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Oxalic acid production of fifteen brown-rot fungi in copper citrate- treated southern yellow pine

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Abstract

Non-arsenical copper-based wood preservatives have grown in number since the 1980's as a response to environmental concerns posed by arsenicals. Interest in copper tolerant decay fungi has increased accordingly. Oxalic acid (OA) production by brown-rot fungi has been proposed as one mechanism of copper tolerance. Fifteen brown-rot fungi representing the genera *Postia, Wolfiporia, Serpula, Gloeophyllum, Laetiporus, Coniophora, Antrodia,* and *Tyromyces* were evaluated for OA production bi-weekly in southern yellow pine (SYP) blocks treated with 1.2% AI copper citrate (CC). Ten fungi were designated copper tolerant and produced 2 to 17 times more OA in the CC-treated blocks than in untreated SYP after 2 weeks. Weight losses ranged from 20 to 55% in CC-treated SYP after 10 wks. Five fungi were copper-sensitive, producing low levels of OA and low weight losses on CC-treated blocks. In this study, early induction of OA appears to closely correlate with copper tolerance. We conclude that brown-rot fungi able to exceed and maintain an OA concentration of $\geq$400 mM in this study effectively decayed SYP treated with copper citrate.

Keywords: oxalic acid, copper tolerance, copper citrate, brown-rot fungi

Introduction

Interest in copper-based wood preservatives has increased in recent years in response to public concern about the environment. Copper exhibits good biocidal activity (Nicholas and Schultz, 1997), but a major requirement of any formulation of copper-based wood preservative is efficacy against copper-tolerant fungi. Fungi can be extremely tolerant of toxic metals (Gadd, 1993; Schmidt and Ziemer, 1976). Early reviews of preservative tolerance by Zabel (1954) and Cowling (1957) reported comparative tolerances of economically important decay fungi to current preservatives. Cowling reported unusual tolerance to one or more preservatives in 14 of 18 fungi tested. Up to forty times the amount of preservative needed to control the most susceptible fungi was required to prevent decay by the most tolerant fungi. Brown-rot wood decay fungi in the genus *Poria* (Pers. ex Gray), and other genera related to *Poria,* such as *Serpula* [(Pers.) Gray], *Antrodia* (D. C.:Fr.) Ryv., and *Wolfiporia* (Schw syn. *Poria cocos*), are known to be copper tolerant (Davidson, 1954; Collet 1992; Stephan *et al.* 1995; Schmidt and Moreth, 1996; Tsunoda *et al.* 1997). Duncan (1955; 1958) was the first to report resistance to copper naphthenate by *Poria cocos.* She suggested that variations in preservative tolerance within a species may equal that between species. DeGroot and Woodward (1999) recently showed that the decay capacity of certain *Wolfiporia cocos* isolates in samples of copper-treated

wood was greater than in untreated controls, but considerable variation among individual isolates was noted. A subsequent study by Clausen *et al.* confirmed the variation within *Wolfiporia cocos,* but was unable to demonstrate a statistical correlation between decay capacity for this group of fungi and the amount of oxalic acid produced in copper-treated wood. Collet (1992) reported that isolates of *Antrodia vaillantii* differed significantly in their tolerance to copper. Variation in preservative tolerance among isolates of individual fungal species has not been adequately investigated (Collet 1992).

A relationship between copper tolerance and oxalic acid production has been implicated, due to copper oxalate crystal formation in decayed wood (Murphy and Levy, 1983; Sutter *et al.,* 1984). Rabanus (193 1) and Shimazano and Takubo (1952) suggested that tolerance of brown-rot fungi is linked to oxalic acid production, which presumably precipitates copper into the insoluble form of the oxalate, rendering the copper metabolite inert. Both groups concluded that lowering of the pH by oxalic acid had more to do with copper tolerance than low solubility of copper oxalate. In support of this hypothesis, Young (196 1) studied *Wolfiporia cocos,* isolated from failed copper naphthenate-treated fence posts. He demonstrated a striking increase in tolerance to copper when the pH of agar medium supplemented with copper sulfate was lowered from pH 6 to pH 2.

Chou (197 1) reported that copper oxalate crystals could not be detected in wood infested with *Fibroporia vaillantii* (syn. *Antrodia vailantii*) (D.C.) Cke., reportedly one of the most copper tolerant *Poria* species. He concluded that fungal metabolism may differ in wood from that in artificial medium. Clausen *et al.* (1994) also noted fungal physiological differences in wood versus an artificial medium for *Postia placenta.* More recent studies by Tsunoda *et al.* (1997) and Sutter *et al.* (1983; 1984) have closely examined copper tolerance in wood decay fungi. Both studies, involving copper (II) sulfate and copper naphthenate, concluded that copper tolerance in *Poria* species is a function of the precipitation of copper oxalate. These studies examined decay capacity and microscopic copper oxalate crystal formation, but did not correlate oxalic acid production with an increase in decay capacity.

