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A rapid method to screen fungi for resistance to toxic chemicals

Bruce C. Alleman,[1] Bruce E. Logan[2] & Robert L. Gilbertson[3]
[1] *Environmental Technology Department, Battelle Memorial Institute, Columbus, Ohio 43201–2693, USA;* [2] *Environmental Engineering Program, Department of Civil Engineering and Engineering Mechanics, University of Arizona, Tucson, AZ 85721, USA;* [3] *Department of Plant Pathology, University of Arizona, Tucson, AZ 85721, USA*

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Abstract

Although many species of fungi are able to degrade highly toxic chemicals, only a few species have been evaluated for resistance to toxic effects of these chemicals. In this paper we demonstrate the successful application of a method to rapidly screen several species of fungi for toxicity to chemicals or mixtures of chemicals using pentachlorophenol (PCP) as a model toxic compound. Cellulose antibiotic assay disks were soaked in solutions containing different concentrations of PCP (5, 10, 25, 50, and 80 mg l^{-1}) and then placed in a triangular pattern outside the growing edge of the mycelia of eighteen species of white rot fungi. The plates were incubated and observed for development of inhibition zones (non-growth areas) around the disks. The short-term (24 h) growth of all eighteen species of fungi was inhibited by 5–10 mg-PCP l^{-1}, a range similar to that observed using previously reported techniques. Long-term growth studies using this screening method were not useful since PCP diffused from the disk into the agar, decreasing the applied dose.

Introduction

White-rot fungi are capable of degrading a wide variety of environmental pollutants including complex multi-ring compounds such as anthracene, chlorinated aromatic compounds such as pentachlorophenol (PCP), several alkyl halide insecticides, and complex chloro-organics found in pulp mill wastewaters (Bumpus et al. 1985; Hammel et al. 1986; Bumpus & Aust 1987a, b; Mileski et al. 1988; Pellinen et al. 1988; Kennedy et al. 1990). Fungi are able to degrade these chemicals extracellularly using ligninase and manganese-dependent enzymes (Michel et al. 1991; Perie & Gold 1991). It has been proposed to use white-rot fungi for *in situ* bioremediation of contaminated soils, but most research has focused on the use of only one species, *Phanerochaete chrysosporium* (Ryan & Bumpus 1989; George & Neufeld 1990; Lamar & Dietrich 1990). However, a large number of fungi can potentially be used to treat contaminated wastewaters and soils. These fungi differ in optimum growth conditions such as temperature, pH, production rate of manganese and lignin peroxidases, and their resistance to toxic chemicals (Rucksdeschel & Renner 1986; Alleman 1991; Alleman et al. 1992a; Alleman et al. 1992b; Lamar et al. 1990).

Typical procedures used to screen fungi for resistance to toxic levels of chemicals can require a large number of flasks and can be very time consuming (Ruckdeschel & Renner 1986). In addition, tests of fungal growth in liquid media may not reflect the ability of mycelia to penetrate and overgrow surfaces and particles contaminated with chemicals. This

latter condition could be more typical of mycelial growth in contaminated soils treated with fungi during site bioremediation than mycelial growth in liquid media. In order to identify species of fungi most resistant to chemicals under a variety of temperatures, pHs and soil conditions, we examined the usefulness of a screening procedure originally developed to screen microorganisms for antibiotic resistance. We found that this procedure was much simpler than more recent procedures developed by others (Lamar et al. 1990) since the chemicals used did not need to be dissolved in hot agar, and the toxic doses determined in our tests were comparable to previously reported values (Mileski et al. 1988; Lamar et al. 1990).

Materials and methods

Eighteen species of white-rot fungi were selected for testing based on exceptional ligninase activity (Table 1). *P. chrysosporium* ME-446 and *Trametes versicolor* 697 were obtained from the U.S. Forest Products Laboratory, Madison, Wisconsin. All other species were obtained from the University of Arizona Mycological Collection, Dept. of Plant Pathology, University of Arizona, Tucson. Cultures were maintained at room temperature by aseptically cutting and transferring a piece of agar (2% malt extract) from a mature slant with a dissecting needle to fresh media every 30 to 60 days. Fungal mats used in experiments were developed on agar starter plates, containing, per liter of distilled water: 20 g glucose, 15 g l^{-1} agar, 2.5 g sodium citrate, 5.0 g K_2SO_4, 2.0 g NH_4NO_3, 0.068 g $CaCl_2$, 0.2 g $MgSO_4$, and 1 mg of thiamine (Kirk et al. 1977). Starter plates were inoculated from slants by placing each fungus in the plate center and allowing the cultures to grow radially outward. Plates were used after the cultures grew to approximately 30 mm in diameter.

