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Biodegradation of Pentachlorophenol (PCP) - Treated Ammunition Boxes Using White-Rot Fungi

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reported weight losses by these organisms in nontreated 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.

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EXECUTIVE 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%) was caused by *T. hirsuta*, and the smallest decrease was caused by *C. subvermispora* (37%). 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 nontreated 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.

1.0 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 [17]. Although PCP has been classified as a priority pollutant, PCP-treated wood products are currently disposed of as ordinary solid (nonhazardous) wastes. Nonregulated 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) [1] 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 [10] 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 [6, 7] and in the field [5].

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.

2.0 MATERIALS AND METHODS

2.1 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 ponderosa 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. 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 3 successive days.

2.2 Chemicals

N,N-Dimethylformamide (DMF) WAS obtained from Sigma Chemical Co., St. Louis, MO. PCP (purity > 99%) and Na₂SO₄, anhydrous, purity 99%, were obtained from Aldrich Chemical Co., Milwaukee, WI. Acetone and hexane were B & J Brand high-purity solvents obtained from Baxter Healthcare Corporation, McGaw Park, IL. Pentachloroanisole (PCA) was prepared by reaction of PCP with diazomethane in ether. All other chemicals were reagent grade.

2.3 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* [12]) were tested. The two *Phanerochaete* species were also evaluated for decomposition of hardwood materials.

Each fungus was grown and maintained on yeast malt peptone glucose (YMPG) agar on slants. The YMPG medium was composed of glucose $10\text{ g}\cdot\text{L}^{-1}$, malt extract $10\text{ g}\cdot\text{L}^{-1}$, Bacto-Peptone $2\text{ g}\cdot\text{L}^{-1}$, yeast extract $2\text{ g}\cdot\text{L}^{-1}$, asparagine $1\text{ g}\cdot\text{L}^{-1}$, KH_2PO_4 $2\text{ g}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ $1\text{ g}\cdot\text{L}^{-1}$, thiamine $1\text{ g}\cdot\text{L}^{-1}$, and Bacto Agar $23\text{ g}\cdot\text{L}^{-1}$. For each fungus, inoculum plates were prepared by aseptically transferring pieces of fungal mycelium from YMPG slants to 2% malt agar plates (100mm x 20mm). 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.

2.4 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 an 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.

2.5 Dry Weight Loss

Percentage dry weight loss of chips was determined using the following formula: $[(\text{initial chip dry weight} - \text{chip harvest dry weight}) / \text{initial dry weight}^{-1}] \times 100$. 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.

2.6 Analytical procedure

Concentrations 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. Approximately 100 mg of $\text{Na}_2\text{S}_2\text{O}_4$ 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 pentachloroanisole.

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 nonderivatized 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, N₂. The column was a 30-m by 0.321-mm DB-5 fused silica capillary column, film thickness 0.25 μ m (J & W Scientific, Folsom, CA). The temperature program was initial 60°C, hold for 1 min, split on for 1 min, ramp A, 10° min⁻¹ for 9 min (60°C to 150°C), ramp B, 2° min⁻¹ for 20 min (150°C to 190°C), and hold at 190° for 5 min.

2.7 Experiment 1

In Experiment 1, 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 nonsterile chips were supplemented with 5000 ppm glutamine and inoculated or left noninoculated. Cultures were incubated at 30°C. Initial concentrations of PCP and PCA were determined on 10 replicate samples from each batch of sterile or nonsterile 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.

2.8 Experiment 2

In Experiment 2, softwood chips were inoculated with *T. hirsuta* or *C. subvermispora*. Weight loss and PCP and PCA concentrations were evaluated. These fungi were chosen for testing because of their proven ability to effect large weight losses, in contrast to most other white-rot fungi, in softwood (Otjen et al. 1987). 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 noninoculated 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.

2.9 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 $\mu\text{g g}^{-1}$ 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 $\mu\text{g g}^{-1}$), glycerin (5251.79 $\mu\text{g g}^{-1}$), NH_4Cl (3661.2 $\mu\text{g g}^{-1}$), glutamine (5000 $\mu\text{g g}^{-1}$), KNO_3 (2478.67 $\mu\text{g g}^{-1}$), or no supplement. Initial concentrations of PCP and PCA were determined on 5 replicate samples from each batch of chips. Three inoculated and two noninoculated 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 3 weeks.

2.10 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 ($\alpha = .05$).

