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Pentachlorophenol degradation by the fungus *Leptosphaeria maculans*

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Abstract. In the present investigations, biodegradation of pentachlorophenol (PCP) by the fungus, *Leptosphaeria maculans* was demonstrated by the disappearance and mineralization of [^{14}C]PCP in nutrient nitrogen-limited culture. Mass balance analyses demonstrated the formation of water-soluble metabolites of [^{14}C]PCP during degradation. Toxicity studies showed that PCP concentrations above 4 mg/liter (15 μM) prevented growth when fungal cultures were initiated by inoculation with pycnidiospores. The lethal effects of PCP could, however, be circumvented by allowing the fungus to establish a mycelial mat before adding PCP. With this procedure, the fungus was able to grow and degrade [^{14}C]PCP at concentrations as high as 500 mg/liter (1.9 mM).

Key words: Biodegradation; Degradation; *Leptosphaeria maculans*; Pentachlorophenol.

Introduction

Pentachlorophenol (PCP) has been used extensively as a wood preservative, fungicide, bactericide, herbicide, molluscicide, algicide and insecticide (Crosby, 1981). Although numerous reports have shown that PCP undergoes biodegradation, its biodegradation in the environment is, in fact, often slow (Arjmand and Sanderman, 1985; Bumpus and Aust, 1987 a, b, and c; Bumpus & Brock, 1988; Bumpus *et al*, 1985 and Eaton, 1985). This, coupled with its extensive use, has led to the contamination of many terrestrial and aquatic ecosystems worldwide. The white rot fungus *Phanerochaete chrysosporium* has been shown to degrade a wide variety of environmentally persistent organopollutants, including a number of organohalides (Eaton, 1985). In the present study, biodegradation of pentachlorophenol (PCP) by a fungus, *Leptosphaeria maculans* is reported.

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Materials and Methods

Fungus

Leptosphaeria maculans was isolated from the stubbles collected from Canola fields near Glenlea, Agriculture Canada Research Branch, Winnipeg. The fungus was maintained on V_8 agar slant cultures at 4°C and was subcultured every 30 days.

Chemicals

Pentachlorophenol (PCP) was obtained from Aldrich Chemical Co., Milwaukee, Wisc., U.S.A. ^{14}C-labeled PCP (10.57 mCi/mmol) and D-glucose (1.88 mCi/mmol) were obtained from Pathfinder Laboratories, Inc., St. Louis, Missouri, U.S.A.

Culture Conditions

To determine PCP disappearance, mineralization, or toxicity, stationary-phase cultures of *L.*

maculans were incubated at 28±2°C in 10 ml of nutrient nitrogen-limited culture medium containing 56 mM glucose, trace elements, thiamine-HCl (1 mg/l) and 1.0 mM di-ammonium tartarate in 20 mM 2,2-dimethylsuccinate buffer, pH 4.5. Nutrient nitrogen-sufficient cultures were grown in the same medium except that the ammonium tartarate concentration was 10 mM.

Cultures were incubated with 1 ml of pycnidiospore suspension (1.6 x 10^5 pycnidiospores/ml) grown under ambient atmosphere for 4 days, and then flushed every 4 days with oxygen (99.9%). In one experiment [^{14}C] PCP was added to the cultures immediately after inoculation. All other experiments with PCP were initiated by adding the PCP in a small amount of acetone (100 μl) to cultures after 7 days of growth in order to overcome toxicity of PCP to pycnidiospore germination, which occurs at levels above 4 mg/l (15 μM). Cultures were grown in medium bottles (250 ml) sealed with Teflon-lined screw-caps.

Mineralization of PCP and Respiration of Glucose

To measure the evolution of $^{14}CO_2$ from cultures containing either [^{14}C]PCP or [^{14}C]glucose, the caps of the incubation flasks were modified to include gas exchange manifolds as described (Bumpus *et al.*, 1985). Every 4 days the headspaces of the incubation flasks were flushed for 20 min. with oxygen (99.9%) and the CO_2 evolved was trapped in 10 ml of a solution containing ethanolamine-methanol-Safety-Solve scintillation cocktail (1:4:5). The amount of $^{14}CO_2$ trapped was determined by liquid scintillation spectrometry.

