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Anthracene and mycorrhiza affect the activity of oxidoreductases in the roots and the rhizosphere of lucerne (*Medicago sativa* L.)

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Abstract

DAF (2,7-diaminofluorene)-peroxidases were the principal oxidative system in roots and rhizosphere of lucerne. The expression of these enzymes was stimulated by mycorrhiza as well as by anthracene (500 mg kg^{-1} added to the growth medium). Inversely, infection by mycorrhizal fungi repressed laccase activities. Electrophoretic analysis of the DAF-peroxidases revealed similar isoenzyme patterns for all treatments but in presence of anthracene some of the isoenzymes had more intensive bands.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent compounds which are ubiquitous in terrestrial and aquatic ecosystems. These organic pollutants result essentially from the incomplete combustion of fossil fuels but also from industrial processes and forest fires. The low water solubilities of these compounds lead to their accumulation and persistence in the environment, and thereby present a health risk because of the toxic, mutagenic and carcinogenic properties of many of them. Various processes have been developed for the removal of PAHs from contaminated soils, including soil excavation and incineration. Due to the high cost of such techniques, bioremediation technologies have been explored to clean up polluted soils. Among these technologies, phytoremediation exploits the processes that take place in the rhizosphere, and constitutes an inexpensive and efficient approach (Günther *et al.* 1996, Reilley *et al.* 1996, Leyval & Binet 1998). Stimulation of microbial populations, plant uptake, abiotic degradation, mineralization, leaching and irreversible sorption have been suggested to contribute to the dissipation of PAHs in the rhizosphere. As a result of these processes, Reilley *et al.* (1996) showed that PAH degradation increased by 30 to 44% in vegetated soils compared to unvegetated soils.

Arbuscular mycorrhizal (AM) fungi are ubiquitous symbiotic microorganisms associated with plants of most families of angiosperms and gymnosperms (Smith & Read 1997). They play an essential role in plant nutrition and stress tolerance, including stress imposed by PAH contamination (Leyval & Binet 1998, Joner & Leyval 2000). The mechanisms behind mycorrhizal stress alleviation under these conditions are not known, but may be related to expression of various oxidative enzymes. Arbuscular mycorrhizas are known to modify both extracellular and root enzymes (activity, isoforms, etc.), including peroxidase activity (Salzer *et al.* 1999). Since the transformation of PAHs by plants and microorganisms is catalyzed by various oxidoreductases (e.g., peroxidases, laccases, dioxygenases, monophenol monooxygenases), we wanted to quantify the activity of these enzymes in roots and associated rhizosphere as influenced by a three ring PAH (anthracene) and the presence or absence of a mycorrhizal fungus.

Material and methods

Experimental design

Pots with 250 g heat-sterilized sand (<2 mm) were sown with four seeds of lucerne (*Medicago sativa* L. cv. Europe) and watered by weight with a low P nutrient solution containing 1 mM $Ca(NO_3)_2$; 1 mM NH_4NO_3; 1.0 mM K_2SO_4; 0.8 mM $MgSO_4$; 0.1 mM Na_2HPO_4; 25 μm Fe(III)NaEDTA; 25 μm H_3BO_3; 5 μm $MnSO_4$; 2 μm $ZnSO_4$; 0.5 μm $CuSO_4$; 0.1 μm Na_2MoO_4 and 4 nm $CoCl_2$, to maintain the equivalent to 70% of the water holding capacity of the sand. Plants were kept in a growth chamber with a 16–8 h light-dark cycle at 21–16 °C (day-night), and an average photon flux density of 300 μmol m^{-2} s^{-1}. The experiment consisted of four treatments: an unamended control (C), a mycorrhizal treatment (M), an anthracene treatment (ANT) and a combined mycorrhiza-anthracene treatment (M-ANT), all with four replicate pots per treatment. Prior to sowing, 2 kg sand was amended with 500 mg anthracene (ANT) kg^{-1} by dissolving 1 g ANT in 250 ml chloroform, adding it to 1 kg sand in a dessicator and evaporating the solvent under vacuum. When dry, anthracene-amended sand was mixed with 1 kg heat sterilized sand moistened to 15% (w/w) water and added to pots with or without mycorrhizal inoculum. Mycorrhizal inoculum consisted of 60 spores of *Glomus mosseae* P2 (BEG 69; Banque Européenne de Glomales) added at half depth during preparation of pots. Similar pots were also prepared without ANT.

Harvest

Plants were harvested after 8 weeks, separating roots from sand by gentle shaking, washing off remaining sand, mixing the root pieces in water after cutting the roots in 0.5–1 cm pieces. Sub-samples (0.5 g fresh weight) of roots were taken for determination of mycorrhizal colonization according to Kormanik & McGraw (1982).

Preparation of extracts – Enzyme assays

Roots (1 g) were ground in a mortar with liquid N_2 and suspended in 10 ml 0.2 M Bis/Tris buffer (pH 6.0) with 1 g polyvinylpolypyrrolidone (PVPP) and 0.05% Tween 80. The homogenate was centrifuged (12 000 *g*, 20 min) and the supernatant filtered (0.2 μm) and dialyzed for 48 h against 2 mM Bis/Tris buffer.