Percentage decreases in the PCP concentration were determined by using the concentration found in identical noninoculated cultures as the base.

3.0 RESULTS

3.1 Concentrations of PCP and PCA

In Experiment 1 inoculation of sterile and nonsterile, 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. 3.1). No decreases in PCP concentrations were observed in either sterile or nonsterile noninoculated chips, indicating that observed PCP decreases were due to the activities of the 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 nonsterile softwood chips (Fig. 3.1). Most depletion in sterile hardwood and softwood and nonsterile hardwood chips occurred during the second week of incubation. In nonsterile 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 nonsterile softwood and hardwood chips resulted in only a 50% and 45% decrease in the PCP concentration, respectively, after 42 days (Fig. 3.1). However, the PCP concentration in both hardwood and softwood chips that had been sterilized was decreased by ca. 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 nonsterile 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. 3.2). No accumulation of PCA was observed in noninoculated cultures, indicating that accumulation in inoculated chips was

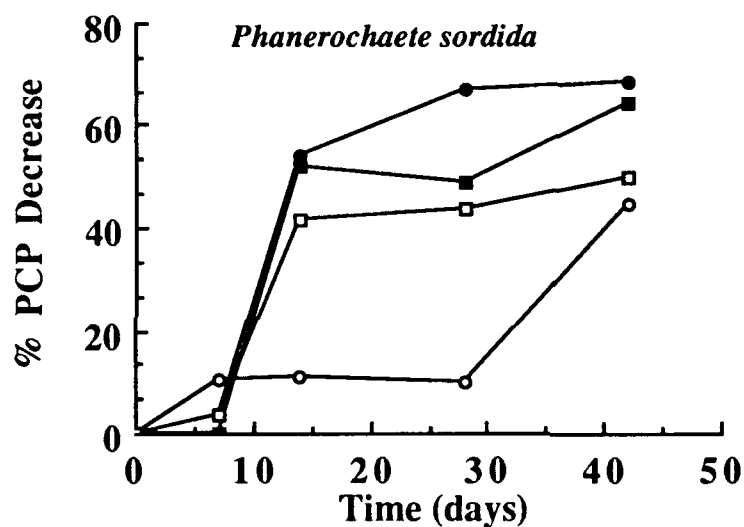
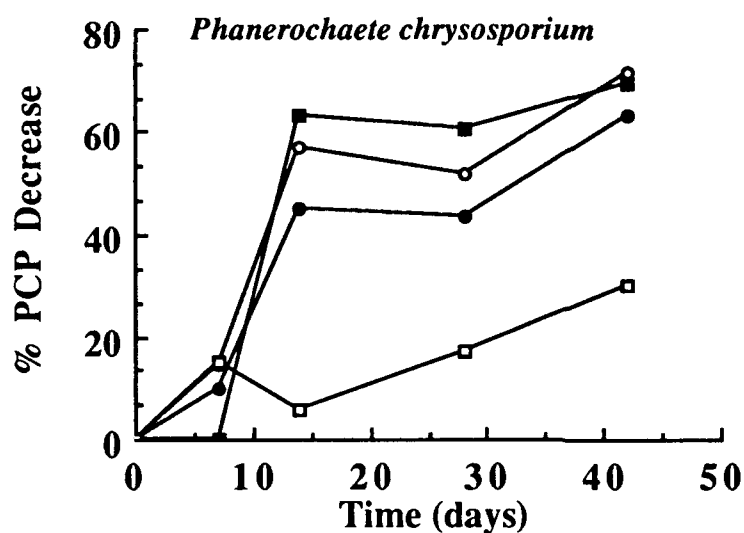


Figure 3.1. Percentage decrease in the PCP concentration of sterile hardwood (●) and softwood (■) and nonsterile hardwood (○) and softwood (□) chips inoculated with *P. chrysosporium* or *P. sordida*. Percentage decrease was determined for each treatment at each sample time using the following formula: $([\text{PCP}] \text{ in noninoculated chips} - [\text{PCP}] \text{ in inoculated chips} / [\text{PCP}] \text{ in noninoculated chips}) \times 100$.

