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Screening of ectomycorrhizal fungi for degradation of polycyclic aromatic hydrocarbons

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Abstract Ectomycorrhizal fungi belonging to 16 species (27 strains) were tested for their ability to degrade polycyclic aromatic hydrocarbons (PAHs): phenanthrene, chrysene, pyrene and benzo[*a*]pyrene. Cultivated on a complex liquid medium, most of the fungi tested were able to metabolise these compounds. Approximately 50% of the benzo[*a*]pyrene was removed by strains of *Amanita excelsa*, *Leccinum versipelle*, *Suillus grevillei*, *S. luteus*, and *S. variegatus* during a 4-week incubation period. The same amount of phenanthrene was also metabolised by *A. muscaria*, *Paxillus involutus*, and *S. grevillei*. The degradation of the other two PAHs was, for the most part, less effective. Only *S. grevillei* was able to remove 50% of the pyrene, whereas *Boletus edulis* and *A. muscaria* removed 35% of the chrysene.

Introduction

The widespread prevalence and high toxicity of polycyclic aromatic hydrocarbons (PAHs) make these compounds one of the most important groups of environmental pollutants (see, e.g., review by Edwards 1983). Accidental disposal of distillation wastes from the chemical industry has resulted in severe, long-lasting contamination of the soil in many areas. Such locations are usually confined and are characterised by a very high level of contamination at their centres. Surrounding regions display a slightly lower contamination level resulting from diffusion and leakage of PAHs. In addition, a good deal of PAHs has been spread through the environment via aerosols, e.g. adsorbed on coal dust; this has resulted in low-level contamination but in deposits over large areas. Many on-site physico-chemical techniques and also biological remediation methods (Wilson and Jones 1993; Shuttleworth and Cerniglia 1995) have been successfully tested for cleaning up the highly contaminated sites. The choice of method depends strongly on the proposed usage of any given detoxification area. In contrast, many large regions with lower concentrations of PAH cannot be detoxified in an economical way by removal of the contaminated soil or other on-site decontamination techniques; therefore, in-situ methods must be applied in such cases.

The in-situ activation of soil micro-organisms is limited by their inability to degrade highly condensed PAHs effectively. The organisms which have overcome this problem, wood-degrading white rot fungi (Bumpus et al. 1985; Hüttermann et al. 1988; Holroyd and Caunt 1994; Eggen and Majcherczyk 1998) cannot be maintained in the soil in-situ for the length of time necessary for decontamination. Thus, there is still a need for long-term but low cost in-situ biological methods.

The ability of ectomycorrhizal fungi to degrade lignin (Trojanowski et al. 1984; Haselwandter et al. 1990), has given rise to the possibility of applying this group of organisms to the biodegradation of aromatic pollutants (Donnelly and Fletcher 1994). This supposition has been confirmed in the past few years under laboratory conditions through the successful application of mycorrhizal fungi to metabolise chlorinated soil contaminants such as chlorpropham (Rouillon et al. 1989), atrazine, 2,4-dichlorophenoxyacetic acid (Donnelly et al. 1993; Gaskin et al. 1997), and polychlorinated biphenyls (PCB) (Donnelly and Fletcher 1995). The ability of these fungi to metabolise 2,4,6-trinitrotoluene (TNT) has also been reported (Meharg et al. 1997).

In the present, preliminary study we report on the ability of ectomycorrhizal fungi belonging to 16 species (27 strains) isolated from the Middle European forest to degrade four PAHs (phenanthrene, pyrene, chrysene and benzo[*a*]pyrene) in a pure, liquid culture.

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Materials and methods

Fungal cultures

Most of the cultures belong to the collection of the Institute of Forest Botany, University of Göttingen. Other fungi were isolated from newly collected fruiting bodies (several fungi were deposited by DSM) and still others were kindly donated by the Institute of Microbiology, University of Jena (Germany). Stock cultures of fungi were grown on modified Melin-Norkrans medium (Norkrans 1949; Marx 1969) solidified by 2% agar.

