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Effect of phenanthrene and *Rhodotorula glutinis* on arbuscular mycorrhizal fungus colonization of maize roots

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Abstract The effect of the polycyclic aromatic hydrocarbon (PAH) phenanthrene and the yeast *Rhodotorula glutinis* on the arbuscular mycorrhizal fungus (AMF) *Glomus geosporum* colonizing maize roots, was studied. During a 90-day experiment, the highest *G. geosporum* colonization values were found in control plants. Mycorrhiza root length, measured both on the basis of percentage of root colonization and on the activity of succinate dehydrogenase, showed similar patterns in different phenanthrene treatments. The presence of phenanthrene in the substrate reduced *G. geosporum* intraradical colonization. The presence of *R. glutinis* did not enhance AMF colonization in the presence of phenanthrene. The biomass of the external mycelium estimated on the basis of the fatty acid 16:1 ω5 concentration showed a progressive increase through time, and the amounts of this fatty acid differed among treated and untreated substrates. However, this increase was found to be lowest in the phenanthrene and *Rhodotorula* treatment at 60 days. There was less phenanthrene accumulation in roots of maize inoculated with AMF and the yeast than in roots inoculated only with AMF. A similar pattern was observed in the phenanthrene content of *G. geosporum* spores collected after 90 days.

Keywords *Glomus geosporum* · Polycyclic aromatic hydrocarbons · Pollution · Arbuscular mycorrhizal fungus development · Fatty acid 16:1 ω5

Introduction

Phenanthrene is one of the most abundant polycyclic aromatic hydrocarbons (PAH) in the environment (Cerniglia 1992). Although it is not genotoxic, this substance has been used as an indicator for monitoring PAH-contaminated wastes because the molecular structure of phenanthrene is found in potent carcinogenic PAHs (Pothuluri and Cerniglia 1994).

Pollutants affect the natural environment. In recent years, as environmental pollution has increased, the role of plants in the biodegradation of xenobiotics in polluted soils has aroused interest. Plants growing in a polluted habitat with a mycorrhizal association often may be more tolerant of the chemical stress than those growing without the symbiotic fungi. Thus, arbuscular mycorrhizal fungi (AMF) may improve plant growth even in the presence of a toxicant in the soil (Lindsey et al. 1977; Khan 1981; Call and McKell 1982, 1984; Cabello 1995, 1997). Therefore, the estimation of AMF biomass in polluted soils is of importance. The signature fatty acid 16:1 ω5 provides a tool for the estimation of this biomass (Olsson et al. 1997).

Mycorrhizal symbiosis and its interactions with other microorganisms present in the rhizosphere can have beneficial effects on plant growth beyond that caused by mycorrhizas alone (Ocampo 1993). Mycorrhizas increase the biological activity around plant roots. Treatments with both plants and microorganisms may be the best strategy to improve seedling survival and growth in substrates polluted with hydrocarbons (Cabello 1999). Hydrocarbon-degrading microorganisms interacting with AMF might provide an important biotechnological tool for bio- and phytoremediation processes. Yeasts are the most abundant fungi in polluted environments (Berdicevsky et al. 1993). One of these is *Rhodotorula glutinis* which was able to grow in a liquid medium containing the PAH phenanthrene without a co-substrate (Romero et al. 1998). This yeast could transform all the phenanthrene present in the liquid medium.

The aim of the present work was to study the effects of the association among *Glomus geosporum*, *Zea mays*, and *R. glutinis* on the bioaccumulation of phenanthrene in maize plants. Another point of interest was to study the development of the external and internal phases of *G.*

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geosporum, and the influence of *R. glutinis* in the mycorrhizosphere-polluted system.