The screening method for examining the tolerance of fungal mats to PCP was developed by modification of a procedure used to test for antibiotic resistance (Pelczar et al. 1977). Cellulose antibiotic assay disks (No. 740-E, Schleicher & Schuell) were soaked in solutions containing 5, 10, 25, 50, and 80 mg-PCP l^{-1}. A 5-mm plug of fungi was cut from the growing edge of a culture and transferred to fresh plates containing agar and media. When plated cultures developed to approximately 4 cm in diameter, three PCP-saturated disks (at a single concentration) were placed in a triangular pattern outside the growing edge of the mycelium. The plates were incubated at room temperature (approximately 25 °C) and observed for development of inhibition zones (non-growth areas) around the PCP-saturated disks. Additional disks soaked in media containing 80 mg-PCP l^{-1} were incubated on sterile agar plates for 24 days in order to monitor abiotic PCP losses.

PCP was measured using high performance liquid chromatography (HPLC, Beckman) equipped with a C-18 reverse phase column (4.6 × 250 mm, 5µm-Econosphere C-18, Alltech Assoc.). PCP was eluted with a 75:25:0.125 mixture of acetonitrile:water:acetic acid, monitored at 238 nm, and concentrations determined by comparing resulting peak area counts to a standard calibration curve (Mileski et al. 1988). PCP, acetonitrile, and methanol were obtained from Aldrich Chemical Company (Milwaukee, WI).

Table 1. Species selected for PCP toxicity testing.

Species	Isolate
Phanerochaete chrysosporium	ME 446
Trametes versicolor	MAD 697
Ganoderma colossum	NHC 2959
Ganoderma zonatum	JPL 1847
Ganoderma lobatum	RLG 16384
Ganoderma sp. (on mesquite)	RLG 16295
Ganoderma oregonense	RLG 16381
Ganoderma lucidum	RLG 16400
Inonotus rickii	RLG 16385
Inonotus dryophilus	RLG 16297
Inonotus arizonicus	RLG 16346
Phellinus badius	JEA 1047
Phellinus texanus	JEA 1049
Phellinus robustus	RLG 16373
Perenniporia fraxinophila	JF 17898
Perenniporia ohiensis	JF 17894
Perenniporia medulla-panis	JEA 832
Perenniporia phloiophila	MB 2405

