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Use of lignin-degrading fungi in the disposal of pentachlorophenol-treated wood

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SUMMARY

The lignin-degrading fungi *Phanerochaete chrysosporium*, *P. sordida*, *Trametes hirsuta*, and *Ceriporiopsis subvermispora* were evaluated for their ability to decrease the concentration of pentachlorophenol (PCP) and to cause dry weight loss in PCP-treated wood. Hardwood and softwood materials from PCP-treated ammunition boxes that were chipped to pass a 3.8-cm screen were used. All four fungi caused significant weight losses and decreases in the PCP concentration. The largest PCP decrease (84% in 4 weeks) was caused by *T. hirsuta*, and the smallest decrease was caused by *C. subvermispora* (37% in 4 weeks). After 4 weeks, the fate of spiked ^{14}C[PCP] in softwood chips inoculated with *T. hirsuta* was as follows: 27% was mineralized, 42.5% was non-extractable and bound to the chips, 23.5% was associated with fungal hyphae, and 6% was organic-extractable. Decreases of PCP by *P. chrysosporium* and *P. sordida* averaged 59% and 57%, respectively. PCP decreases caused by *Phanerochaete* spp. were not significantly affected by wood type or sterilization and were primarily due to methylation of PCP that resulted in accumulation of pentachloroanisole. Softwood weight losses caused by *T. hirsuta*, *P. chrysosporium* and *C. subvermispora* were respectively, 24, 6.5, and 17%, after 4 weeks. These weight losses are comparable to reported weight losses by these organisms in non-treated softwood. Nutrient supplementation significantly increased weight loss but not percentage decrease of PCP. The results of this research demonstrate the potential for using lignin-degrading fungi to destroy PCP-treated wood.

INTRODUCTION

Pentachlorophenol (PCP) in its technical grade formulation, Penta, dissolved in petroleum oil, has been used as a wood preservative since the 1930s. Penta has been used to treat poles, timbers, lumber, crossties, fence posts, land- and freshwater pilings, and wood used for homes and other buildings [20]. Although PCP has been classified as a priority pollutant, PCP-treated wood products are currently disposed of as ordinary solid (non-hazardous) wastes. Non-regulated disposal of these materials is allowed because concentrations of PCP in extracts from PCP-treated wood products such as poles and crossarms, determined by the Toxicity Characteristic Leaching Procedure (TCLP) [3] have been shown to be well below the 100 mg/l^{-1} PCP criterion used to classify these materials as hazardous wastes under the Resource Conservation and Recovery Act [2]. However, recent research demonstrating that technical grade mixtures of PCP are carcinogenic to B6 mice [12] may result in a decrease in the allowable limits of PCP to levels that would in turn result in reclassification of PCP-treated wood products as hazardous wastes. Indeed, PCP-treated ammunition boxes are currently stockpiled rather than disposed of by the U.S. Army in anticipation of this reclassification. Thus, cost-effective and environmentally benign treatment methods need to be developed for destruction and disposal of PCP-treated wood products.

Wood-degrading fungi that degrade lignin (white-rot fungi) have been shown to degrade a number of hazardous organic compounds, including PCP. The organisms are filamentous fungi that share the ability to metabolize lignin and the polysaccharide components of wood. They are the major degraders of fully lignified tissues (lignin content $\geq$20%) and therefore play a vital role in the recycling of photosynthetically-fixed carbon. We reported that two lignin-degrading fungi, *Phanerochaete chrysosporium* and *P. sordida*, were capable of rapidly depleting PCP in soils in the laboratory [8,9] and in the field [7].

The combined ability to degrade wood and PCP makes these organisms attractive candidates for use in destroying PCP-treated wood products. The objective of this research was to assess, on a laboratory-scale, the ability of lignin-degrading fungi to destroy PCP-treated wood from discarded ammunition boxes.

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MATERIALS AND METHODS

PCP-treated materials

Six PCP-treated ammunition boxes were used. Three were nailed pine boxes constructed mostly of lodgepole (*Pinus contorta* Dougl. ex Loud.) or pondersosa pine (*Pinus ponderosa* Dougl. ex Loud.) panels and yellow poplar (*Liriodendron tulipifera* L.) end cleats, and three were wire-bound, constructed of hardwood, most blackgum (*Nyssa sylvatica* Marsh.) and sweetgum (*Liquidambar styraciflua* L.) panels with yellow poplar end cleats. The boxes were disassembled and the hardwood and softwood materials separated. The materials were chipped using a hammer mill to pass a 3.8 cm screen and stored in plastic bags at 4 °C. In general, sterile chips were prepared by adjusting the moisture content of the chips to 60% with distilled water and autoclaving at 121 °C for 30 min on three successive days.

Chemicals

[UL-^{14}C]PCP with a specific activity of 4.55×10^8 Bq · mmol^{-1} and chemical purity >99%, *N,N*-dimethyl formamide (DMF), and 2,5-diphenyloxazole (POP) spectrophotometric grade were obtained from Sigma Chemical Co., St. Louis *p-bis*-[2-(5-Phenyl-oxazolyl]benzene (POPOP), spectrophotometric grade, was obtained from Research Products International, Mount Prospect. PCP (purity >99%); ethanolamine, 99 + %; $Na_2S_2O_4$, technical grade, purity approximately 85%; and Na_2SO_4, anhydrous, purity 99%, were obtained from Aldrich Chemical Co., Milwaukee. Acetone and hexane were B & J Brand high-purity solvents obtained from Baxter Healthcare Corporation, McGaw Park. Pentachloroanisole (PCA) was prepared by reaction of PCP with diazomethane in ether. All other chemicals were reagent grade.