Culture media

The degradation of PAH was studied in liquid batch cultures using 15 ml medium in 500-ml Erlenmeyer flasks. Cultures were inoculated with three 0.5-cm^2 pieces of fungal agar cultures, closed with a cotton plug, and incubated at 22 °C for 4 weeks in the dark. All experiments were performed in five repetitions using three different media. Medium A was modified Moser medium (Moser 1958; Trojanowski et al. 1984), medium B was the same as the former but without peptone and medium C was a modified Melin-Norkrans medium.

Degradation of PAHs

Acetone solutions of PAHs (phenanthrene, pyrene, chrysene, and benzo[*a*]pyrene), each containing 1500 ppm of the respective compound, were sterilised by filtration through a 0.2-µm filter. 11.3 ml of this solution were mixed with 88.7 ml of the desired, sterile filtered medium containing 42.5% Tween 89.2 ml of these stock solutions were added to 4-week-old fungal cultures, resulting in 20-ppm final concentrations of each PAH. Control samples were left uninoculated. Cultures were incubated for another 4 weeks.

Extraction of PAHs from liquid cultures

The cultures were transferred to 100-ml bottles with Teflon-lined screw caps and flasks were rinsed twice with 3 ml acetone with the aid of an ultrasonic bath. PAHs were extracted by 10 ml isooctane containing 5 ppm fluoranthene and perylene as internal standards. After the addition of 20 ml water the samples were homogenised for 30 s using a high-speed homogeniser (Ultraturax TP 18/10, rod 10 mm, Janke und Kunkel, Germany); finally, the emulsion was shaken for 1 h. Aliquots of the samples were centrifuged and 0.5 ml of the isooctane phase was withdrawn for analysis.

Analysis of PAHs

Samples were analysed by gas chromatography and mass spectrometry (GC-MS) (GC 5890, MSD 5970B, Hewlett Packard, Palo Alto, USA). The column was a 16-m × 0.25-mm capillary with 5% phenyl-methyl-silicon (0.32 µm). Detection was by monitoring single ions (phenanthrene 178, 179, 186; pyrene: 100, 101, 202; chrysene: 226, 228, 229; benzo[*a*]pyrene: 125, 252, 253). One microlitre of the sample was injected using a KAS II cold injection system (Gerstel, Mülheim an der Ruhr, Germany). The initial GC temperature of 80 °C was maintained for 2 min, raised at a rate of 10 °C/min to 300 °C and then maintained for 1 min. The carrier gas was helium at 1 ml/min; the detector temperature was 280 °C.

Results

All fungi were cultivated and tested on three semi-defined media. Most of the fungi grew very slowly, and a pre-incubation period of 4 weeks was necessary to obtain a sufficient amount of biomass. Accordingly, the degradation time of 4 weeks was chosen to detect significant removal of PAHs. The semi-defined media selected were previously reported on in the context of mycorrhizal fungi cultivation. Medium A was a carbon- and nitrogen-rich medium and medium B was simply a modified version of A with nitrogen depletion. Medium C was a carbon-rich medium with additional glucose and only an inorganic nitrogen source. No optimisation of media for the individual fungus was performed; therefore, it was not surprising that the growth of the organisms differed among the media, with no clear, general preference.

The degradation ability did not correspond to the visible growth of mycelia in all cases. The best overall results were obtained on a complex medium (medium A, Fig. 1) with peptone and yeast extract: more than 50% of the fungi were able to degrade benzo[*a*]pyrene. The best degraders, *Amanita excelsa* and *Suillus luteus*, removed over 45% of this compound during a 4-week incubation period. Phenanthrene was removed by over 70% of the fungi, and the most efficient of them (*Amanita muscaria* strain VI) removed up to 50% of this compound. Over 65% of the tested ectomycorrhizal fungi degraded pyrene and over 55% degraded chrysene; 15% and 35% of these compounds were removed, respectively. No single best degrader was detected in the case of pyrene; the best degraders of chrysene were *Boletus edulis* and *A. muscaria* strain VI.

