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Effect of birch (*Betula* spp.) and associated rhizoidal bacteria on the degradation of soil polyaromatic hydrocarbons, PAH-induced changes in birch proteome and bacterial community

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Birch can enhance degradation of PAH compounds in the rhizosphere.

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ABSTRACT

Two birch clones originating from metal-contaminated sites were exposed for 3 months to soils (sand–peat ratio 1:1 or 4:1) spiked with a mixture of polyaromatic hydrocarbons (PAHs; anthracene, fluoranthene, phenanthrene, pyrene). PAH degradation differed between the two birch clones and also by the soil type. The statistically most significant elimination ($p \leq 0.01$), i.e. 88% of total PAHs, was observed in the more sandy soil planted with birch, the clearest positive effect being found with *Betula pubescens* clone on phenanthrene. PAHs and soil composition had rather small effects on birch protein complement. Three proteins with clonal differences were identified: ferritin-like protein, auxin-induced protein and peroxidase. Differences in planted and non-planted soils were detected in bacterial communities by 16S rRNA T-RFLP, and the overall bacterial community structures were diverse. Even though both represent complex systems, trees and rhizoidal microbes in combination can provide interesting possibilities for bioremediation of PAH-polluted soils.

1. Introduction

Polyaromatic hydrocarbons (PAHs) develop by incomplete combustion or pyrolysis of organic compounds. Main sources of PAHs are exhaust fumes, petroleum industry and forest fires (Finlayson-Pitts and Pitts, 1997). Most PAHs are toxic, mutagenic and/or carcinogenic and thus present threats to human health (Mastrangelo et al., 1997).

Phytoremediation is an environmental technology, in which plants are used for decontamination of soils from organic and inorganic pollutants. Phytoremediation of soils from PAHs has been studied mainly using grasses and annual herbs, and less with trees (Binet et al., 2002; Davis et al., 2002; Mattina et al., 2003; Mueller and Shann, 2006). Plants have been shown to enhance the elimination of PAHs from soils, but only in the presence of rhizospheric microbes (Davis et al., 2002). Trees have extensive root systems and provide nutritionally rich microenvironments for microbes. Plant exudates and decaying fine roots increase soil nutrient levels, promoting microbial proliferation and activity (Olson et al., 2003). Rhizoidal communities can also be enriched with microbes that actively degrade PAHs (Binet et al., 2002). PAHs are often bound in soil organic matter, which reduces their bioavailability (Kipopoulou et al., 1999; Meudec et al., 2006) and plant root epidermis can also bind PAHs. Since plants do not have cellular uptake mechanisms for PAHs, accumulation is low and correlates with soil PAH concentration (Gao and Zhu, 2004).

Plants have evolved tolerance to various environmental stresses. Tolerance to PAHs is not easily defined since the bioavailability, rate of PAH metabolism, formation of toxic metabolites and microbial communities cannot be standardized readily in the soil. However, differences in PAH tolerance of various plant species and in the

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elimination of PAHs have been demonstrated (Kucerová et al., 2001; Sverdrup et al., 2003; Gao and Zhu, 2004). It is of interest that tolerance to one pollutant can significantly improve tolerance to other pollutants and abiotic stresses (Wu et al., 2007). A wide stress tolerance is a prerequisite for plants aimed for phytoremediation of soils contaminated with multiple pollutants.

In this study, performance of two metal-tolerant birch clones representing two *Betula* species were compared in a 3-month greenhouse experiment in soils spiked with a PAH mixture (anthracene, phenanthrene, fluoranthene, and pyrene). In addition to PAH degradation in the soils, changes in the protein complement of the plants and in the microbial communities were explored.

2. Materials and methods

2.1. Experimental design

Two micropropagated birch clones were used. The Cu-tolerant clone W008 (here called WA, *Betula pendula*) originates from spoil heaps of disused Pb/Zn mine near Aberystwyth, Wales. The Ha02 clone (here called HA, *B. pubescens*) originates from a Cu/Ni smelter area in Harjavalta, Finland, and is also moderately tolerant to Cu (Utriainen et al., 1997; Kopponen et al., 2001). Micropropagation of the birches was carried out as described by Utriainen et al. (1997). After 4 weeks in micropropagation, the plantlets were transferred to soil which consisted of fertilized sphagnum peat, sand and steam-sterilized garden soil, 1:1:1 (v/v/v), in mini-greenhouses, where the plants were grown for 2 months before PAH exposure.