Extracellular enzymes in rhizospheric sand were extracted according to Criquet *et al.* (1999) by shaking 200 g sand in 200 ml 0.1 M $CaCl_2$ with 0.05% Tween 80 and 10 g PVPP. After centrifugation, the supernatant was dialyzed as above and concentrated in a cellulose dialysis tube, with a 10 kDa molecular mass cut-off, covered with polyethylene glycol until a final volume of 5–10% of the initial volume.

Enzymatic activities in root and soil extracts were measured spectrophotometrically. A final volume of 3.0 ml reaction mixture contained 10–500 μl enzyme extract diluted in an appropriate buffer and added with the substrate of the enzyme studied (Gramss & Rudeschko 1998; Criquet *et al.* 2000). Peroxidase activity was measured using 23 μM 2,7-diaminofluorene or 0.4 mM pyrogallol or 0.6 mM of guaiacol as substrates diluted in 0.1 M phosphate buffer (pH 6.0) with 4.4 mM H_2O_2. Oxidation rates of the different substrates were monitored at 600, 430 and 436 nm, respectively. The activity of manganese peroxidase (MnP) was measured in 0.2 mM $MnSO_4 \cdot H_2O$, 0.2 mM H_2O_2, 50 mM sodium-malonate and 0.1 M lactate buffer pH 4.5 (A_{270}). The activity of lignin peroxidase (LiP) was measured in 3.3 mM veratryl alcohol, 0.44 mM H_2O_2 and 0.1 M tartaric buffer at pH 3.0 (A_{310}).

Laccase activity was measured in 23 μM 2,7-diaminofluorene (DAF) or 16 μM syringaldazine or 50 μM 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulphonate) (ABTS) and phosphate buffer at pH 6.0. Oxidation rates of the different substrates were monitored at 600, 525 and 420 nm, respectively. The activity of monophenol monooxygenase (tyrosinase) was measured in 14 mM 3,4-dihydroxy-DL-phenylalanine (DL-DOPA) and phosphate buffer at pH 6.0 (A_{475}).

Aromatic-ring cleavage dioxygenases were measured using 0.1 mM catechol and 1.35 mM EDTA at pH 7.0 (catechol 1,2-dioxygenase; A_{260}), or 0.35 mM catechol at pH 7.5 (catechol 2,3-dioxygenase; A_{375}). All the activities are expressed in enzyme units (U), defined as μmol oxidized substrate min^{-1} and g^{-1} of root fresh wt (FW) or sand.

Electrophoretic analysis

Root extracts were subjected to electrophoretic analysis to determine peroxidase isoenzymes present in the different treatments. Non-denaturing PAGE was used according to Laemmli (1970). For the analysis of peroxidase isoenzymes, polyacrylamide gels (7.5%) layered with 4% stacking gels were used. The mini-

gels (Mini-Protean II, Biorad) were run at 200 V for 45 min. Gels were stained with 0.1 mM DAF and 8.8 mM H_2O_2.

Results

Mycorrhizal inoculation resulted in colonization of 30–60% of the roots, without any significant difference between treatments. No mycorrhizal structures were detected in non-inoculated plants.

Peroxidases (DAF-, guaiacol- or pyrogallol-types) were the dominant enzyme species detected in both root and sand extracts. Peroxidase activities were considerably higher in roots than in the soil, and ranged from 1.6 to 142 U g^{-1} FW in root extracts, and from 2.0×10^{-4} to 2.55×10^{-2} U g^{-1} sand in sand extracts (Figure 1). Among these peroxidases, the DAF-peroxidase showed the highest activity in both root and sand extracts, and the activity decreased in the order: mycorrhizal (M), mycorrhizal/ANT-amended (M-ANT), non-mycorrhizal/ANT-amended (ANT) and control (C). In soil extracts, DAF-peroxidase activities were similar for all treatments, except for the M treatment which had a 5 times higher activity.

Laccases (DAF-, ABTS- or syringaldazine types) were detected in roots, but not in sand extracts. The highest value for laccase activity was recorded with syringaldazine as substrate, though differences were not significant between the different substrates. No laccase activity was detected in mycorrhizal roots (M and M-ANT), and no significant differences were established between C and ANT, except for DAF-laccase which was absent in the ANT treatment. Neither tyrosinase nor catechol 1,2- or 2,3-dioxygenase nor LiP or MnP activity was detected in either treatment.

Electrophoretic analysis of DAF-peroxidases of roots revealed the presence of 8 isoenzymes with R_f-values of 0.2, 0.3, 0.4, 0.45, 0.5, 0.55, 0.8 and 0.85. The isoenzyme patterns were similar for all treatments, but for the ANT treatment the isoenzymes with Rf-values of 0.4, 0.45, 0.5 and 0.55 had more intensive bands.