Our previous study examined the relationship of oxalic acid production with the decay capacity of nineteen isolates of copper tolerant *Wolfiporia cocos* in wood treated with ammoniacal copper citrate (CC) after 2 weeks incubation In this study, our objective was to evaluate the relationship of oxalic acid production with the decay capacity of known copper-tolerant and copper-sensitive brown-rot fungi at two-week intervals in wood treated with ammoniacal copper citrate.

Materials and methods

Fungal culture

Brown-rot fungi evaluated in this study were maintained on 2% malt extract agar (Difco Laboratories, Detroit, MI). Fungal isolate designations are given in Table 1.

Preservative treatment and decay test
Southern yellow pine (SYP) sapwood blocks (19 x 19 x 19mm) were conditioned to 6% equilibrium moisture content and weighed. The blocks were then treated with 1.2% solution of ammoniacal copper citrate (CC) giving an average retention of approximately 8.5 kg/m^3. After treatment, blocks were conditioned for four weeks, reweighed, and steam sterilized. Untreated (control) and treated blocks were subjected to fifteen brown-rot fungi in a soil-block test (ASTM 1994) following the guidelines of AWPA Standard E-10 (AWPA 1997). Soil-bottle cultures were incubated at 27°C/70 % RH for 10 weeks. Six replicate blocks were removed at two-week intervals, dried to constant weight at 60°C and reweighed before testing. Percentage of weight loss was calculated for each group of blocks.

Oxalic acid production
Each block was extracted in 3 ml 0.1M phosphate buffer, pH 7.0, for 2 hours with mixing. Oxalic acid for each extracted sample was determined by microassay using a commercial test kit (Sigma, St. Louis, MO).

Results and discussion

The decay capacity of fifteen brown-rot fungi in untreated SYP was compared to that in copper citrate (CC)-treated SYP. Ten fungi were copper-tolerant (Table 1); tolerance is defined as the relative capacity of an organism to grow or thrive when subjected to an unfavorable environment. Mean percent weight loss (n=6) in CC-treated SYP ranged from 32 to 57%. Copper citrate-treatment of the wood stimulated the decay capacity in two of the Cu-tolerant decay fungi tested, namely *Antrodia radiculosa* and *Serpula incrassata* TFFH-294. A 23% and 8% increase in weight loss, respectively for treated wood was seen compared to untreated wood for those isolates.

Table 1 also shows the maximum values of oxalic acid produced and weight loss for each fungus in control SYP and CC-treated SYP. These values represent the maximum OA and weight loss achieved during the 2 to 10 week evaluation period for each fungus. Generally, maximum weight loss was achieved at 10 wks, but in certain cases, maximum OA was achieved in 2 wks (Figure 1).

Figure 1 shows the time course of OA production and weight loss for each fungus. Overall, OA peaked after 10 wk in control SYP, whereas high levels of OA were achieved in 2 weeks in the CC-treated blocks exposed to Cu-tolerant fungi and remained high throughout the 10-wk incubation. Clearly, the copper citrate induced high levels of OA early in the decay process. Oxalic acid measured in this study represented the soluble free acid, as copper oxalate and/or calcium oxalate form an insoluble precipitate in the wood blocks.

Oxalic acid values in untreated blocks were generally lower at early time points, despite the fact that maximum OA values were above 400 mM for *five* fungi. Only *Antrodia radiculosa* and *Serpula incrassata* TFFH-294 produced and sustained high levels of OA throughout the 10-wk experiment (Figure 1). At 6 wks, *Tyromyces palustris* Typ 6137, had the highest average OA reading (581 mM) in the untreated control blocks and exceeded the OA in CC-treated blocks. In a previous study by Clausen *et al.* (2000), a group of nineteen copper-tolerant *Wolfiporia cocos*

produced 4 to 40-fold more oxalic acid at 2 wks in the presence of copper citrate than on untreated wood. In the broad genera of the ten copper tolerant brown-rot fungi evaluated in this study, the OA produced in the CC-SYP was 2 to 17 times greater at 2 wks than the OA produced on the untreated SYP.

Oxalic acid production and a rapid lowering of pH by decay fungi is important in the initial stages of brown-rot (Green *et al.* 1991). Schmidt *et al.* (1981) implicated oxalic acid in nonenzymatic wood decay by brown-rot fungi. Oxalic acid production is not always directly related to the ability of fungi to decay wood (Micales and Highley, 1988). Micales (1995) demonstrated that a nondegradative isolate of *Postia placenta* (ME-20) produced oxalate decarboxylase, which rapidly broke down OA. Studies on remediation of treated wood by Clausen and Smith (1998) and Clausen (2000) showed that exposure of CCA-treated wood to oxalic acid is effective at removing 81% copper. In this study, copper-tolerant brown-rot fungi showed rapid production of oxalic acid in CC-treated SYP compared to untreated control blocks. This increase was noticeable within 2 weeks exposure to CC-treated blocks. It was also noted that these same fungi were able to produce and maintain oxalic acid at a level of 400 mM over 10 wks.

Conclusions

In biological systems, the interaction of a number of diverse factors such as growth rate, pH, production of OA, and decay capacity all contribute to copper tolerance. However, in this study, copper induced rapid oxalic acid production in the ten copper-tolerant brown-rot fungi. Since it has been shown that oxalic acid is effective at chelating significant amounts of copper from treated wood, we believe oxalic acid is clearly a key component in the successful colonization and degradation of treated wood by copper-tolerant fungi.