Materials and methods

Biological material and treatments

The AMF strain used was *Glomus geosporum* (Gerdemann and Nicolson) Walker [La Plata Spegazzini Herbarium (LPS) culture no. Be14]. It was isolated using the trap culture method from soils with a long record of pollution (Cabello 1999). The fungal inoculum consisted of soil, roots of *Sorghum vulgare* L. colonized by the AM fungus and AM fungus spores. The yeast *Rhodotorula glutinis* (Fresenius) Harrison LPS culture no. 585 was originally isolated from sediments obtained from a stream heavily contaminated with hydrocarbons near a petroleum refinery in La Plata (Argentina). This strain has been used for studies of phenanthrene degradation (Romero et al. 1998). *R. glutinis* was grown in mineral basal salts (MBS) containing (per litre distilled water): 1,000 mg $(NH_4)_2SO_4$, 800 mg K_2HPO_4, 200 mg KH_2PO_4, 200 mg $MgSO_4.7H_2O$, 100 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, and 20 g glucose (pH adjusted to 6.5) for 5 days at 25±1°C.

Seeds of maize (*Zea mays* L.) were surface-sterilized with sodium hypochlorite (10% v/v) for 10 min and thoroughly rinsed with sterilized water. Maize was used as an experimental subject because of its ability to grow in polluted soils (Weissenhorn et al. 1995). After germination, seedlings were grown in nursery beds with and without *G. geosporum*. Thirty-day-old maize seedlings inoculated or not with *G. geosporum* were transplanted to pots of each treatment. Plants were grown in a greenhouse at 24±1°C day/20±10°C night, and a 16-h photoperiod provided by halogen lamps for 30, 60 and 90 days. During growth experiments, plants were watered from below using a capillary system.

Four treatments were used in the experiment: (1) inoculated with *G. geosporum* without pollutant; (2) inoculated with *G. geosporum* with the addition of phenanthrene to the substrate; (3) inoculated with both *G. geosporum* and *R. glutinis*; (4) inoculated with both *G. geosporum* and *R. glutinis*, with the addition of phenanthrene to the substrate. All treatments had three replicates for each harvest. Plants were grown in pots (5 l) filled with a steamed mixture of soil and vermiculite (1:2 v/v). Soil characteristics were described by Cabello (1999). The substrate corresponding to treatments 2 and 4 was polluted with 0.1 g phenanthrene/kg (Sigma) dissolved in ethanol. After thorough mixing with the substrate, the ethanol was allowed to evaporate for 2 weeks. Three hundred millilitres of MBS with *R. glutinis* (1×10^4 cells/ml) was used as the yeast inoculum in treatments 3 and 4, and 300 ml MBS without *R. glutinis* was applied to treatments 1 and 2 in order to obtain similar nutrient conditions.

Plants were harvested 30, 60, and 90 days after transplanting, and the dry mass was determined. After the plants were harvested, the root system in each of the three replicates per treatment was divided into two portions to record the following: (1) mycorrhizal root length: part of the root system was cleared and stained (Phillips and Hayman 1970) and the percentage of root colonization was measured by Giovannetti and Mosse's gridline intersection method (Giovannetti and Mosse 1980); (2) mycorrhizal fungus succinate dehydrogenase (SDH) activity detected in the fungus mycelium by the reduction of tetrazolium salts at the expense of added succinate (Kough et al. 1987).

To evaluate the growth of *R. glutinis* during the experiments rhizosphere soils were sampled 5, 30, 60 and 90 days after transplanting. About 1 g rhizosphere soil was taken from each of the experimental pots. Then a tenfold dilution series was prepared for each sample. The number of yeast colony-forming units at suitable dilutions were counted on malt agar medium plus 0.5% streptomycin and 0.25% chloramphenicol.