Discussion

This study shows that peroxidases constitute the principal oxidative system in lucerne roots and its rhizosphere, and that the activities of these enzymes may

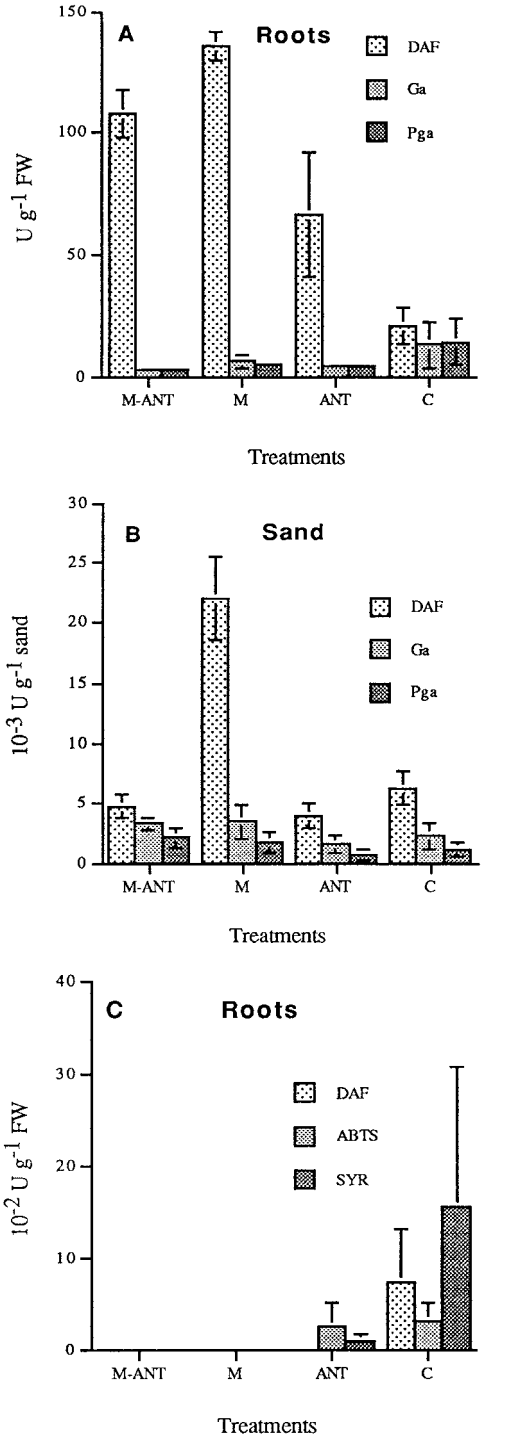

Fig. 1. Activities of peroxidases (A, B) and laccases (C) in root and sand extracts of the different treatments: mycorrhizal (M); mycorrhizal/anthracene-amended (M-ANT); non-mycorrhizal/anthracene-amended (ANT); control (C). DAF: 2,7-diaminofluorene; Ga: guaiacol; Pga: pyrogallol; SYR: syringaldazine; ABTS: 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulphonate). The values represent the averages of four samples, and bars represent standard error of means.

be stimulated by mycorrhiza and/or anthracene. Arbuscular mycorrhizal (AM) colonization constitutes a root cell invasion that provokes various defence reactions (Gianinazzi-Pearson *et al.* 1996), including enhanced activity of peroxidases (Salzer *et al.* 1999). Root contact with toxic chemicals like PAH also induces peroxidase activity which may have an intracellular function as part of a defence mechanism and/or a direct effect on degradation of aromatics in the external medium (Kraus *et al.* 1999). The activity of extracellular oxidoreductases may also be enhanced in roots upon colonization by AM fungi (McArthur & Knowles 1992). Enhanced oxidoreductase activities in roots and rhizosphere may thus explain recent observations that AM counteracts the adverse effects of PAHs on plant growth and survival (Leyval & Binet 1998, Joner & Leyval 2000), and contributes to an enhanced dissipation of PAH during phytoremediation (Joner E., Portal J.M., Leyval C. unpublished results). Whether the DAF-peroxidase activity detected in the rhizosphere of mycorrhizal plants originated from the plant or the fungus could not be determined with the present experimental set-up, but may be elucidated in compartmented pots where hyphae connected to the host plant are spatially separated from the roots (see, e.g., Joner *et al.* 1995).

Guaiacol peroxidase measurements did not show significant variations between treatments, a result that is consistent with the study of Fieldes & Gerhardt (1998) who argued that this enzymatic assay may lead to misinterpretations of stress effects on plants. Infection by AM fungi repressed laccase activity in roots, while it stimulated peroxidase expression. This indicates a modified metabolism of aromatic substances in roots as a result of plant mycorrhization, a finding that is consistent with modifications of expressed oxidoreductases in ectomycorrhizal roots (Münzenberger *et al.* 1997).

The present study did not include PAH analyses, and may thus not correlate extracellular activity of oxidoreductases to PAH dissipation. Such information would ultimately demonstrate if AM is implicated in PAH degradation, or restricted to intracellular plant protection. Many fungi are known as potent mediators of PAH degradation due to the action of their oxidoreductases (Gramss *et al.* 1999, Rama *et al.* 1998, Rama-Mercier *et al.* 1998), but their growth in soil may be slow and limit their action (Eggen & Sveum 1999). The use of symbiotic fungi whose growth is independent of carbon availability in soil may thus be advantageous, and justify their inclusion in phytoremediation schemes.

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