Some fungi did not show visible growth on medium B so these samples were not analysed. The remaining fungi metabolised benzo[*a*]pyrene even more efficiently in this medium: 60% of the fungi showed significant removal of this compound (Fig. 2). The metabolisation of approximately 50% was detected in cultures of *Leccinum versipelle* (DSM 13000), *Suillus grevillei* (strain I), *S. luteus* and *S. variegatus*. Only 30% of the fungi were able to metabolise phenanthrene in medium B; however, the best degraders (*Paxillus involutus* strain IV and *S. grevillei* strain I) removed over 50% of the latter compound. Pyrene and chrysene were metabolised by only 35% and 25% of the fungi, respectively. Only *S. grevillei* (strain I) removed as much as 50% of the pyrene; other degrading fungi showed less than 20% metabolisation of both these PAHs. Very low-grade removal of all PAHs was detected in medium C; in the best case no more than 10% of the compounds was removed (data not presented).

Discussion

The screening presented here demonstrates the ability of most of the ectomycorrhizal fungi tested to metabolise the polycyclic aromatic compounds independently of their condensation grade. No direct correlation was detected between the number of aromatic rings and the metabolisation. The removal of PAHs depends strongly

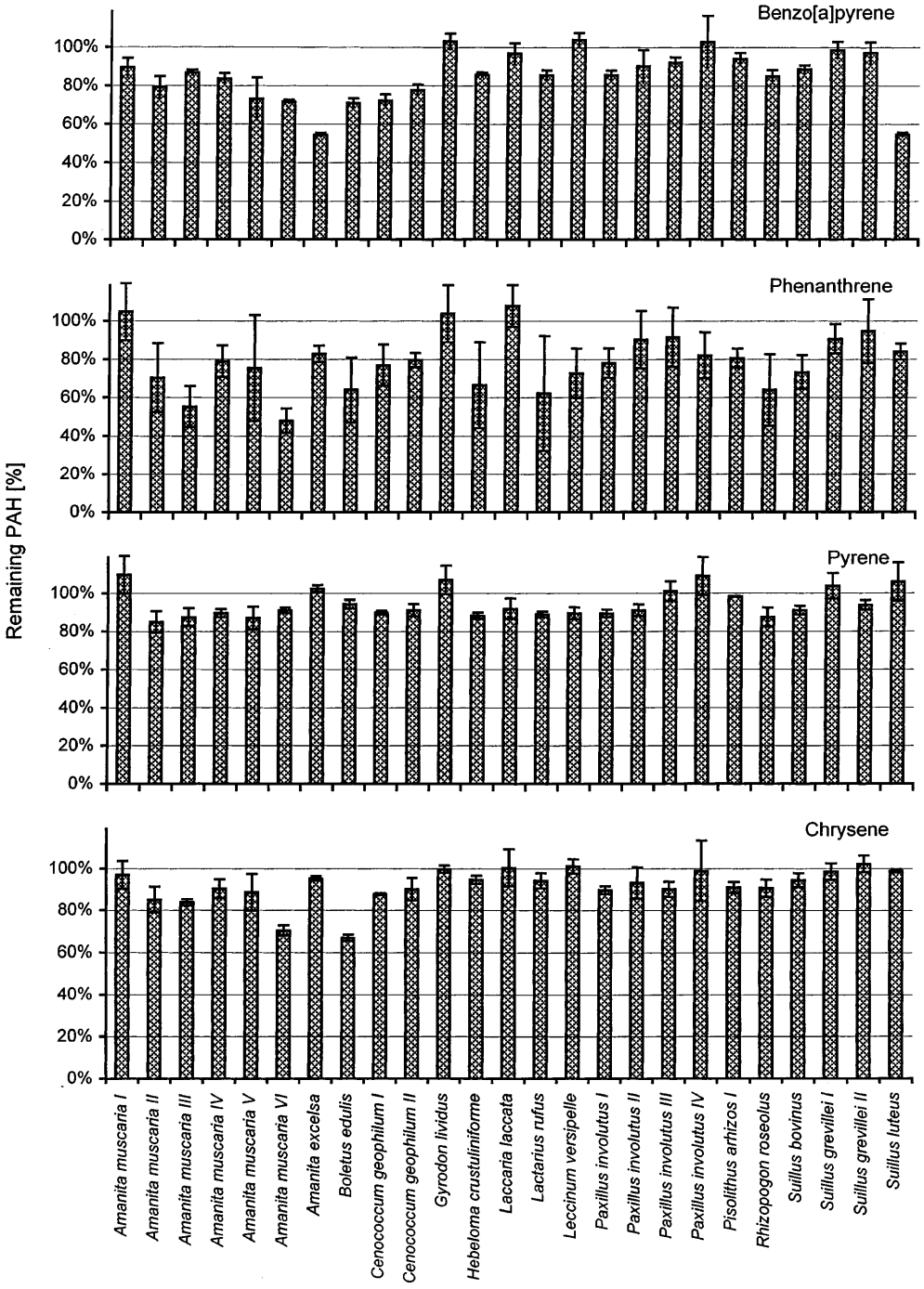

Fig. 1 Degradation of polycyclic aromatic hydrocarbons (PAHs) by ectomycorrhizal fungi: medium A

on the culture medium; however, no general, significant correlation between nitrogen depletion and degradation was found for the fungi tested.

The slow but very efficient metabolisation of the most toxic PAH, benzo[*a*]pyrene, was comparable to results obtained by experiments with white rot fungi (Higson 1991; Schützendübel et al. 1999). This result may correspond to the low redox potential of this compound; however, no attempt was made at this stage to differentiate between the extracellular and intracellular degradation processes. The surprisingly high rate of metabolisation of phenanthrene in comparison to pyrene could indicate that at least the former compound could be metabolised intracellularly, as is the case with other basidiomycetes (Hammel et al. 1992; Sutherland et al. 1993). The degree of mineralisation and metabolites were not studied in the present work; the commonly employed extraction procedure used here excludes the losses of PAHs by physical adsorption at the mycelia.

The ability to degrade various litter and humus components such as hemicellulose, cellulose and lignin has often been reported, as has the hydrolysis of chitin