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Table 1. Maximum oxalic acid and weight loss in CC-treated and untreated SYP over 10 weeks in soil block bottles'. Copper tolerance is designated + or - for fifteen brown-rot fungi.

	Copper citrate			Control	
Fungal ID	**OA (max)** (mM)	**wt. Loss (max)** (%)	**Cu tolerance**	**OA (max)** (mM)	**Wt. Loss (max)** (%)
***Antrodia vaillantii* FP90877**	624	32	+	380	38
***Antrodia radiculosa* FP90848T**	580	49	+	490	26
***Laetiporus sulphureus* Boat 206**	518	32	+	438	54
***Serpula incrassata* TFFH-294**	486	45	+	426	37
***Wolfiporia cocos* MD106R**	474	57	+	126	61
***Postia placenta* MAD 698**	468	39	+	403	65
Serpula incrassata Mad 563	446	51	+	148	59
***Tyromyces palustris* Typ 6137**	442	51	+	581	42
***Postia placenta* TRL2556**	429	55	+	386	63
***Wolfiporia cocos* FP97438sp**	405	32	+	99	50
Tyromyces *palustris* L15755sp	373	39	+/-	45	35
***Coniophora puteana* MAD 515**	239	6	-	291	34
***Serpula lacrymans* Bam Ebers 315**	178	2	-	169	53
***Gloeophyllum trabeum* MAD 617**	73	0	-	36	50
***Serpula lacrymans* Harm 888-R**	49	5	-	27	15

[1]n=6

OA_{max} and wt $loss_{max}$ for *T. palustris* L15755sp only 6 wk data

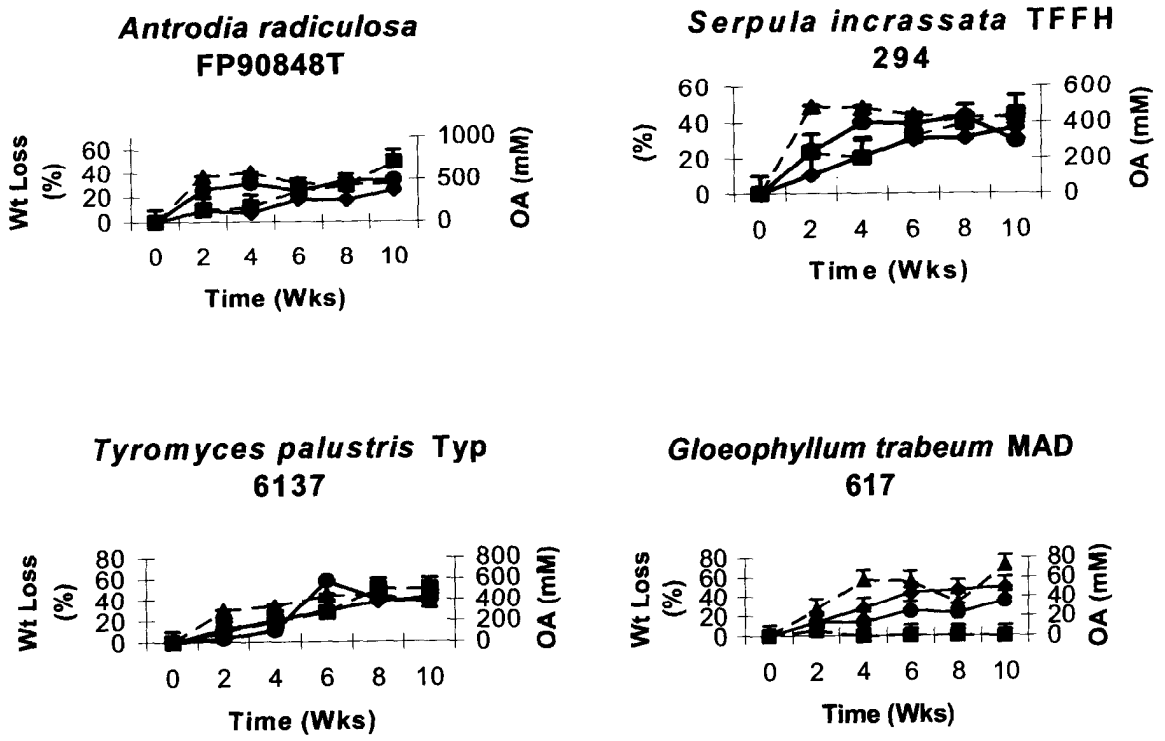

Figure 1. Oxalic acid and weight losses on CC-treated and untreated SYP for representative copper-tolerant fungi (*Antrodia radiculosa* FP 90848T, *Serpula incrassata* TFFH-294, and *Tyromyces palustris* Typ 6137) and a copper-sensitive fungus (*Gloeophyllum trabeum* Mad 617) are shown over a 10 wk incubation:---■---CC-treated wt loss, --- ▲---CC-treated OA, —♦—untreated wt loss, —●—untreated OA.