Fungi

For decomposition of softwood materials, *Phanerochaete chrysosporium* Burds. (BKM-F-1767), *Phanerochaete sordida* (Karst.) Erikss. & Ryv., *Ceriporiopsis subvermispora* (Pila't) Gilbn. & Ryv. and *Trametes hirsuta* (Wulf.: Fr.) Pila't (called previously *Dichomitous squalens* [14]) were tested. *Ceriporiopsis subvermispora* and *T. hirsuta* were chosen for testing because of their proven ability to effect large weight losses, in contrast to most other white-rot fungi, in softwood [14]. The two *Phanerochaete* species were also evaluated for decomposition of hardwood materials. All fungal strains used in this study are maintained in the culture collection of the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wisconsin.

Each fungus was grown and maintained on yeast malt peptone glucose (YMPG) agar on slants. The YMPG medium was composed of (g/l^{-1}): glucose 10; malt extract 10; bacto-peptone 2; yeast extract 2; asparagine 1; KH_2PO_4 2; $MgSO_4 \cdot 7H_2O$ 1; thiamine 1; and bacto agar 23. For each fungus, inoculum plates were prepared by aseptically transferring pieces of fungal mycelium from YMPG slants to 2% malt agar plates (100 mm × 20 mm). The fungi were kept at their incubation temperature until colony growth completely covered the plates. Incubation temperatures were as follows: *P. chrysosporium* 39 °C; *P. sordida* 30 °C; and *Trametes hirsuta* and *Ceriporiopsis subvermispora* 27 °C. Incubation temperatures for experimental cultures were the same except for *P. chrysosporium*, which was incubated at 30 °C.

Culture preparation

Chip cultures were prepared by aseptically placing ca. 10 g chips (dry weight) in an aluminum foil-covered 125-ml Erlenmeyer flask (experiment 1) or in a 272-ml canning jar with a modified cover (experiments 2 and 3). Covers were modified to allow adequate air exchange by gluing a piece of microporous material over a 0.32-cm hole on the inside of the cover. Erlenmeyer flasks and canning jars were sterilized by autoclaving at 121 °C for 15 min. Canning jar covers were sterilized by fumigation with methyl bromide (methyl bromide 98%, chloropicrin 2%). Fungal cultures were prepared by aseptically adding pieces of 2% malt agar, infested with the appropriate fungus, to the chips. Approximately half of the agar from an inoculum plate (ca. 8 g) was added per culture.

Dry weight loss

Percentage dry weight loss of chips was determined using the following formula [(initial chip dry weight – chip harvest dry weight) · initial dry weight^{-1}] × 100. Harvest wet weight of chips from fungal inoculated-cultures was determined after removing mycelium from chip surfaces. Harvest dry weight was then determined after drying a sample of the chips at 105 °C for 24 h to determine moisture content gravimetrically.

Analytical procedure

Concentrations of PCP and pentachloroanisole (PCA) in chips were determined on organic solvent extracts, prepared as follows: for experiment 1, ca. 5 g of chip subsamples were placed in 25- by 150-mm culture tubes with teflon-lined screw caps. For experiments 2 to 4, chips were ground in a commercial coffee grinder prior to extraction. Approximately 4 g (wet weight) of ground chips were then placed in the culture tubes. Replicate determinations were performed per culture for PCP and PCA analysis. A sample was also taken to determine moisture content of the chips or ground chips, gravimetrically. Ap-

proximately 100 mg of $Na_2S_2O_4$ were added to each tube. Chip samples were then extracted for 1 h on a rotating tumbler shaker with two 20-ml volumes of a mixture of hexane-acetone (1 : 1) acidified to pH 2 with concentrated H_2SO_4. The extracts were pooled in a clean tube and dried by passing them through a column of anhydrous Na_2SO_4. The Na_2SO_4 was prepared by muffling for 4 h at 400 °C and storing over dessicant. Drying tubes and culture tubes were muffled for 1 h at 450 °C prior to use. Culture tubes containing the extracts were placed in a Tubovap LV evaporator held at 30 °C and the extracts evaporated to approximately 5 ml under nitrogen. The 5 ml was then transferred with hexane rinse to a 10-ml volumetric flask and the extract volume adjusted to 10 ml with hexane. Extracts were stored at − 20 °C under nitrogen in amber vials with teflon-lined screw caps.

Extracts were analyzed by gas chromatography for PCP and PCA. Pentachlorophenol was analyzed as the trimethylsilyl derivative and quantified with derivatized standards. The derivatizing reagent was Sylon BTZ (Supelco Inc., Bellefonte, PA). Pentachloroanisole was quantified non-derivatized with authentic standards. Gas chromatographic analyses of extracts were performed on a Hewlett Packard Model 5890 equipped with ^{63}Ni electron capture detector, Model 7673A autosampler, Model 3396A reporting integrator, and split-splitless capillary column injection port. Operating temperatures were injector 220 °C and detector 300 °C, carrier gas, He; and make-up gas, N_2. The column was a 30-m by 0.321-mm DB-5 fused silica capillary column, film thickness 0.25 μm (J & W Scientific, Folsom, CA). The temperature program was initial 60 °C, hold for 1 min, split on for 1 min, ramp A, 10 °C min^{-1} for 9 min (60 to 150 °C), ramp B, 2 °C min^{-1} for 20 min (150 to 190 °C), and hold at 190 °C for 5 min.

Experiment 1. In experiment 1, the effects of wood type, chip sterilization and fungal species on concentrations of PCP in wood chips were evaluated. PCP-contaminated softwood and hardwood chips were inoculated with *P. chrysosporium* or *P. sordida*. Weight loss and concentrations of PCP and PCA were determined. Sterile and non-sterile chips were supplemented with 5000 ppm glutamine and inoculated or left non-inoculated. Cultures were incubated at 30 °C. Initial concentrations of PCP and PCA were determined on 10 replicate samples from each batch of sterile or non-sterile hardwood and softwood chips. Percentage dry weight losses and concentrations of PCP and PCA were determined at 1, 2, 4, and 6 weeks. In addition, percentage dry weight losses were determined at 9 weeks. Analyses were performed in duplicate on five cultures per treatment at each sample time.

