* means significant difference between them under the least significant difference (LSD) test ($p < 0.05$). Each value is mean ± SD (n = 3)

root to the environment stress, such as nutrient deficiencies, heavy metal tolerance, salt and alkaline stress, plant-microbe interactions, etc. (José et al. 2000). Researches proved that environmental stress could induce the quantity and composition changes of organic acids both in plant accumulation and root exudates (Landsberg 1981; Alhendawi et al. 1997; Yan et al. 2006).

In this experiment, oxalic acid was the dominant composition of organic acids accumulated in ryegrass root, it made up 97% of the total organic acids in root, other acids been detected including malic, lactic and acetic acids only accounted for 3% together. Under PHE treatments (range from 1 to 8 mg/L), the composition did not have significant change. Data showed that, the oxalic acid accumulated in root increased significantly at 1 mg/L PHE, and then there was a decrease as PHE further increasing, while SP (oxalic acid secreted by root/oxalic acid accumulated in root) increased continuously as PHE rising, considering the increased oxalic acid in root exudates, the decrease of oxalic acid in root may be caused by the enhanced secretion. Using TA as a index of oxalic acid metabolism in root, we found that TA varied from 23.57 to 28.95 mg/g, the peak value also appeared at 1 mg/L PHE treatment, we presumed that oxalic acid metabolism is enhanced by relatively low concentration of PHE, this may be associated with the promotion of plant growth, evidence also shown in plant biomass. However, as a whole, PHE treatment in this study seemed to have little impact on root oxalic acid metabolism.

The composition of organic acids in root exudates had the same character with which accumulated in root, oxalic acid made up 98% of the total organic acids. Oxalic acid in root exudates increased significantly under PHE stress than control (ranged from 28.7% to 106.8%), simultaneously, the secrete proportion of oxalic acid also increased as PHE concentration rising (ranged from 13.9% to 23.9%), oxalic acid secretion by root was enhanced as the PHE concentration increasing. Data analysis showed that there was a significant positive relationship between oxalic acid in root exudates and PHE concentration ($r = 0.882$, $p < 0.01$), SP (secrete proportion) also had a significant positive relationship with PHE ($r = 0.929$, $p < 0.01$). This result suggested that PHE application could enhance the organic acids secretion by root, but more researches were needed to reveal the mechanism.

Because of the physicochemical property of PAHs, its transport and bioavailability may be drastically determined by organic compounds in soil, humic acids are the major part of natural organic matter (NOM) and dominant sorbent of organic pollutants in soil, Ke et al. (2003) revealed that adding humic acid into contaminated sediments decrease both the pyrene dissipation in sediments and plant uptake. According to researches, organic acids in plant root exudates are able to disaggregate soil organic fractions into low apparent molecular size and mobilize the soil organic fractions (Nardi et al. 1997; Jones 1998). Evidences also show that organic acids and their salts can increase desorption and bioavailability of organic pollutants in soil (White 2002; Luo et al. 2006). Ample researches have already proved that root exudates are able to enhance the dissipation of organic pollutants in soil (Haby and Crowley 1996; Burken and Schnoor 1996; Yoshitomi and Shann 2001), most of the researchers attributed this enhancing to the stimulation of microbial degradation by root exudates, as a supplement, we speculated that the increased organic acids secretion may also play an important role in organic pollutant phytoremediation via enhanced organic pollutants desorption.

As the data given by Table 3, the root TTC reduction activity varied slightly at spiked PHE treatments. At 4 mg/L PHE treatment, root TTC reduction activity increased to 0.82 mg/h g FW, and under other concentrations, they had no differences compared to control.

The metabolism of PAHs in organism can generate reactive oxygen species (ROS), which may induce the oxidative damages (Choi and Oris 2000; Babu et al. 2001). To evaluate the damage of PHE treatment on ryegrass growth and tissue viability, root TTC reduction activity and plant biomass were used as indexes in this experiment. TTC reduction is performed by the mitochondrial dehydrogenases in cells (Rich et al. 2001; Tanaka et al. 2005); it was regarded as an indicator of mitochondrial capacity and plant tissue viability. Researches also proved that, TTC reduction activity is a sensible indicator of oxidative damage; it can indicate the lipid peroxidation and cell membrane permeability sensitively (Upadhyaya and Cladwell 1993; Nayyar et al. 2005). In this study, the root TTC reduction activity varied slightly from 1 to 8 mg/L PHE treatment, it varied from 0.72 to 0.82 mg/g h FW, which

Table 3 2,3,5-Triphenyltetrazolium chloride (TTC) reduction activity of the root

Phenanthrene concentration (mg/L)	0	1	2	4	8
2,3,5-Triphenyltetrazolium chloride (TTC) reduction activity (mg/h g FW)	0.77 ± 0.05a	0.74 ± 0.07a	0.76 ± 0.04a	0.82 ± 0.04b	0.72 ± 0.02a

Values marked by different letter in a column mean significant difference between them under the least significant difference (LSD) test ($p < 0.05$)

Each value is mean ± SD (n = 3)

were all had no significant decrease compared with control (0.77 mg/g h FW). These data indicated the viability of ryegrass root was not inhibited by PHE and there was no significant membrane damage of root cell under PHE treatments at the concentrations set in this study. The plant appearance also suggested ryegrass had high tolerance of PHE.

Lacking of research on the root exudates diversification under PAHs stress, this experiment was just a preparatory work, further research will be conducted to reveal not only the mechanism of increased organic acids secretion but also the relationship between organic acids and PAHs desorption.

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