PCP Concentration

The amount of PCP remaining in cultures was determined by HPLC with C-18 reverse column (4.6 x 250 mm, Waters Associate Inc., U.S.A.) R-Sil.-C -18, after homogenization and extraction the method of which are described later.

PCP was eluted from the column with acetonitrile -H_2O-glacial acetic acid (65:35:0.125) at a flow rate of 1 ml/min. Elution was monitored at 238 nm and quantitated by peak area with a Hewlett Packard digital integrator. A standard curve for PCP was determined daily by plotting the peak area obtained from the integrator versus known standard amounts of PCP injected.

At the time of harvest, 1 μmol of sodium azide was added to the cultures to inhibit enzyme activity in the fungus. The harvested cultures were stored at -20°C until they were homogenized in a blender. Culture flasks were rinsed with ethanol (10 ml) followed by water (20 ml), and the rinses were combined with the homogenates and stored at -20°C. PCP was extracted from 0.5 - 5 ml samples of culture homogenates with n-hexane (0.5 to 2 ml) following the addition of 1 ml of H_2O saturated with NaCl.

Mass Balance Experiments

Nutrient nitrogen-limited and nutrient nitrogen -sufficient cultures of *L. maculans* were established in 250 ml bottles equipped with a gas exchange manifold and allowed to grow for 4 days under an atmosphere of air and 4 days under an atmosphere of O_2, then after which time [^{14}C]PCP (13.6 nmol) was added in 10 μl acetone. The cultures were then flushed with oxygen and incubated for 4 more days, after which the amount of $^{14}CO_2$ evolved was assayed as described above. The mycelium was separated from the aqueous fraction by filtration. The cultures were homogenized and homogenized material was then extracted with hexane (50 ml; 2 x), hexane portions were pooled. Following the hexane extraction, the aqueous phase was acidified to pH 2.0 with conc. HCl and extracted with methylene chloride (50 ml, 2 x), acidic methylene chloride fractions were pooled. Safety-Solve (10 ml) was then added to the recovered mycelium in a scintillation vial. Safety-Solve (10 ml) was also added to 1 ml samples of the hexane, methylene chloride, and aqueous fractions. The radioactivity of all fractions was determined by liquid scintillation spectrometry.

Results

It is obvious from the experimental findings (Fig. 1) that PCP underwent rapid and extensive degradation in nutrient nitrogen-limited cultures of *L. maculans*. At an initial concentration of 1.1 mg/l (4.1 μM), more than 90% decrease in PCP concentration was observed in 96 h of incubation relative to uninoculated controls.

PCP biodegradation by *L. maculans* was also demonstrated by degradation of [^{14}C]PCP. Fig. 2 shows that 21% of the [^{14}C]PCP had been degraded after 28 days of incubation in nutrient-nitrogen limited cultures of *L. maculans*. It should be noted that the