Experiment 2. In experiment 2, the ability of *T. hirsuta* or *C. subvermispora* to deplete PCP in chips was evaluated. Softwood chips were inoculated with *T. hirsuta* or *C. subvermispora*. Weight loss and PCP and PCA concentrations were evaluated. Evaluation of each fungus was performed using a separate batch of softwood chips. The chips were sterilized by autoclaving, amended with 5000 ppm glutamine and inoculated with *T. hirsuta* or *C. subvermispora* or left non-inoculated for controls. Initial concentrations of PCP and PCA were determined on 10 replicate samples for each batch of chips. Percentage dry weight loss and concentrations of PCP and PCA were determined after 2 and 4 weeks of incubation. Replicate analyses were performed on six cultures per treatment at each sample time.

Experiment 3. In experiment 3, the effects of different carbon and nitrogen source supplementations on the concentrations of PCP and PCA in softwood chips inoculated with *P. chrysosporium* were investigated. The final concentration of the supplements in $\mu g\ g^{-1}$ of chips was based on the equivalent amount of either carbon or nitrogen supplied by 5000 μg glutamine g $chips^{-1}$. Each of six batches of chips taken from a common chip batch was supplemented with either glucose (5136.25 $\mu g\ g^{-1}$), glycerin (5251.79 $\mu g\ g^{-1}$), NH_4Cl (3661.2 $\mu g\ g^{-1}$), glutamine (5000 $\mu g\ g^{-1}$), KNO_3 (2478.67 $\mu g\ g^{-1}$), or no supplement. Initial concentrations of PCP and PCA were determined on 5 replicate samples from each batch of chips. Three inoculated and two non-inoculated cultures were prepared for each treatment. Weight losses and concentrations of PCP and PCA in sterile softwood chips were determined on duplicate samples from each culture after three weeks.

Experiment 4. In experiment 4, the fate of PCP in sterile softwood chips inoculated with *T. hirsuta* or left non-inoculated as control was determined. In this experiment sterile chips were prepared as follows: the moisture content of the chips was adjusted to 60% with deionized water after which the chips were autoclaved at 121 °C. The next day the chips were aseptically placed in sterile 125-ml Erlenmeyer flasks (ca. 4 g $chips\ flask^{-1}$). The chips in each flask were then spiked with approximately 600 000 dpm (9.99×10^3 Bq) of ^{14}C-labeled PCP in 0.5 ml acetone and autoclaved a second time. On the day following the second autoclaving, fungal and control cultures were prepared. Fungal cultures were inoculated as in previous experiments. Control cultures consisted of non-inoculated chips. Six cultures were prepared for each treatment. Cultures were incubated at 30 °C for 28 days.

To assess losses of PCP by mineralization or volatilization and to aerate the cultures, the 125-ml Erlenmeyer flasks were fitted with inlet-outlet ports. Ports were protected from contamination by sterile, silanized glass wool traps. Outlet ports were connected to ORBO-42 volatile traps (Supelco, Inc., Bellefonte, PA) that were connected

in turn to manifold assemblies that directed evolved CO_2 into 10 ml of CO_2-trapping scintillation mixture. The mixture was composed of toluene cocktail, methanol, and ethanolamine (v:v:v, 5:4:1). Toluene cocktail contained 4 g l^{-1} POP and 0.1 g l^{-1} POPOP in toluene.

Headspaces of all cultures were flushed with humidified air every three to four days. The first flushing was conducted three days after inoculation. After each flushing, the amount of trapped $^{14}CO_2$ was determined by transferring the 10 ml of scintillation mixture to a 20-ml scintillation vial for counting. [^{14}C]volatiles were determined at the termination of the experiment by measuring the ^{14}C contents of two 0.25-ml aliquots of a 2-ml hexane extract of the ORBO tube packing material. The aliquots were transferred to 10 ml of Ecolume scintillation cocktail (ICN Biomedicals, Inc., Irvine, CA). All liquid scintillation counting was performed on a Pharmacia 1214 RackBeta Liquid Scintillation Counter (Wallac Oy, Turku, Finland).

Initial concentrations of PCP and PCA were determined on 10 ca. 4-g samples of chips that were not amended with ^{14}C[PCP] but that were taken from a common batch and treated identically to the chips used to prepare the cultures. Final concentrations of PCP and PCA were determined as described in the analytical section with the following exception. The amount of organic extractable ^{14}C was determined by measuring the amount of ^{14}C associated with two 1-ml aliquots from the pooled hexane-acetone extract volume, prior to evaporation. Non-extractable ^{14}C (^{14}C associated with the organic extracted chips) was determined by combustion of 2 ca. 0.1 g samples from each sample of previously extracted chips in a Harvey Model OX-600 Biological Oxidizer (R.J. Harvey Instrument Corp., Hillsdale, NJ). Radioactivity from the evolved CO_2 was determined by liquid scintillation counting. The ^{14}C content of the fungus infested malt agar pieces was determined by allowing the agar to air dry, followed by combustion and liquid scintillation counting as described for the chips.

Statistical analysis

Data for concentrations of PCP and PCA and percentage weight loss were analyzed by analysis of variance and differences among means were determined using Scheffe's test ($\alpha = 0.05$). Percentage decreases in the PCP concentration were determined by using the concentration found in identical non-inoculated cultures as the base.