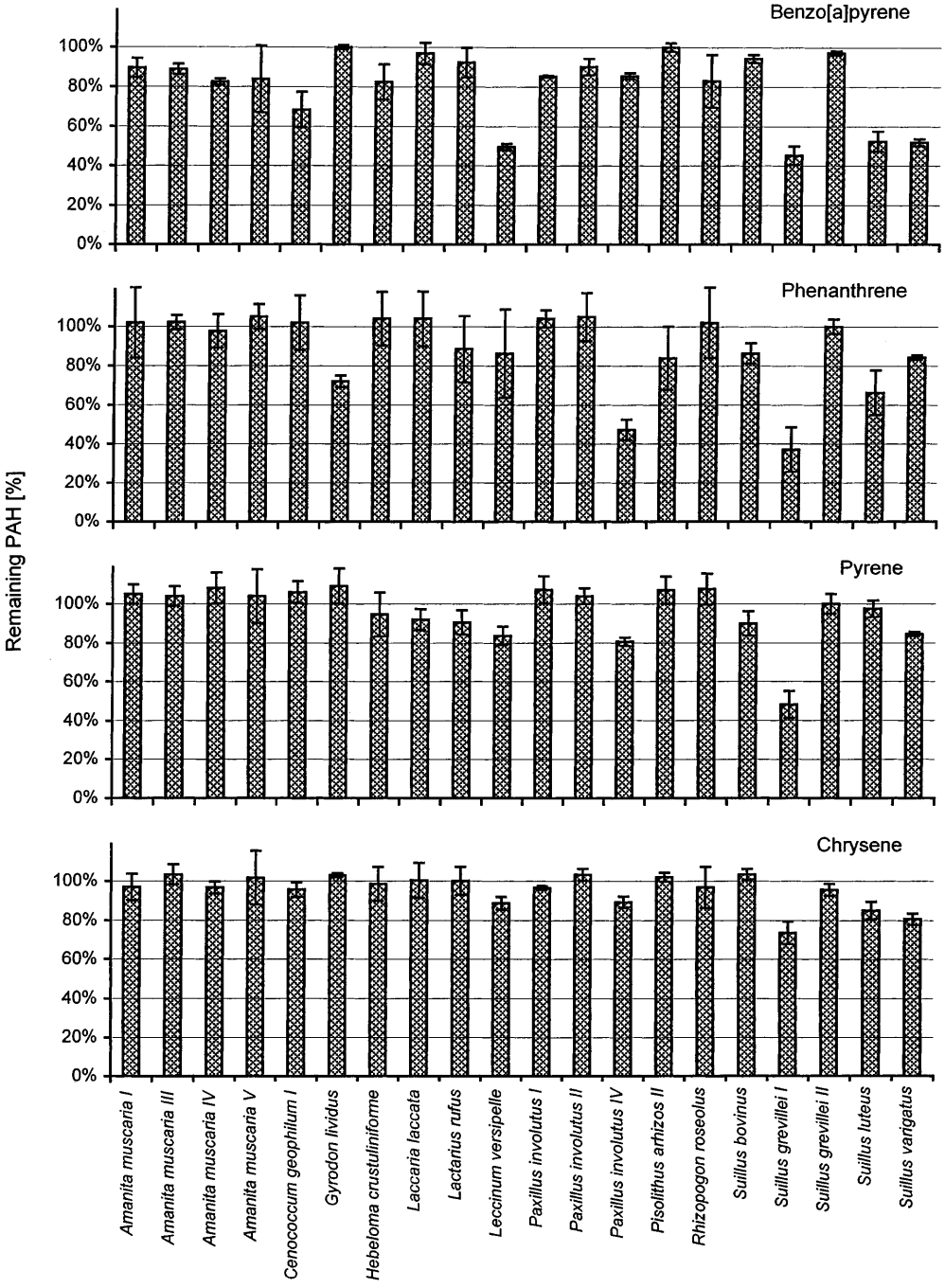

Fig. 2 Degradation of PAHs by ectomycorrhizal fungi: medium B

and proteins, but nevertheless this ability does not seem to be universal among all mycorrhizal fungi (Hutchison 1990; Durall et al. 1994; Kerley and Read 1995). Numerous ectomycorrhizal fungi grown in pure culture secrete oxidative enzymes such as polyphenoloxidases, laccases, tyrosinases or peroxidases (Cairney and Burke 1994; Colpaert and Van-Laere 1996; Bending and Read 1997; Gramss et al. 1998). The production of the latter group of enzymes has been reported as a possibility but has not yet been convincingly proved (Cairney and Burke 1998). The activity of carbohydrate oxidases, production of H_2O_2 and hydroxyl (Fenton) radicals ($OH^{\cdot}$) may also play an important role in the degradation of lignocellulose (Burke and Cairney 1998) and aromatic pollutants. Phenol oxidising enzymes were also secreted by fungi grown in symbiosis with plants (Gramss et al. 1998). The involvement of the extracellular oxidative enzymes in the degradation of environmental pollutants has not been proved but seems probable. This assumption is similar to that made with regard to wood-rotting basidiomycetes.

The potential of the ectomycorrhizal fungi to degrade PAHs and their ability to inhabit soil as symbionts of plants could be very promising in the recultivation and