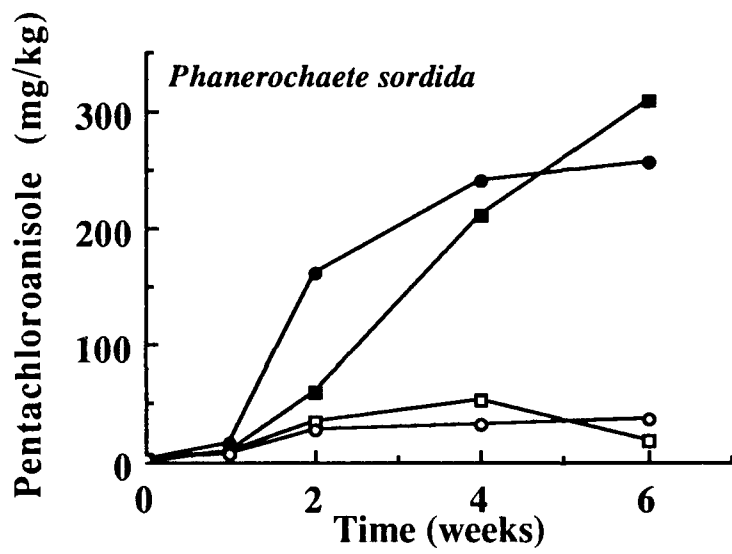
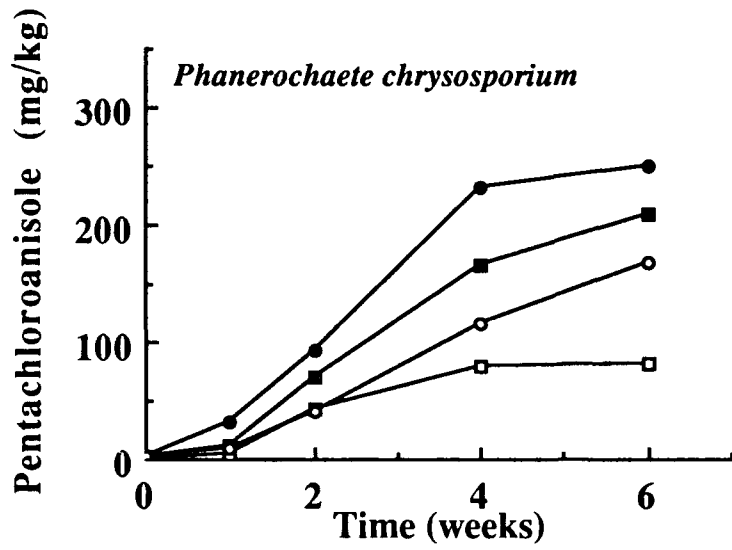


Figure 3.2. Accumulation of PCA in sterile hardwood (●) and softwood (■) and nonsterile hardwood (○) and softwood (□) chips inoculated with *P. chrysosporium* or *P. sordida*.

due to the activity of the fungi. Accumulation of PCA in sterile cultures was much greater than in nonsterile cultures of both fungi. This was particularly true for cultures inoculated with *P. sordida*. Only 7% and 19% of the PCP decrease in nonsterile 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 nonsterile 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 $367 \mu\text{g g}^{-1}$, except in chips supplemented with glutamine (Table 3.1). Extraction of chips supplemented with glutamine appeared to have a significantly higher initial PCP concentration. However, after 3 weeks, the extractable PCP concentration in noninoculated chips increased in all cases, except in chips supplemented with glutamine. 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 $\geq$ glutamine = no supplement $\geq$ glycerin $\geq$ KNO_3 = NH_4Cl .

Table 3.1. Initial and final (3 weeks) concentrations of PCP and PCA in chips supplemented with different carbon and nitrogen sources and inoculated or not with *P. chrysosporium* ^a.

Supplement	PCP ($\mu\text{g g}^{-1}$)			Decrease (%)	PCA ($\mu\text{g g}^{-1}$)		
	Initial	Inoculated	Non-inoculated		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	6.1b
NH_4Cl	337.9b	295.1c	539.2a	45.3	5.3b	189.0ab	4.1bcd
Glutamine	551.3a	175.7ab	529.8a	66.8	4.1b	221.1a	4.0cd
KNO_3	333.8b	237.3bc	441.3a	46.2	3.9b	181.9b	5.8bc
Nothing	394.2b	196.7ab	509.5a	61.4	3.9b	198.6ab	3.1d

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

Decreases in PCP concentration were always accompanied by increases in PCA concentration (Table 3.1). However, the percentage of the total decrease in the PCP concentration as a result of PCA formation varied greatly among the treatments (Fig. 3.3). 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 $\mu\text{g g}^{-1}$ to 145 $\mu\text{g g}^{-1}$ (Table 3.2). This represented a 62% decrease in the amount of PCP found in noninoculated cultures after 4 weeks. This percentage decrease is similar to and greater than the amount of PCP removed by *P. chrysosporium* and *P. sordida*, respectively, after 4 weeks. However, the decrease affected by *T. hirsuta* was not due to conversion of PCP to PCA as it was in chips inoculated with *P. chrysosporium* or *P. sordida*. Although the amount of PCA increased slightly in inoculated cultures (Table 3.2), the amount of accumulation represented only a fraction of a percent of the amount of PCP removed.