RESULTS

Concentrations of PCP and PCA

In experiment 1 the initial concentrations of PCP in the chips were affected by autoclaving ($P = 0.0644$) but not by wood type ($P = 0.8600$). The concentration of PCP was higher in non-autoclaved than in autoclaved chips (Table 1). Inoculation of sterile and non-sterile, softwood or hardwood chips with either *P. chrysosporium* or *P. sordida* resulted in decreases in the PCP concentrations of the chips that ranged from 30 to 72% after 6 weeks (Fig. 1). No decreases in PCP concentrations were observed in either sterile on non-sterile non-inoculated

TABLE 1

Initial concentrations of PCP in autoclaved and non-autoclaved hardwood and softwood chips

Wood type	PCP (μg g^{-1})		
	Autoclaved	Non-autoclaved	Mean
Hardwood	281.7	355.8	338.7
Softwood	233.7	428.5	379.8
Mean	257.7	390.2	

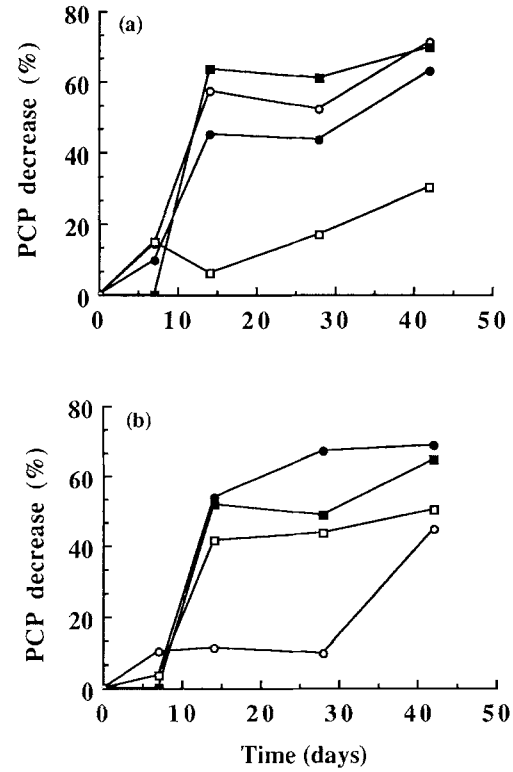

Fig. 1. Percentage decrease in the PCP concentration of sterile hardwood (●) and softwood (■) and non-sterile hardwood (○) and softwood (□) chips inoculated with *P. chrysosporium* (a) or *P. sordida* (b). Percentage decrease was determined for each treatment at each sample time using the formula: ([PCP] in non-inoculated chips – [PCP] in inoculated chips/[PCP] in non-inoculated chips) × 100.

chips, indicating that observed PCP decreases were due to the activities of the inoculated fungi. Decreases of PCP by either fungus were not significantly influenced by wood type.

Decreases in the PCP concentration in hardwood and softwood chips inoculated with *P. chrysosporium* were rapid and extensive (63 to 72% decrease after 6 weeks), except in non-sterile softwood chips (Fig. 1). Most depletion in sterile hardwood and softwood and non-sterile hardwood chips occurred during the second week of incubation. In non-sterile softwood chips, depletion of PCP was relatively slow and resulted in only a 30% decrease after 42 days. However, a slow but steady increase occurred in the percentage PCP decrease between days 14 and 42.

Depletion of PCP by *P. sordida* was greatly affected by sterilization. Inoculation of non-sterile softwood and hardwood chips resulted in only a 50 and 45% decrease in the PCP concentration, respectively, after 42 days (Fig. 1). However, the PCP concentration in both hardwood and softwood chips that had been sterilized was decreased by approximately 66% by *P. sordida* after 42 days. As was observed with *P. chrysosporium*, most of the PCP decrease occurred during the second week of incubation, except in non-sterile hardwood chips where the majority of the decrease occurred between days 28 and 42.

Depletion of PCP was always accompanied by formation of PCA (Fig. 2). No accumulation of PCA was observed in non-inoculated cultures, indicating that accumulation in inoculated chips was due to the activity of the fungi. Accumulation of PCA in sterile cultures was much greater than in non-sterile cultures of both fungi. This was particularly true for cultures inoculated with *P. sordida*. Only 7 and 19% of the PCP decrease in non-sterile softwood and hardwood chips, respectively, was due to conversion of PCP to PCA. However, this low rate of conversion was associated with relatively low amounts of total PCP depletion.

In non-sterile hardwood and softwood chips inoculated with *P. chrysosporium*, 65 and 72%, respectively, of the PCP decrease was due to conversion of PCP to PCA. In sterile chips inoculated with either fungus, virtually all of the PCP decrease was due to conversion to PCA.

In experiment 3 the initial PCP concentration in the chips averaged 367 $\mu g\ g^{-1}$, except in chips supplemented with glutamine (Table 2). Extraction of chips supplemented with glutamine appeared to have a significantly higher initial PCP concentration. However, after three weeks, the extractable PCP concentration in non-inoculated chips increased in all cases, except in chips supplemented with glutamine.

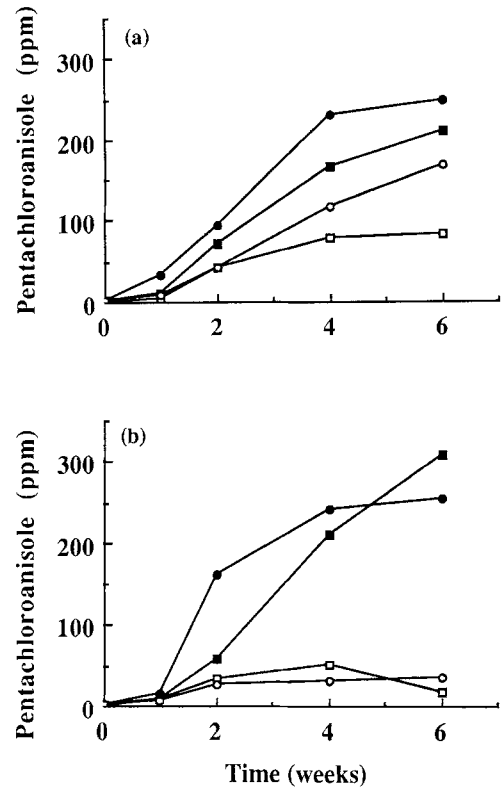

Fig. 2. Accumulation of PCA in sterile hardwood (●) and softwood (■) and non-sterile hardwood (○) and softwood (□) chips inoculated with *P. chrysosporium* (a) or *P. sordida* (b).

There was a decrease in the PCP concentration in inoculated chips regardless of supplement treatment. Rank of percentage decrease in terms of supplement received was glucose ≥ glutamine = no supplement ≥ glycerin ≥ KNO_3 = NH_4Cl.