Isolation, detection and identification of phenanthrene metabolites

The roots from each of the experimental pots were washed 3 times with ethyl acetate and ground with a mortar in liquid nitrogen. They were extracted by sonication 6 successive times with 100 ml ethyl acetate. The extracts were dried over anhydrous sodium sulphate, and the solvent was evaporated under reduced pressure at 40°C. The residue was dissolved in methanol and analysed in a Merck Hitachi HPLC (Merck, Darmstadt, Germany) equipped with a L-6,200 pump. Detection was performed by absorption at 250 nm using a L-4,200 UV-VIS Detector. A 5-µm RP 18 column, 250 mm by 4.6 mm inner diameter (LiChrospher 100, Merck) was used to separate phenanthrene using a 40-min programmed methanol/water linear gradient (50–95%, vol/vol) with a flow rate of 1 ml/min. Identification of phenanthrene and its metabolites was achieved using standards (Sigma) run under the same conditions.

Spores from treatments 2 and 4 were collected on day 90 by the wet sieving and decanting method (Gerdemann and Nicolson 1963), then washed with ethyl acetate in an ultrasonic cleaner. The phenanthrene content of spores was measured following the method described above for roots.

Fatty acid analysis

In order to detect the presence of AMF with the molecular marker fatty acid 16:1 ω5, total lipids in soil samples were extracted by sonication with a 2:1 chloroform:methanol mixture from the different treatments. The methanolic phase was discarded, and the chloroform phase containing the total lipids was dried, concentrated, and saponified with 10% KOH in ethanol at 80°C for 45 min in a N_2 atmosphere. The fatty acids were extracted with hexane after acidification, and esterified with boron trifluoride 10% in methanol. Fatty acid methyl esters were analysed by GLC on a 30-m DB-23 phase J & W Scientific capillary column in a Hewlett Packard model 6890 GC equipped with a flame ionization detector. The column temperature was programmed for a linear increase of 3°C/min from 16°C to 220°C. The chromatographic peaks were identified as described earlier (Gaspar et al. 1994).

Statistical analysis

One-way ANOVAs with Tukey's honestly significant difference contrasts were performed to study differences ($P\leq0.05$) among groups.

Results

The percentage of the root length of maize plants colonized by *G. geosporum* decreased significantly when phenanthrene was present in the substrate (Fig. 1). Also the *G. geosporum* mycelium with succinate dehydrogenase activity was affected by the presence of phenanthrene in the rhizosphere (Fig. 2). This reduction of internal hyphae growth was maximized when *R. glutinis* was present.

AMF external mycelium development varied with the addition of both *R. glutinis* and phenanthrene to the substrate, as revealed by the concentration of fatty acid 16:1 ω5 in the soil (Fig. 3). *R. glutinis* without phenanthrene enhanced the external mycelium growth 60 days after transplanting. In contrast, when both phenanthrene and *R. glutinis* coexisted with *G. geosporum* hyphae, the percentage of fatty acid 16:1 ω5 on day 60 after transplanting fell to one half the value obtained for the control.

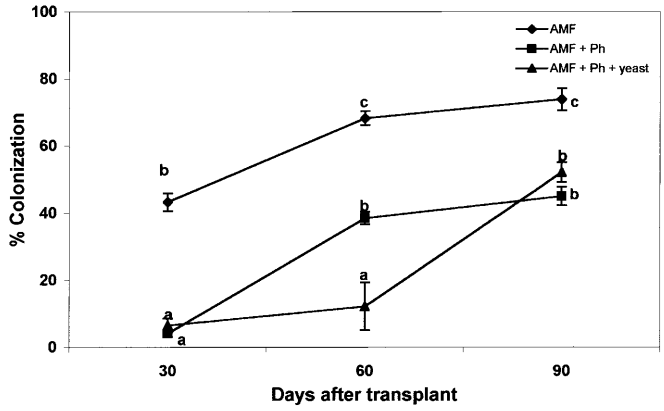

Fig. 1 Percentage root length colonized in maize plants by *Glomus geosporum*. ◆ Maize plants in nontreated substrate. ■ Maize plants grown in a substrate polluted with phenanthrene (*Ph*). ▲ Maize plants grown in a polluted substrate with *Rhodotorula glutinis* added. Percent root colonization was not assessed for the *G. geosporum* plus *R. glutinis* treatment in the absence of Ph. Values are means ±SD for three separate harvests. The *same letter above the symbol* indicates that values do not significantly differ across time or treatment according to ANOVA and Tukey's honestly significant difference (HSD) test ($P<0.05$). *AMF* Arbuscular mycorrhizal fungus