Table 3.2. Concentrations of PCP and PCA in sterile softwood chips inoculated with *Trametes hirsuta* or left noninoculated.^a

Fungus	PCP ($\mu\text{g g}^{-1}$)			Day	PCA ($\mu\text{g g}^{-1}$)		
	0	14	28		0	14	28
<i>T. hirsuta</i>	381.69a	240.98b	145.39c		2.80a	2.27a	4.41c
Noninoculated	381.69a	353.27a	355.46a		2.80a	1.75ab	1.30b

^aMeans followed by the same letter are not significantly different according to Scheffe's test ($\alpha = 0.05$)

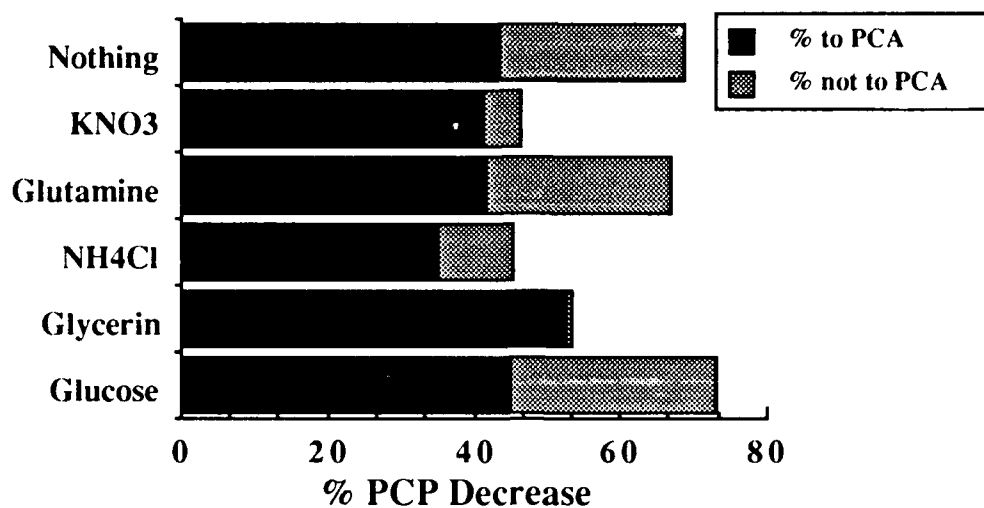


Figure 3.3. Percentage decrease of PCP after 3 weeks in PCP-contaminated softwood chips supplemented with different sources of nitrogen and/or carbon or not and inoculated with *P. chrysosporium*.

Inoculation of PCP-contaminated softwood chips with *C. subvermispora* resulted in a decrease in the concentration of PCP from 448 $\mu\text{g g}^{-1}$ to 266 $\mu\text{g g}^{-1}$ after 4 weeks (Table 3.3). 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 conversion of PCP to PCA (Table 3.3).

Table 3.3. Concentrations of PCP and PCA in sterile softwood chips inoculated with *Ceriposiopsis subvermispora* or left noninoculated.^a

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

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

3.2 Dry Weight Loss

After 9 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 always greater in sterile than in nonsterile chips, except in softwood chips inoculated with *P. chrysosporium* (Table 3.4). No weight loss was observed in noninoculated 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 3.5). 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.

Table 3.4. 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 noninoculated, after 9 weeks.^a

Fungus	Dry weight loss (%)				
	Hardwood		Softwood		Overall mean
	+	-	+	-	
<i>P. chrysosporium</i>	21.3	14.0	18.0	18.1	17.8
<i>P. sordida</i>	13.6	9.2	14.9	14.5	11.4
Noninoculated	0.0	0.4	0.1	0.6	0.3

^a + is sterile; - is nonsterile.

Table 3.5. 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

^aMeans followed by the same letter are not significantly different according to Scheffe's multiple comparison test ($\alpha = 0.05$).