Decreases in PCP concentration were always accompanied by increases in PCA concentration (Table 2). However, the percentage of the total decrease in the PCP concentration as a result of PCA formation varied greatly among the treatments (Fig. 3). When chips were supplemented with glycerin, virtually all (99.05%) of the PCP decrease was due to conversion to PCA. In chips receiving inorganic sources of nitrogen, the majority (77 to 89%) of the PCP loss was due to conversion of PCP to PCA. Finally, in chips supplemented with glucose or glutamine and in chips receiving no supplement, slightly less than two-thirds (61 to 63%) of the PCP decrease was due to conversion to PCA.

In experiment 2 inoculation of sterile PCP-contaminated softwood chips with *T. hirsuta* resulted in a decrease in the PCP concentration from 382 $\mu g\ g^{-1}$ to 145 $\mu g\ g^{-1}$ (Table 3). This represented a 62% decrease in the amount of PCP found in non-inoculated cultures after

TABLE 2

Initial and final (three weeks) concentrations[a] of PCP and PCA in softwood chips supplemented with different carbon and nitrogen sources and inoculated with *P. chrysosporium* or left non-inoculated

Supplement	PCP ($\mu g\ g^{-1}$)				PCA ($\mu g\ g^{-1}$)		
	Initial	Inoculated	Non-inoculated	Decrease (%)	Initial	Inoculated	Non-inoculated
Glucose	403.7b	125.5a	463.1a	72.9	5.3b	207.3ab	8.4a
Glycerin	366.6b	195.4ab	418.2a	53.3	11.3a	220.7a	6.1ab
NH_4CL	337.9b	295.1c	539.2a	45.3	5.3b	189.0ab	4.1b
Glutamine	551.3a	175.7ab	529.8a	66.8	4.1b	221.1a	4.0b
KNO_3	333.8b	237.3bc	441.3a	46.2	3.9b	181.9b	5.8ab
Nothing	394.2b	196.7ab	509.5a	61.4	3.9b	198.6ab	3.1b

[a] Means within columns followed by the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$).

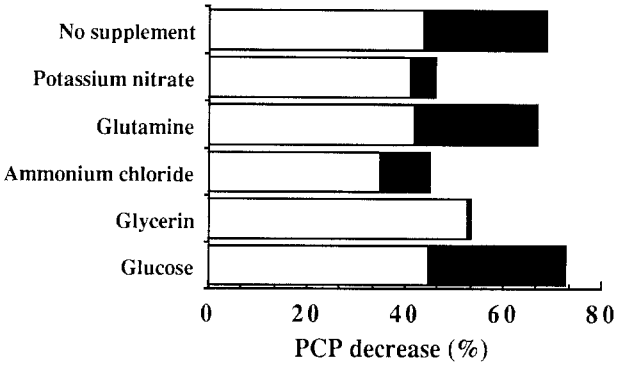

Fig. 3. Percentage decrease of PCP (■) and percentage decrease of PCP caused by methylation (□) after 3 weeks in PCP-contaminated softwood chips supplemented with different sources of nitrogen and/or carbon or receiving no supplementation and inoculated with *P. chrysosporium*.

4 weeks. This percentage decrease is similar to and greater than the amount of PCP removed by *P. chrysosporium* and *P. sordida*, respectively, after four weeks. However, the decrease affected by *T. hirsuta* was not due to transformation to and accumulation of PCA as it was in chips inoculated with *P. chrysosporium* or *P. sordida*. Although the amount of PCA increased slightly in inoculated cultures (Table 3), the amount of accumulation represented only a fraction of a percent of the amount of PCP removed.

Inoculation of PCP-contaminated softwood chips with *C. subvermispora* resulted in a decrease in the concentration of PCP from 448 $\mu g\ g^{-1}$ to 266 $\mu g\ g^{-1}$ after 4 weeks (Table 4). This 37% decrease was the least of any of the fungi evaluated. However, no accumulation of PCA in inoculated cultures indicated that, as was observed with *T. hirsuta*, the decrease was not due to transformation to and accumulation of PCA (Table 4).

Fate of PCP in sterile softwood chips inoculated with Trametes hirsuta

In experiment 4, inoculation of sterile PCP-contaminated softwood chips with *T. hirsuta* resulted in an 84% decrease in the PCP concentration over what was found in non-inoculated chips after four weeks (Table 5). As was observed previously with this fungus, the decrease in the PCP concentration was not due to transformation to and accumulation of PCA. Indeed, the level of PCA significantly decreased in inoculated compared to non-inoculated chips (Table 5).

Total recoveries of ^{14}C in the mass balance analysis

TABLE 3

Concentrations of PCP and PCA in sterile softwood chips inoculated with *Trametes hirsuta* or left non-inoculated[a]

Fungus	PCP ($\mu g\ g^{-1}$)			PCA ($\mu g\ g^{-1}$)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
T.hirsuta	381.7a	241.0b	145.4c	2.8a	2.3a	4.4c
Non-inoculated	381.7a	353.3a	355.5a	2.8a	1.8ab	1.3b

[a] Means followed by the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$).

TABLE 4

Concentrations of PCP and PCA in sterile softwood chips inoculated with *Ceriposiopsis subvermispora* or left non-inoculated[a]

Fungus	PCP ($\mu g\ g^{-1}$)			PCA ($\mu g\ g^{-1}$)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
C. subvermispora	448.0a	300.0b	266.1c	5.1a	4.4a	6.2a
Non-inoculated	448.0a	408.9a	418.9a	5.1a	4.1a	3.6a

[a] Means within compound followed by the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$).