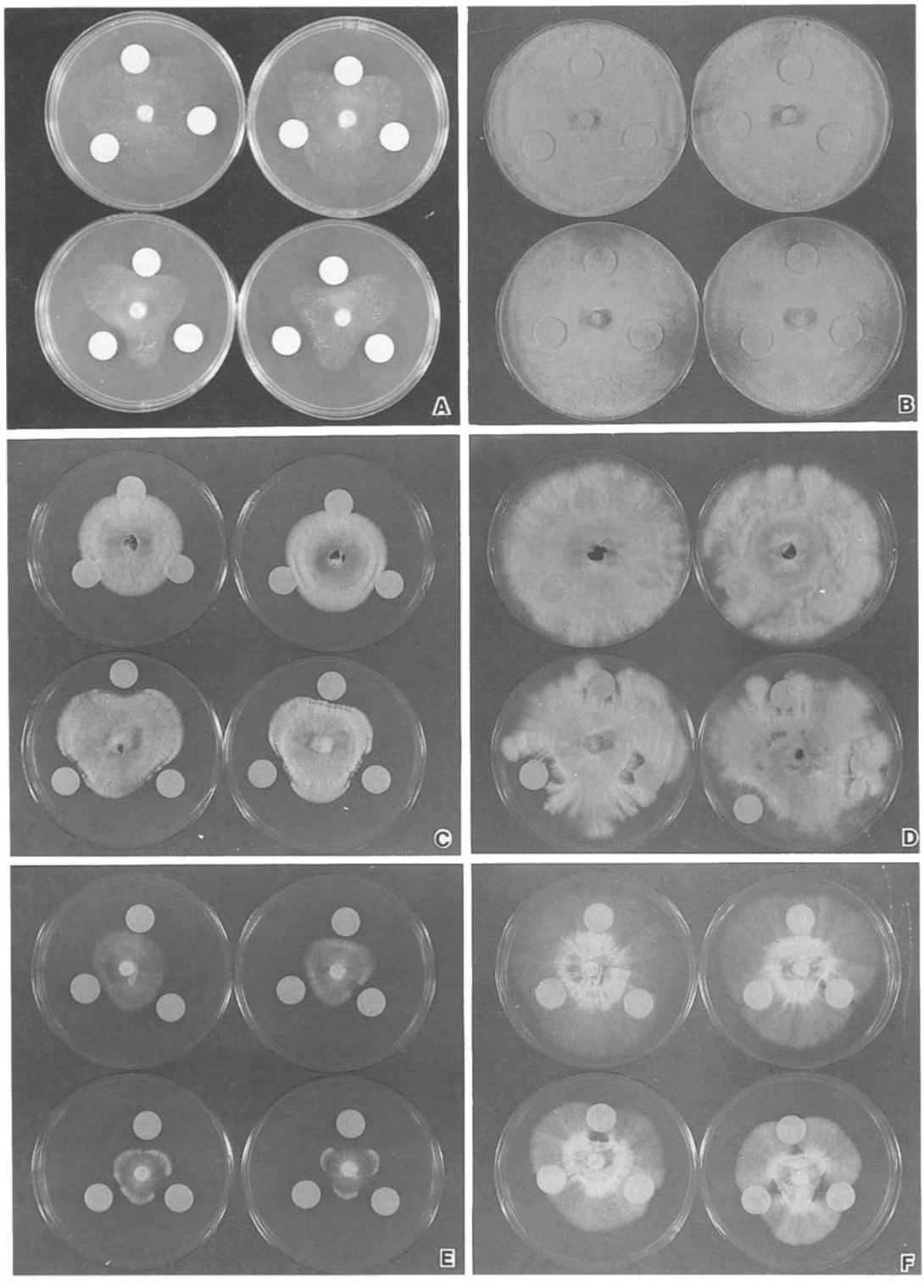

Fig. 1. Agar plates showing inhibition zones after 24 h for (A) *Phanerochaete chrysosporium*, (C) *Trametes versicolor*, and (E) *Ganoderma zonatum*; same, but after 2 weeks for (B) *P. chrysosporium*, (D) *T. versicolor*, and (F) *G. zonatum*.

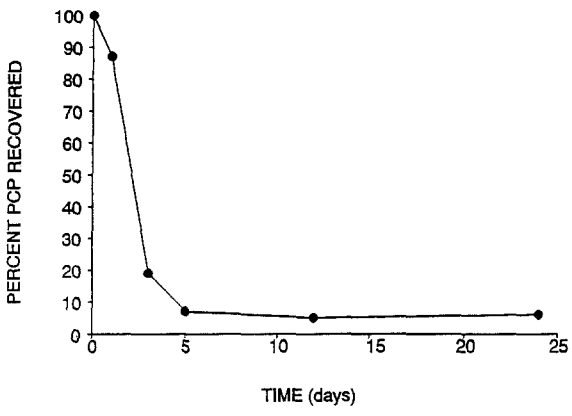

Fig. 2. The percent of PCP recovered from disks soaked in 80 mg-PCP l^{-1} and incubated on agar plates at room temperature (25 °C).

Results and discussion

All 18 species of fungi were able to overgrow disks containing 5 mg-PCP l^{-1} within 24 h. However, the spread of all species was inhibited when disks were saturated with 10 mg l^{-1} or more of PCP. Inhibition of growth was evidenced by the presence of clear zones around the disks (Fig. 1). Within 14 days after inhibition zones were observed, 17 of the 18 species had overgrown the disks at every PCP concentration ($\leq$ 80 mg l^{-1}). Only *Ganoderma lobatum* (not shown) failed to overgrow disks with PCP at 10 mg l^{-1} or higher. For this species, zones of inhibition remained apparent around all of the disks, indicating that this species was the most sensitive to PCP. Increased biomass production on the PCP disks was noticed for several species after 14 days and is believed to be due to the utilization of the cellulose in the disks (Fig. 1b, d).

This screening procedure is limited to short-term studies of chemical toxicity since even non-volatile chemicals like PCP can diffuse into the agar. Analysis of residual PCP on control disks (disks soaked in media containing 80 mg-PCP l^{-1} and incubated in plates without fungi) showed that there was rapid loss of PCP from the disks (Fig. 2). By approximately the fifth day, a constant PCP distribution was established between the disks and agar medium. The eventual overgrowth of the PCP-disk area after 14 days is probably due to lower, and less toxic, concentrations of PCP in the agar with time.

The inhibition of growth at a critical concentration of 5 to 10 mg-PCP l^{-1} is similar to critical concentrations identified by others, although results between investigators vary somewhat due to the different strains and techniques used. Mileski (1988) reported that spore germination of *P. chrysosporium* was prevented at PCP concentrations as low as 4 mg l^{-1}. Lamar et al. (1990) found several species of white rot fungi were unable to grow on a 2% malt agar impregnated with 5 mg-PCP l^{-1}, but that *P. chrysosporium* and *P. sordida* were able to grow in agar containing 25 mg-PCP l^{-1}. Recent studies have shown that it is the PCP dose (μg-PCP mg-mycelia^{-1}), and not the concentration (μg l^{-1}) that determines whether fungi can grow and degrade PCP (Alleman et al. 1992b). Therefore, the use of different masses of mycelia will produce some variability in results from different laboratories.

Screening of different fungi is essential to optimize degradation of compounds in contaminated soils. Most of the fungal biodegradation studies have focused on fast-growing fungi, such as *P. chrysosporium*. However, the present study suggests that many slower-growing species of fungi may also be suitable for use. Further examination of dose-response relationships for alternative strains of fungi is being conducted to improve removals of mixtures of chemicals under a variety of field conditions (i.e. pH, temperature, and presence of metals) likely to be encountered in field bioremediation projects.

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