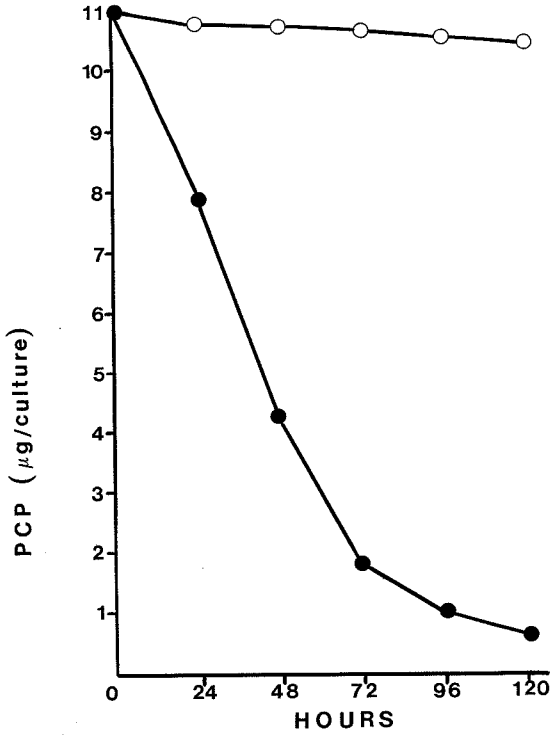

Fig. 1. PCP degradation by cultures of *L. maculans*. Nutrient-nitrogen-limited (1.0 mM ammonium tartarate) cultures of *L. maculans* (●—●) containing PCP were incubated for the specified periods. (○—○) uninoculated sterile control containing PCP.

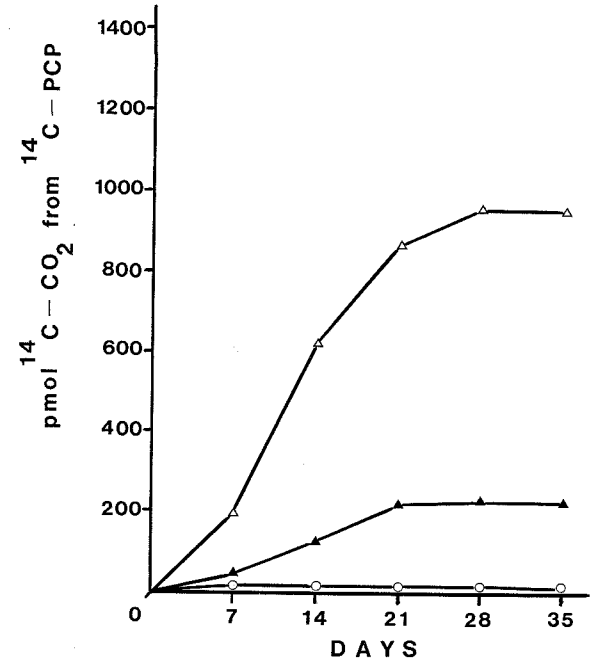

Fig. 2. Effect of nutrient nitrogen on PCP mineralization by *L. maculans*. Cultures containing either 10 mM ammonium tartarate (▲: nitrogen sufficient) or 1.0 mM ammonium tartarate (△: nutrient nitrogen limited) were incubated with 5.0 nmol of [^{14}C]PCP. Values are the means for triplicate cultures. (○) Uninoculated sterile control incubated with 5.0 nmol of [^{14}C]PCP.

amount of $^{14}CO_2$ evolved from [^{14}C]PCP in nutrient-nitrogen-limited cultures was variable between experiments. In some experiments the amount of $^{14}CO_2$ evolved was greater than 45%. This type of variation is similar to that observed during [^{14}C]DDT mineralization (Bumpus and Aust, 1987b) under these conditions. In addition, degradation of [^{14}C]PCP was suppressed when nutrient nitrogen was not limiting.

Mass balance experimental results (Table 1) showed that, in nutrient nitrogen-limited cultures, extensive degradation of [^{14}C]PCP occurred, as evidenced by the fact that 48.2% of the recovered radioactivity was present as $^{14}CO_2$ and 22.5% was present as water-soluble metabolites of [^{14}C]PCP. Only 2.3% of the recovered radioactivity had been incorporated into the fungal mycelium. Mass balance results also showed that substantial biodegradation of [^{14}C]PCP occurred in nutrient nitrogen-sufficient cultures of *L. maculans*, since 9.5% of the recovered radioactivity was present as $^{14}CO_2$ while 24.8% was present as water-soluble metabolites and 7.6% had been incorporated into fungal mycelium.