bioremediation of contaminated sites. Purposeful afforestation with trees inoculated with the desired fungi could establish the mycorrhiza in the soil and allow gradual decontamination over a period of several years. This could be especially important in the case of low-level contaminated soil if there is no acute human risk and slow, long-term decontamination is acceptable. The lignolytic activity of ericoid mycorrhizal fungi has been reported to be even higher than that of the ectomycorrhizal organisms (Haselwandter et al. 1990; Bending and Read 1997). The studies on the degradation of environmental pollutants cited here should be expanded to include this group of fungi and their symbiosis.

The detoxification of pollutants such as PAHs is expected to proceed even more readily in complex cultures that prevent an accumulation of intermediate or dead-end metabolites and increase the degradation rate of the contaminants (Brodkorb and Legge 1992; in der Wiesche et al. 1996). The contamination originating from industrial sources is often very complex and may also involve different chemical classes of compounds, e.g. aromatic and aliphatic contaminants, simultaneously. The application of mycorrhizal fungi for biodegradation as symbionts of plants could help in establishing suitable complex, degradative consortia in the root area. An excellent example of such a system was recently presented by Sarand et al. (1998), who reported that the colonisation of hydrocarbon-contaminated soil by fungal hyphae emanating from mycorrhizal roots resulted in the development of a pseudoparenchymatous patch supporting a hydrocarbon-degrading bacterial biofilm.

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