TABLE 5

Concentrations of PCP and PCA in sterile softwood chips inoculated with *Trametes hirsuta* or left non-inoculated[a]

Fungus	PCP ($\mu g\ g^{-1}$)		PCA ($\mu g\ g^{-1}$)	
	Day 0	Day 28	Day 0	Day 28
T. hirsuta	212.0a	39.9b	3.2a	1.7b
Non-inoculated	212.0a	255.0a	3.2a	3.2a

[a] Means within compound followed by the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$).

averaged 98.65% from inoculated cultures and 96.36% from non-inoculated cultures (Table 6). In inoculated cultures, 27% of the PCP was mineralized, 43% was non-extractable and associated with the chips, 24% was associated with the residual pieces of malt agar and fungal hyphae, and the remaining 6% was organic extractable. In non-inoculated cultures, the bulk (80%) of the ^{14}C was found in the organic extract. Approximately 16% was non-extractable and associated with the chips. The amount of ^{14}C lost by volatilization was negligible in both inoculated and non-inoculated cultures.

Dry weight loss

After nine weeks, the percentage dry weight loss of wood chips was significantly affected by fungal species ($P = 0.0314$) but not by sterilization treatment ($P = 0.1276$) or by wood type ($P = 0.9520$). Overall, *P. chrysosporium* caused an average 17.8% weight loss compared to 12% for *P. sordida*. Although sterilization treatment did not affect percentage weight loss significantly, weight loss was greater in sterile than in non-sterile chips, except in softwood chips inoculated with *P. chrysosporium* (Table 7). No weight loss was observed in non-inoculated chips.

Supplementing softwood chips with different sources of carbon and nitrogen, in all but one case (glycerin), increased the percentage dry weight loss effected by *P. chrysosporium* over that observed in chips receiving no supplement (Table 8). The extent of the percentage weight loss varied greatly with the supplement. The greatest percentage dry weight loss was obtained in chips supplemented with glutamine. However, the weight loss obtained with supplemental glutamine was not significantly different than that obtained when glucose or NH_4Cl was used as a supplement. Percentage dry weight loss was very low when KNO_3 or glycerin was used as a supplement and when the chips were not supplemented.

Inoculation of softwood chips with *T. hirsuta* resulted

TABLE 6

Percentage recoveries[a] of ^{14}C[PCP] from sterile softwood chips inoculated with *T. hirsuta* or left non-inoculated

	^{14}C Recovered (%) fraction					
	Mineralization[b]	Volatilization	Organic extractable[c]	Chips[d]	Fungal hyphae	Total
Inoculated	27.23	0.00	5.48	42.49	23.45	98.65
Non-inoculated	0.01	0.00	80.21	16.14	NA	96.36

[a] Percentage recoveries represent the means of six replicates.
[b] Evolved and collected as ^{14}C–CO_2.
[c] Extracted in a mixture of hexane : acetone (50 : 50) acidified to pH 2.
[d] Residual ^{14}C associated with the chips after organic extraction.

in a 25% weight loss after four weeks (Table 9). This weight loss was much greater than those obtained from inoculation with *P. chrysosporium* or *P. sordida* after nine weeks (Table 7). After four weeks, *C. subvermispora* decreased the dry weight of the PCP-contaminated softwood chips by 17% (Table 9). This loss was greater than those obtained from inoculation with *P. chrysosporium* or *P. sordida* (Table 7) but less than that obtained with *T. hirsuta*. No weight loss was observed in non-inoculated chips (Table 9).

TABLE 7

Effect of wood type and sterilization on the percentage dry weight loss of PCP-contaminated wood chips inoculated with *Phanerochaete chrysosporium* or *P. sordida* or left non-inoculated, after 9 weeks[a]

Fungus	Dry weight loss (%)				
	Hardwood		Softwood		Overall mean
	+	−	+	−	
P. chrososporium	21.3	14.0	18.0	18.1	17.8
P. sordida	13.6	9.2	14.9	14.5	11.4
Non-inoculated	0.0	0.4	0.1	0.6	0.3

[a] + is sterile; − is non-sterile.

TABLE 8

Effect of different carbon and nitrogen sources on percentage dry weight loss after 3 weeks on PCP-contaminated softwood chips inoculated with *P. chrysosporium*[a]

Carbon or nitrogen source	Dry weight loss (%)
Glutamine	11.68a
NH_4Cl	9.49ab
Glucose	8.33ab
KNO_3	4.53bc
Glycerin	1.46bc
No supplement	2.50bc

[a] Means followed by the same latter are not significantly different according to Scheffe's multiple comparison test ($\alpha = 0.05$).

TABLE 9

Pertage dry weight loss of sterile softwood chips 14 and 28 days after inoculation with *Trametes hirsuta* or *Ceriporiopsis subvermispora* or left non-inoculated. Figures represent the mean and (standard deviation) of six replicates

Fungus	Weight loss (%)	
	Day 14	Day 28
Trametes hirsuta	12.4 (4.4)	24.5 (6.7)
Non-inoculated	0.7 (0.5)	0.8 (1.1)
Ceriporiopsis subvermispora	6.3 (1.9)	17.4 (5.2)
Non-inoculated	0.1 (1.2)	1.6 (2.2)

DISCUSSION

Utilizing lignin-degrading fungi to dispose of PCP-treated wood products would seem to be contradictory, because the PCP is applied to wood for the purpose of preventing the growth of these organisms. However, the results of this research demonstrate that once the protective barrier of PCP in the wood is disrupted (for example, by chipping the treated wood to expose the non-treated portion) lignin-degrading fungi have the ability to colonize the wood and to rapidly deplete a large percentage of the PCP. Along with their ability to transform PCP, a key factor contributing to the depletion of PCP by these organisms is their rapid and extensive colonization of the wood. This colonization gives them access to the PCP that is deposited in the wood cell walls and cell lumens during application.

Phanerochaete chrysosporium and *P. sordida* were chosen for inclusion in this study because of the ability of *P. chrysosporium* to transform PCP in aqueous media [9–11] and of both organisms to transform PCP in soil [7–9]. The results reported here demonstrate that these organisms are also able to decrease PCP, quite rapidly and extensively, in PCP-treated wood. However, the decrease by *Phanerochaete* spp. was generally due to methylation of PCP resulting in accumulation of PCA. We reported previously that PCA was a major PCP transformation product of these fungi in some soils but not in others [7,9], and that both organisms had the ability to mineralize PCA in liquid culture [9]. However, no evidence existed for decreases of PCA after it had accumulated in wood chips inoculated with *P. chrysosporium* or *P. sordida*.