Preliminary results showed that PCP concentration of 4 mg/l (15 μM) or higher prevented growth when cultures of *L. maculans* were initiated with pycnidiospores. However, it was found that if cultures were allowed to establish a mycelial mat before the addition of PCP, the lethal effects of PCP could be circumvented. To study possible toxicity of PCP on fungal respiration (as measured by metabolism of [^{14}C] glucose to $^{14}CO_2$), cultures of *L. maculans* were incubated with [^{14}C] glucose and concentrations of PCP between 0 and 500 mg/l (1.9 mM) (Table 2). It is obvious from the results that, except for nitrogen-limited cultures containing 500 mg/l of PCP, increasing PCP concentration had remarkably little ability to inhibit fungal respiration, as determined by measuring the total amount of [^{14}C]glucose metabolized to $^{14}CO_2$

Table 1. *Mass balance analysis of* ***Leptosphaeria maculans*** *cultures for 7 days after addition of* [^{14}C]*PCP to 7-day-old cultures*

Fraction	% of radioactivity recovered*	
	N-limited Cultures	N-sufficient Cultures
Hexane	7.8	48.2
Acidic CH_2Cl_2	12.9	3.8
Aqueous	22.5	24.8
Mycelium	2.3	7.6
CO_2	48.2	9.5

*The total radioactivity recovered from nutrient nitrogen-limited and nutrient nitrogen-sufficient cultures was 75 and 69%, respectively.

Table 2. *Effect of PCP on respiration of* ***Leptosphaeria maculans***[a]

PCP conc. mg/l (mM)	Mean amount of [^{14}C]glucose converted to $^{14}CO_2$ during 28 days of incubation[b]	
	N-limited Cultures	N-sufficient Cultures
0 (0)	29.7±10.8	36.9±4.5
1 (0.0038)	40.8± 7.6	39.5±2.1
10 (0.038)	26.2± 4.9	37.8±1.8
50 (0.190)	25.9± 2.8	38.6±2.9
100 (0.38)	23.8± 4.5	39.1±2.1
500 (1.9)	0.5± 0.25	39.4±4.1

[a] [^{14}C]glucose (53.2 nmol, 100 nCi) and the indicated amount of PCP were added to 7 day-old cultures. Cultures were then incubated for another 28 days.

[b] Values (nanomoles) represent means±the standard deviations for triplicate cultures.

Table 3. *Effect of initial PCP concentration on the rate of its degradation by* ***Leptosphaeria maculans***

Initial conc. of PCP, mg/l (mM)*	Mean amount (nmol±S.D.) of PCP degraded in 28 days of incubation	
	N-limited Cultures	N-sufficient Cultures
1 (0.0038)	18.0± 2.1	3.8± 0.25
10 (0.038)	170.4±11.5	52.4± 5.8
50 (0.190)	460.2±13.8	123.8± 7.9
100 (0.38)	820.8±30.5	276.2±11.9
500 (1.9)	1260.5±23.9	878.5±12.1

*The indicated amount of PCP (containing 155 nCi of [^{14}C]PCP was added to 7 day-old cultures. Cultures were then incubated for another 28 days.

during the 28-day incubation period. The amount of PCP degraded increased with increasing PCP concentrations under both nutrient nitrogen-limited and sufficient conditions (Table 3).

Discussion

Experimental results indicate that *L. maculans* is able to degrade PCP. Biodegradation was demonstrated by disappearance, intermediary product formation, degradation of [^{14}C]PCP by mass balance analyses. It is interesting that, relative to other environmentally persistent organohalide pollutants [^{14}C]PCP underwent a greater degree of degradation under the same culture conditions (Bumpus and Aust, 1987c). It is also interesting that even under nutrient nitrogen-sufficient conditions, substantial degradation of [^{14}C]PCP occurred. In fact, the amount of [^{14}C]PCP mineralized in nutrient nitrogen-sufficient cultures typically was similar in magnitude to the amount of degradation observed for a number of other environmentally persistent chemicals under nutrient nitrogen-limited conditions. PCP has been shown to be present in wood treatment plant wastewater effluents at concentrations ranging from 25 to 150 mg/l (about 95 to 570 μM) (Rao, 1978 and Rochkind *et al.*, 1986). Concentrations of upto 500 mg/Kg of a technical mixture of chlorophenols in soil from a wood treatment facility have been reported (Thompson & Dust, 1971; Valo, *et al.*, 1984 and Kirk *et al.*, 1989) and even higher concentrations may be present in soils at other wood treatment facilities.