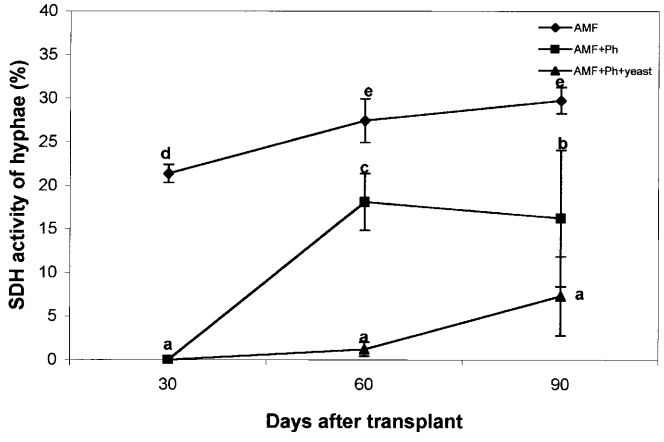

Fig. 2 Effect of phenanthrene and *R. glutinis* on the succinate dehydrogenase (*SDH*) activity of *G. geosporum*. This activity was not assessed for the *G. geosporum* plus *R. glutinis* treatment in the absence of phenanthrene. For notation and abbreviations see Fig. 1

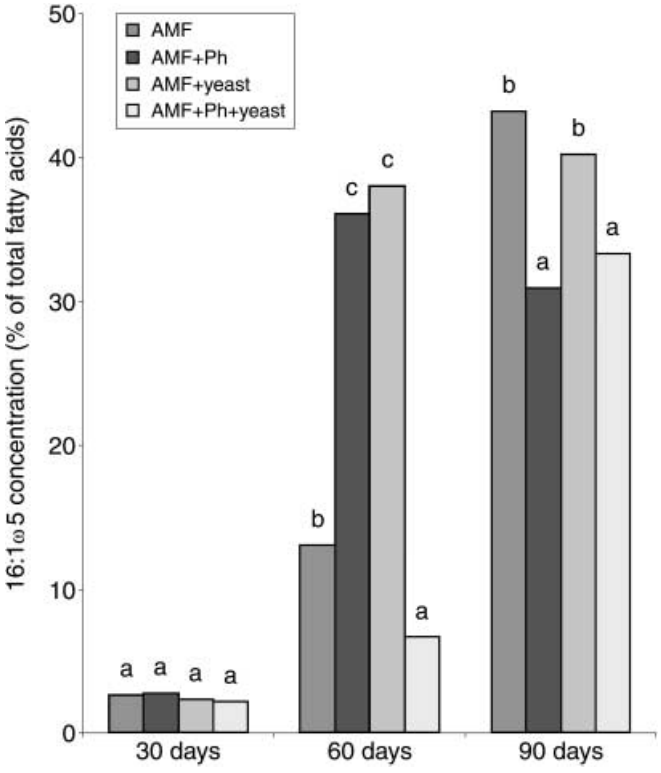

Fig. 3 Percentage of 16:1 ω5 of soil total lipids from the different treatments. Values are means for three separate harvests. The *same letter above bars within a harvest* indicates that the values do not differ significantly by one-way ANOVA and Tukey's HSD test ($P<0.05$). *AMF* Maize roots inoculated with *G. geosporum*, *AMF+Ph* maize roots inoculated with *G. geosporum* with the addition of phenanthrene to the substrate, *AMF+yeast* maize roots inoculated with *G. geosporum* and *R. glutinis*, *AMF+Ph+yeast* maize roots inoculated with *G. geosporum* with the addition of phenanthrene and *R. glutinis* to the substrate; for abbreviations, see Fig. 1