The conditions that influenced methylation of PCP and accumulation of PCA compared to other transformations in chips inoculated with *Phanerochaete* spp. are not known. The percentage PCP decrease caused by methylation by both fungi was less in non-sterile than in sterile chips. Autoclaving changes the chemical and physical environment of the chips. For example, autoclaving decreased the pH of softwood chips from 5.1 to 4.6. The pKa of PCP is ca. 4.8. Thus, most of the PCP in the sterile chips was in the more toxic protonated form. Fungi are known to methylate chlorinated phenolic compounds to the corresponding methylated derivatives [1]. Methylation is thought to be a detoxification mechanism because

investigations on the relative toxicities of PCP and PCA to selected microbes indicate that PCA is less toxic than PCP. For example, PCA was less toxic than PCP to 16 strains of blue-stain and wood-rotting fungi [19]. The fungi growing on the autoclaved chips may have responded to the increased amount of the protonated form by methylation to detoxify PCP. Alternatively, indigenous microbes inhabiting the non-sterile chips that were not able to metabolize PCP, may have been able to metabolize PCA and thus decrease its accumulation.

There is evidence that the lignin-degrading system of *P. chrysosporium* is involved in oxidation and subsequent catabolism of PCP [5,10,11] and 2,4-dichlorophenol [21]. A low rate of mineralization of PCP by this fungus in aqueous culture has also been shown to occur in the absence of the lignin-degrading system [10]. However, in that investigation [10] the rate of PCP mineralization by *P. chrysosporium* was shown to increase with increasing concentration of extracellular lignin-degrading enzymes. The lignin-degrading system of *P. chrysosporium* is expressed only under secondary metabolic conditions that are triggered by depletion of nitrogen, carbon or sulfur in the growth medium [6]. Because wood is nitrogen poor, the natural stimulus for secondary metabolism is most likely nitrogen depletion. Supplementing aspen wood with organic (glutamate) or inorganic (NH_4Cl) nitrogen sources significantly decreased the amount of lignin degradation by *P. chrysosporium* [18] and *Phlebia tremellosa* (Schrad.: Fr.) [17]. Also, glutamine and other complex and inorganic nitrogen sources have been shown to decrease ligninolytic activity in aqueous cultures of *P. chrysosporium* [4]. Softwood chips inoculated with *P. chrysosporium* or *P. sordida* were supplemented with 5000 ppm glutamine (959 ppm N) to stimulate rapid and extensive fungal growth. As the fungi colonized the glutamine supplemented chips, they would have initially encountered the PCP under nitrogen sufficient conditions. These conditions may have suppressed or severely limited expression of the lignin-degrading system until the added nutrient nitrogen was exhausted. In the absence of the lignin-degrading system, methylation may have been the dominant fungal transformation reaction.

We tested the theory that supplementing the chips with a nitrogen source may have suppressed the lignin-degrading system of *P. chrysosporium*, resulting in primarily methylation rather than oxidation of PCP. Softwood chips were supplemented with several different sources of carbon and/or nitrogen and the effects of the supplements on the concentrations of PCP and PCA were determined. The initial concentrations of extractable PCP and PCA were significantly influenced by supplement type. However, in non-inoculated chips the effect of supplement on the concentrations of extractable PCP and PCA had, respectively, disappeared or changed after three weeks. Thus, the differences in the concentrations of PCP and PCA in inoculated chips after three weeks probably reflected the influence of the supplements on fungal growth and activity rather than extraction efficiency.

Supplementing with inorganic nitrogen sources (i.e., KNO_3 or NH_4Cl) or glycerine resulted in significantly less PCP removal and a greater percentage of the removal caused by methylation and accumulation of PCA than when the chips were not supplemented or were supplemented with glucose. However, this was not true for chips supplemented with an organic nitrogen source (i.e., glutamine). Also, a significant percentage of the PCP decrease in chips supplemented with glucose or glutamine or receiving no supplement was also a result of methylation and accumulation of PCA. Thus, the inhibitory effect of nitrogen on expression of the lignin degrading system of *P. chrysosporium* did not appear to play a major role in controlling methylation of PCP vs. alternative transformation reactions. A significant result of this particular study was that nutrient supplementation was not necessary to obtain large PCP decreases.

Trametes hirsuta and *C. subvermispora* were selected for inclusion in this study because of their superior abilities to effect large weight losses in both hardwood and softwood materials [14]. The results reported here demonstrate that these fungi are also able to deplete PCP in treated wood. However, contrary to decreases caused by *Phanerochaete* spp., decreases in the PCP concentration caused by *T. hirsuta* or *C. subvermispora* were not accompanied by accumulations of PCA. Mass balance analysis of ^{14}C[PCP] in softwood chips inoculated with *T. hirsuta* indicated that metabolism of PCP by this fungus resulted in either complete degradation (27% mineralization) or stabilization (42% non-extractable) of most of the PCP. Another 23% of ^{14}C was associated with the fungal infested malt agar pieces used to inoculate the chips. Part of this may have been due to absorption of ^{14}C[PCP] from the chip surface by the agar. However, the cultures were prepared by adding the ^{14}C[PCP] to the chips after which they were thoroughly mixed, autoclaved, then inoculated by placing pieces of malt agar, that were infested with *T. hirsuta* on top of the chips. Thus, it is unlikely that the malt agar would have had access to 23% of the ^{14}C[PCP]. Most of the ^{14}C associated with the malt agar was probably associated with the fungal hyphae growing on the agar and would have eventually been mineralized had the experiment continued.