Inoculation of softwood chips with *T. hirsuta* resulted in a 25% weight loss after 4 weeks (Table 3.6). This weight loss was much greater than those obtained from inoculation with *P. chrysosporium* or *P. sordida* after 9 weeks (Table 3.4). After 4 weeks, *C. subvermispora* decreased the dry weight of the PCP-contaminated softwood chips by 17% (Table 3.6). This loss

was greater than those obtained from inoculation with *P. chrysosporium* or *P. sordida* (Table 3.4) but less than that obtained with *T. hirsuta* . No weight loss was observed in noninoculated chips (Table 3.6).

Table 3.6. Percentage dry weight loss, 14 and 28 days after inoculation, of sterile softwood chips inoculated with *Trametes hirsuta* or *Ceriporiopsis subvermispora* or left noninoculated.

Fungus	Weight loss (%)	
	14 day	28 day
<i>Trametes hirsuta</i>	12.37	24.49
Noninoculated	0.71	0.84
<i>Ceriporiopsis subvermispora</i>	6.32	17.4
Noninoculated	0.06	1.61

4.0 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 nontreated 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 in 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 [7, 8, 9] and of both organisms to transform PCP in soil [5, 6, 7]. 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 [5, 6], and that both organisms had the ability to mineralize PCA in liquid culture [7]. 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 influence fungal methylation of PCP and accumulation of PCA compared to other transformations are not known. The percentage of PCP decrease caused by methylation by both fungi was less in nonsterile than in sterile chips. Autoclaving changes the chemical and physical environment of the chips. For example, autoclaving decreased the pH of the chips from 5.1 to 4.6. Because the pKa of PCP is *ca.* 4.8, most of the PCP in the sterile chips was in the more toxic protonated form. The fungi may have responded to this change by methylation to

detoxify PCP. Alternatively, indigenous microbes inhabiting the nonsterile 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 [3, 8, 9] and 2, 4-dichlorophenol [18]. 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 [8]. However, in that investigation [8] 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 [4]. 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* [16] and *Phlebia tremellosa* (Schröd.:Fr.) Nakas. et Burds.[15]. We supplemented chips inoculated with *P. chrysosporium* or *P. sordida* 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 encountered the PCP under nitrogen sufficient conditions. These conditions may have suppressed or severely limited expression of the lignin-degrading system. Therefore, in the absence of the lignin-degrading system, the fungi may have methylated the PCP to decrease its toxic effects.

To test the theory that the level of nitrogen in the glutamine-supplemented chips suppressed the expression of 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. Supplementing with inorganic nitrogen (*i.e.*, KNO_3 or NH_4Cl) resulted in significantly less PCP removal and a greater percentage of the removal caused by methylation than

when the chips were not supplemented or were supplemented with glucose or glutamine. This supports the premise that a high concentration of nitrogen may have inhibited or delayed expression of the lignin-degrading system and thus catabolism of PCP. However, a significant percentage of the PCP decrease in chips supplemented with glucose or glutamine or in chips receiving no supplements was also a result of methylation. In glycerin supplemented chips, virtually all of the PCP depletion was due to methylation. Thus, rather than influencing the amount of methylation, supplementing with inorganic nitrogen may have simply delayed expression of the lignin-degrading system that resulted in less overall PCP depletion. 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 [12]. The results reported here demonstrate that these fungi are also able to deplete PCP in treated wood. However, contrary to decreases caused by the *Phanerochaete* spp. decreases in the PCP concentration caused by *T. hirsuta* or *C. subvermispora* were not accompanied by accumulations of PCA.

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 [12]. After 4 weeks, percentage weight losses ranged from 6.4% on

nonsterile 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 nontreated 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 [12]. In sterile PCP-treated softwood chips, *T. hirsuta*, *P. chrysosporium* and *C. subvermispora* caused 24%, 6.5%, and 17% weight loss, respectively, after only 4 weeks. Although most of the weight loss caused by the fungi in the treated wood may have been primarily in the nontreated 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 than 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* [15] and by *Phanerochaete chrysosporium* [16]. Supplementing with glutamate or several other complex carbon-nitrogen sources significantly increased percentage dry weight loss of the aspen wood by *P. tremellosa* [15]. 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 [15]. 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 [16]. 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 [14]. 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 [11] 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 [13]. 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 9 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 [12]. Therefore, in a fungal treatment of PCP-treated wood, we assume that hardwood and softwood materials could be treated collectively using a single fungus.

5.0 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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