Table 1 Number of cells of *Rhodotorula glutinis* from the rhizosphere of maize plants inoculated with *Glomus geosporum*, in a substrate polluted or not polluted with phenanthrene. Data are means for three pots

R. glutinis (×10⁴ cells/ml)		
Incubation time (days)	Polluted soil	Non-polluted soil
0	1.03	1.03
5	1.80	3.12
30	0.62	3.60
60	1.25	6.75
90	2.16	11.00

The growth of *R. glutinis* diminished when the substrate was polluted with phenanthrene (Table 1). While the yeast cells showed a progressive increase in non-polluted substrate, the yeast population densities remained low in polluted substrate.

Maize plants inoculated with *G. geosporum* did have phenanthrene inside their roots (Fig. 4). The content of the toxicant in the roots differed with the presence of *R. glutinis* in the substrate. The uptake of phenanthrene by roots was greater when colonized plants were grown alone than when both yeast and *G. geosporum* coexisted in the rhizosphere. In contrast, colonized roots growing in a substrate with phenanthrene and *R. glutinis* took up more intermediate metabolites of phenanthrene degradation than colonized roots growing in a polluted substrate without the yeast. The phenanthrene content of spores collected 90 days after transplanting from polluted substrates inoculated or not inoculated with *R. glutinis* followed the same pattern found for colonized roots. The phenanthrene concentration in spores formed in the absence of yeast was twofold the values found in spores in

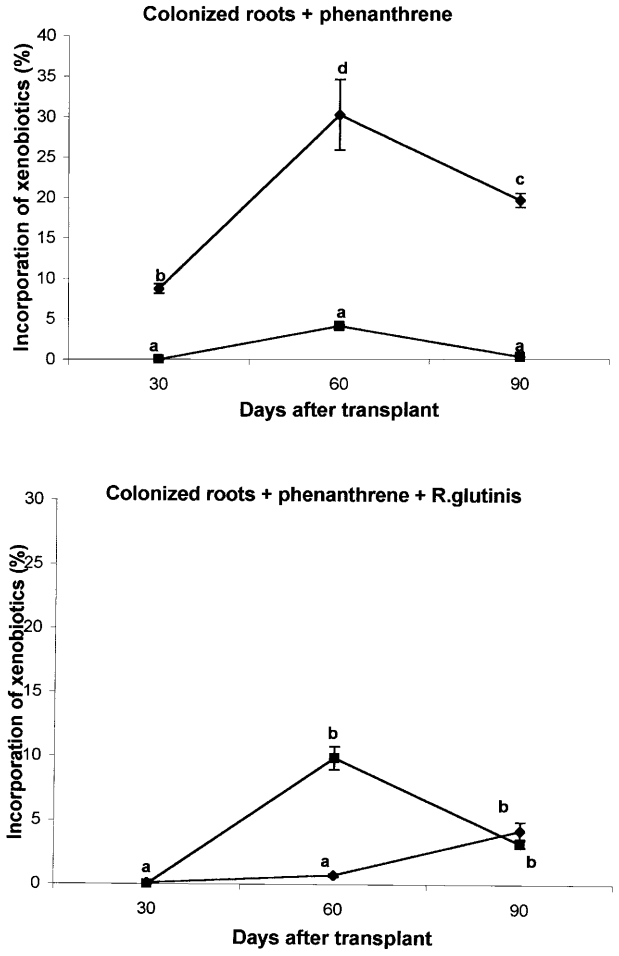

Fig. 4 Uptake of phenanthrene and other metabolites by colonized roots. Roots samples were extracted with ethyl acetate and xenobiotics were eluted by HPLC. ◆ phenanthrene, ■ non-resolved mixture. Values are means ±SD for three separate harvests. The *same letter above symbols* indicates that values do not significantly differ across time or treatment according to ANOVA and Tukey's HSD test ($P<0.05$). For abbreviations, see Fig. 1

polluted substrates inoculated with yeast. Intermediate metabolites of phenanthrene degradation could not be detected in spores.