During the 3-month exposure to PAHs, the plants were grown individually in pots containing 500 g of control (with or without acetone) or PAH-spiked soil and the sprinkling water was recycled. All treatments were done in triplicate. The soil mixtures were: (A) 50% sand (<1.2 mm granules) and 50% peat; and (B) 80% sand and 20% peat. The soils were spiked with a mixture of anthracene, phenanthrene, fluoranthene and pyrene in acetone, 300 mg kg^{-1} each, giving a total PAH concentration of 1200 mg kg^{-1}, respectively. Spiking was performed in hermetically sealed glass bins by adding 100 ml acetone with or without PAHs to 500 g of soil and then mixing in a rotating pot mill for 30 min. The soil was then transferred to a plant pot and vented in a fume chamber for 72 h before planting of birches. Also control soil mixtures with and without acetone were prepared.

Temperature and light/dark cycle were kept at 18 °C and 16/8 h (colours 77 and 965, Osram Fluora and Biolux) to simulate the typical northern conditions in summer. Temperature, air pressure, light, CO_2 concentration and humidity were monitored continuously.

2.2. Sampling of soils and plants

After the PAH exposure, the birches were lifted from the pots. The soil remaining on plant roots was hand-shaken and collected. Six soil sub-samples were pooled from each plant (20 g total) for microbial (Rhiz) analysis. The remaining soil (480 g) was extracted and analyzed for PAHs by HPLC.

The height of the plant stems was measured. The plants were rinsed with tap water, dipped in liquid nitrogen and stored at −70 °C degrees for proteome (roots) and Hsp70 ELISA (leaves) analysis.

2.3. Soil PAH analysis

The soil sample (480 g) was extracted with 1500 ml of 50% acetonitrile in water for 120 min, and 10 ml of the upper phase was filtered through Whatman PVDF microfilter (0.45 μm). PAHs were analyzed according to a modified EPA 500/500.1 method using Hewlett–Packard HPLC (Waldbronn Analytical Division) with a reverse phase C18–RP column (Bondapack, 250 × 4.6 mm, 5 μm particles). The PAHs were separated with gradient elution using water (A) and acetonitrile (B) as follows: 40–60% of A in B 0–2.5 min, 10–90% of A in B 2.5–12 min, and 100% of B 12–20 min. Injection volume was 10 μl, flow rate 1.5 ml min^{-1} and pressure 400 bar. The PAHs were detected at 270 nm, identified based on the retention times of standard compounds and quantified by comparing the peak areas of the samples to those of standards. Standard stock solutions in total PAH concentration range of 200–1200 mg kg^{-1} were used for calibration. Each sample was analyzed in five parallel runs. Recovery was determined for all four PAHs by spiking soil samples with pure standard solutions (200, 800 and 1200 mg kg^{-1}), mixing for 30 min, and storing for 14 days before the extraction and HPLC analysis.

The PAH concentrations remaining in the soil and the differences between treatments were tested with mixed model analysis.

2.4. Hsp70 EIA assay of birch leaves

Hsp70 protein levels were assayed using Hsp70 Immunoassay kit EKS-700 (StressGen Biotechnologies Corp.). Two milligrams of crushed leaves were extracted with 250 μl of Hsp70 extraction reagent. Each sample was analyzed in triplicate.

2.5. Proteomic profiling of birch roots

Total soluble proteins from roots (1.32 to 6.54 g) of each plant (three plants exposed to acetone or 1200 mg kg^{-1} PAH in both soils) were extracted as described by Koistinen et al. (2002). Total protein concentration was analyzed using Bio-Rad Protein Assay Dye reagent. The protein pellets were washed and dissolved in 2-DE sample buffer containing 10 M urea, 2% (w/v) CHAPS, 1% (w/v) DTT and 2% (v/v) Bio-Lyte 3/10 ampholyte (Bio-Rad, Hercules, CA, USA).

2-DE was performed as described by Lehesranta et al. (2005). Approximately 100 μg of root proteins were loaded to 2-DE gels. Image analysis was performed with PDQuest software (Bio-Rad). The data set contained intensity values for 1196 spots. Spots with only 0–2 hits (altogether 164 spots) were removed and the remaining intensity values were transformed with $\log_{10}(\text{int}+1)$. The data set was analyzed with ANOVA and principal component analysis (PCA) using SPSS11.5.