It is obvious from the experimental findings that *L. maculans* may be useful in the degradation of hazardous organochemical wastes in some waste treatment systems. However, it seems paradoxical to suggest use of this fungus to degrade PCP, since PCP has been used quite successfully as a wood preservative for many years by inhibiting the growth of wood-rotting fungi.

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Literature Cited

Arjmand, M. and H. Sandermann, Jr. 1985. Mineralization of

chloroaniline/lignin conjugates and of free chloroanilines by the white rot fungus *Phanerochaete chrysosporium*. J. Agric. Food Chem. **33**: 1055-1060.

Bumpus, J. A. and S. D. Aust. 1987a. Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system. BioEsays **6**: 166-170.

Bumpus, J. A. and S. D. Aust, 1987b. Biodegradation of DDT [1.1.1-trichloro-2,2-bis(4-chlorophenyl)ethane] by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **53**: 2001-2008.

Bumpus, J. A. and S. D. Aust. 1987c. Mineralization of recalcitrant environmental pollutants by a white rot fungus, *In* Proceedings of the National Conference on Hazardous Wastes and Hazardous Materials. Control Research Institute, Silver Spring, Md., p.146-151.

Bumpus, J. A. and B. Brock. 1988. Biodegradation of crystal violet by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **54**: 1143-1150.

Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science **228**: 1434-1436.

Crosby, D. G. 1981. Environmental chemistry of pentachlorophenol. Pure Appl. Chem. **53**: 1051-1080.

Eaton, D. C. 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. Enzyme Microb. Technol. **7**: 194-196.

Kirk T. O'Reilley and R. L. Crawford. 1989. Degradation of Pentachlorophenol by Polyurethane-Immobilized *Flavobacterium* Cells. Appl. Environ. Microbiol. **55(9)**: 2113-2118.

Rao, K. R. (ed.). 1978. Pentachlorophenol: chemistry, pharmacology, and environmental toxicology. Plenum Press, New York.

Rochkind, M. L., J. W. Blackburn, and G. S. Sayler. 1986. Microbial decomposition of chlorinated aromatic compounds. Publ. no. EPA/600/2-86/090. U.S. Environmental Protection Agency Hazardous Waste Engineering Research Laboratory, Cincinnati, Ohio. pp. 86-91.

Thompson, W. S. and J.V. Dust. 1971. Pollution control in the wood preserving industry. I. Nature and scope of the problem. Forest Products J. **21**: 70-75.

Valo, R., V. Kitunen, and M. Salkinoja-Salonen. 1984. Chlorinated phenols as contaminants of soil and water in the vicinity of two Finnish sawmills. Chemosphere **13**: 835-844.

眞菌 *Leptosphaeria maculans* 對 Pentachlorophenol 之分解

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利用碳-14 標示之 pentachlorophenol (PCP) 做材料，可證明此化合物在缺氮源之培養條件下，受*Leptosphaeria maculans*之分解而消失、礦化。質量平衡分析結果顯示有水溶性代謝物之形成。毒性測定證明 PCP 高於 4 毫克/升(15 μM)即抑制此眞菌之腔器孢子(pycindiospores)的萌發生長。但如先讓此眞菌建立菌絲團後再加入 PCP，則 PCP 之致死效力受到限制，故利用此步驟，在 PCP 高達 500 毫克/升(1.9 mM)下，此眞菌仍可將 PCP 加以分解。