Discussion

Microscopic observations of stained roots as well as the detection of the fatty acid 16:1 ω5 in the substrate indicated the ability of *G. geosporum* to grow in a substrate polluted with 0.1 g phenanthrene/kg. However, percentages of colonization were higher in controls than in treated substrates with either phenanthrene or phenanthrene and yeast. These results are in agreement with the data obtained by Cabello (1995, 1997) who found in field experiments that hydrocarbon contamination affects the AMF population associated with plants. Thus, the determination of AMF development in roots could be a sensitive indicator of changes in soil pollutant toxicity.

The hyphal SDH activity observed histochemically was used as an index of fungus metabolic activity. It followed the same pattern reported here for the percentage colonization. Significantly less SDH activity was observed in colonized roots isolated from all substrates polluted with phenanthrene than in roots obtained from non-polluted substrates. This reduction in SDH activity in phenanthrene soils evidenced not only a minor percentage of colonization but also a decline in respiratory activity. The decrease inactive hyphae could be because of a reduction in the transfer of host photosynthate to the fungus. Walton et al. (1994) suggested that when a chemical stress is present in soil, a plant may respond either by increasing or changing exudation (carbon allocation) to the rhizosphere. It is possible that our maize plants responded to the presence of phenanthrene in the soil by expending part of their photosynthate to produce root exudates, thereby diminishing the supply of carbon to mycorrhizal fungi. The fungus could be directly affected by the action of phenanthrene.

The fatty acid 16:1 ω5 has been used by several authors (Olsson et al. 1995, 1999) as a biomarker of *Glomus* sp. in soil. Our results showed that the amount of this fatty acid varied not only through time but also in response to the treatment of the substrates. When *G. geosporum* and *R. glutinis* coexist in soil without the toxicant present, the fungus species shows a progressive increase in external mycelium development. Singh et al. (1991) found that inoculation with yeast cells of *Saccharomyces cerevisiae* enhances symbiotic parameters such as spore number, formation of vesicles and arbuscules, and colonization. Another response is noted when *G. geosporum* lives with *R. glutinis* in soil with phenanthrene. Comparing the values obtained for the external mycorrhizal phase (measured by the biomarker 16:1 ω5) with those registered for the internal phase (measured by SDH activity and percentage of colonization), a decrease in fungus development when polluted soils were inoculated with yeast is revealed. Such mycorrhizal fungus behaviour might be because of the production of inhibitory metabolites by the yeast when it degrades phenanthrene in the soil. Intermediate metabolites of PAHs are more toxic than the parent compounds (Kremer and Anke 1997). Although it is known that some fungi have the capacity to degrade phenanthrene, and what metabolites are generated also are known (Sack et al. 1992, 1997; Cerniglia 1993; Cerniglia et al. 1992), there is only one report about the ability of *R. glutinis* to metabolize phenanthrene (Romero et al. 1998). No one has determined the toxicity of the products generated from phenanthrene biodegradation by *R. glutinis*. We speculate that phenanthrene biotransformation by *R. glutinis* produces metabolites that are toxic to mycorrhizal fungi.

Our results show that maize plants colonized with *G. geosporum* take up phenanthrene from the substrate, and that the presence of *R. glutinis* in the mycorrhizosphere reduces phenanthrene accumulation in roots. The low phenanthrene concentration in spores also suggests minor uptake by the external mycelium when the yeast is present.

In conclusion, we report for the first time that *G. geosporum* can take phenanthrene up from the soil and accumulate it in spores. However, when taken up by the hyphae, the toxin is not cleaved by any enzyme and is retained without modification. This demonstrates that, although *G. geosporum* mycelium cannot degrade phenanthrene, the fungus is able to remove the pollutant from the substrate. Such removal with bioimmobilization of the pollutant in spores may diminish the concentration of the free toxicant around roots in the soil. This could be of interest for the bioremediation of soils.

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