2.6. Mass spectrometric analysis of differentially expressed protein spots

Twenty spots showing differential intensity between birch clones, PAH exposures or both ($p < 0.01$) in ANOVA were selected for identification. To visualize the spots, the gels were silver-stained, and in-gel digestion was performed according to Lehesranta et al. (2005). Tryptic peptides were separated using Ultimate/Famos capillary LC system (LC Packings, Amsterdam, NL). The sample was loaded into a 300-μm i.d. × 5 mm C18 PepMap precolumn (Dionex, CA, USA) with a flow rate of 30 μl min^{-1} of 0.1% (v/v) formic acid and 2% (v/v) ACN. After preconcentration and clean-up, the precolumn was switched in-line with the C18 PepMap (75 μm × 3 μm, 50 mm, Dionex) analytical column. The peptides were eluted starting with 100% buffer A (0.1% formic acid, 5% ACN) from 0 to 3 min. In the next steps, the content of buffer B (0.1% formic acid, 95% ACN) was linearly increased: 3–33 min from 0 to 30%, 33–40 min from 30 to 100%. Between 40 and 50 min the samples were eluted with 100% B, and 50–51 min the percentage of B was linearly decreased to 0%. The column was eluted with 100% A between 70 and 75 min. Flow rate was 200 nl min^{-1}.

The LC was connected to mass spectrometer with nanospray source using distally coated 10 μm PicoTip FS-360-20-10-D-20 emitter (New Objective). The positive TOF mass spectra were recorded on QSTAR XL hybrid quadrupole TOF-instrument (Applied Biosystems, CA, USA) using information-dependent acquisition (IDA). TOF-MS survey scan was recorded for the range m/z 400–2000 followed by MS/MS scans of the two most intense peaks. Typical ion spray voltage was in the range of 2.0–2.4 kV. N_2 was used as collision gas. The peptides were identified with ProID software (Applied Biosystems) using embryophyta database available in NCBI.

2.7. Bacterial community analysis by terminal restriction fragment length polymorphism (T-RFLP)

The DNA was extracted from soil (0.25 g) by chemical and mechanical cell lysis using the Soil DNA Isolation Kit (Mo Bio laboratories, Inc., CA, USA). Portions of the 16S rRNA gene were amplified by PCR with universal bacterial primers 27F and 1492R (Baker et al., 2003). The forward primer was labelled with 6-carboxy-fluorescein (6-FAM; Tag Copenhagen A/S, Denmark). The 50 μl PCR mixture contained 20 pmol of primers, 20 μM dNTPs, 1.2 U DNA-polymerase (Biotools, B&M Labs, Spain), PCR reaction buffer and 1 μl of template. Bovine serum albumin (0.6 μg μl^{-1}) was used to prevent PCR inhibition. The reaction conditions were 5 min initial denaturation followed by 30 cycles of 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. The products were analyzed on 1% agarose gels with ethidium bromide staining.

The PCR amplicons were digested with *Hae*III at +37 °C overnight. The T-RFLP fingerprints (TRFs, terminal restriction fragments) from two replicate Rhiz soil samples were determined by electrophoresis with model ABI 310 automated sequencer (Applied Biosystems Instruments). The base pair lengths of the peaks were defined from three consecutive runs using internal size standard (GeneScan®-500 [TAMRA]™, Applied Biosystems). Electropherogram analysis was performed with GeneScan software. Peaks with intensity of less than 1% of total fluorescence units were excluded from the final results.

3. Results

3.1. Performance of the birch clones

Growth of the birches during the 3-month exposure was estimated by measuring the height of the stems (Fig. 1). In soil A (sand–peat ratio 1:1), growth of the WA clone was reduced statistically significantly ($p = 0.005$) at 1200 mg kg^{-1} PAH exposure compared to the control without acetone. In the more sandy soil B (sand–peat ratio 4:1), the WA clone did not grow at all in the presence of PAHs. Addition of solvent (acetone) in the soil reduced birch growth compared to control in both soils ($p < 0{,}025$). The HA clone did not grow during the experiment regardless of the soil composition, and the leaves were chlorotic.