If all the ^{14}C in the organic extractable fraction was associated with PCP, the total decrease in the PCP concentration, in all but the organic extractable fraction, would have been approximately 93% (Table 6). This is greater than the 84% decrease determined from differ-

ences between concentrations of non-radioactive PCP in inoculated and non-inoculated chips. However, the ^{14}C[PCP] presumably was primarily associated with the chip surfaces and therefore was more available than the non-radioactive PCP, which was deposited in cell walls and lumens. The 84% decrease of PCP in the mass balance experiment is also greater than the 60% decrease caused by *T. hirsuta* in experiment 2. In experiment 2, the cultures were grown using a passive diffusion aeration system. In the mass balance study, the headspaces of the culture flasks were evacuated with air every three or four days. Therefore, the availability of oxygen to the fungi was much greater under the experimental conditions used in the mass balance study. The greater percentage PCP decrease obtained in the mass balance study may have been due to the greater availability of oxygen that allowed enhanced fungal growth and activity, which in turn resulted in more rapid and extensive oxidation of PCP.

Metabolism of PCP by *C. subvermispora* may have been similar to that of *T. hirsuta*. The only evidence for this is that metabolism of PCP by the former fungus did not result in conversion to PCA. However, this particular strain of *C. subvermispora* was inferior to the other fungi in its ability to cause decreases in the PCP concentration and is not a candidate for further study.

Although removal of PCP from treated wood is the primary objective, decreases in weight or volume of the treated materials would be desirable in a disposal process. The percentage weight loss varied considerably among the four fungi examined in this study. These organisms have been shown to vary greatly in their ability to cause weight loss and in their removal of the lignin and carbohydrate constituents [14]. After 4 weeks, percentage weight losses ranged from 6.4% on non-sterile softwood inoculated with *P. chrysosporium* to 24.5% on sterile softwood inoculated with *T. hirsuta*. Percentage weight losses caused by the lignin-degrading fungi in treated wood were comparable to weight losses caused by these organisms in non-treated wood. For example, reported weight losses on sterile pine (*Pinus strobus* L.) wood blocks after 12 weeks for *T. hirsuta*, *P. chrysosporium*, and *C. subvermispora* were 46, 14 and 24%, respectively [14]. In sterile PCP-treated softwood chips, *T. hirsuta*, *P. chrysosporium* and *C. subvermispora* caused 24, 6.5, and 17% weight loss, respectively, after only four weeks. Although most of the weight loss caused by the fungi in the treated wood may have been primarily in the non-treated portions, the level of PCP transformation suggests that the treated portions were well colonized by metabolically active fungi.

Significantly greater weight losses were obtained when the softwood chips inoculated with *P. chrysosporium* were supplemented with carbon and/or nitrogen sources compared to no supplement. Supplementing with glutamine significantly increased the percentage weight loss caused by *P. chrysosporium* after 3 weeks by ca. 10% over that in nonsupplemented chips. However, supplementing with glutamine, which provided both carbon and nitrogen, resulted in only slightly increased dry weight loss in contrast to supplementing with nutrient sources that contained only nitrogen (NH_4Cl) or carbon (glucose). Nutrient supplementation has previously been demonstrated to increase dry weight loss in aspen (*Populus tremuloides* Michx.) wood by *Phlebia tremellosa* [17] and by *Phanerochaete chrysosporium* [18]. Supplementing with glutamate or several other complex carbon-nitrogen sources significantly increased percentage dry weight loss of the aspen wood by *P. tremellosa* [17]. The source of carbon or nitrogen has been shown to be important in influencing the magnitude of the weight loss. For example, although weight loss in aspen wood by *P. tremellosa* was significantly increased when the wood was supplemented with glutamate and several other complex nutrient sources, supplementing with NH_4 acetate or urea significantly decreased weight loss [17]. Similarly, percentage weight loss of aspen wood inoculated with *P. chrysosporium* was significantly increased when supplemented with albumen, peptone, and yeast extract but significantly decreased when supplemented with urea [18]. We also found that the nutrient source greatly influenced the magnitude of the weight losses caused by *P. chrysosporium*. Weight losses were greater in chips supplemented with glucose or NH_4Cl than in chips supplemented with glycerin or KNO_3.

Colonization of lignocellulosic materials by lignin-degrading fungi can be inhibited by the large populations of indigenous microorganisms that germinate and grow rapidly when the substrate is moistened [16]. Therefore, sterilization or pasteurization is usually necessary to actively suppress the indigenous microbes. However, indigenous microbial populations may not be as great on PCP-treated wood because of the presence of the PCP. We found that sterilization increased the amount of weight loss caused by *P. chrysosporium* or *P. sordida*, but the increases were not significant. Therefore, sterilization of PCP-treated materials prior to fungal treatment was not important for weight loss and may not be necessary in practical treatment.

Lignin-degrading fungi are more frequently associated with hardwood hosts [13] and are usually able to cause greater weight losses in hardwood compared to softwood. This preference is thought to be due to the allegedly more refractory nature of softwood lignin compared to hardwood lignin [15]. For example, *P. chrysosporium* caused a 37.5% weight loss in birch compared to a 13.8% weight loss in pine after 12 weeks. However, we found that weight losses of the treated wood inoculated with

P. chrysosporium or *P. sordida* after nine weeks were similar, regardless of wood type. Also, fungi that caused greater weight loss of softwood (i.e., *T. hirsuta* and *C. subvermispora*) are generally also superior in their ability to cause weight loss in hardwood [14]. Therefore, in a fungal treatment of PCP-treated wood, we assume that hardwood and softwood materials could be treated collectively using a single fungus.

CONCLUSIONS

The results of this research demonstrate that utilizing lignin-degrading fungi in the destruction of PCP-treated wood products has potential. Further studies are needed to identify conditions that optimize the rate of PCP removal and effect complete transformation of PCP to innocuous products. The fungal species used in this research varied greatly in their abilities to effect dry weight losses and decreases in the PCP concentration of the chips and in their metabolism of PCP in a chip environment. Further screening may reveal fungi with abilities superior to that of *T. hirsuta* to decrease the PCP concentration and cause weight loss of PCP-contaminated wood. Nutrient supplementation was important for obtaining substantial dry weight losses but not for PCP decreases. Because the primary concern is complete destruction of the PCP, optimization of the fungal treatment process should be focused on PCP removal compared to weight loss.

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