

Copper tolerance of brown-rot fungi: time course of oxalic acid production[☆]

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Abstract

The increase in the use of non-arsenical copper-based wood preservatives in response to environmental concerns has been accompanied by interest in copper-tolerant decay fungi. Oxalic acid production by brown-rot fungi has been proposed as one mechanism of copper tolerance. Fifteen brown-rot fungi representing the genera *Postia*, *Wolfiporia*, *Meruliporia*, *Gloeophyllum*, *Laetiporus*, *Coniophora*, *Antrodia*, *Serpula*, and *Tyromyces* were evaluated for oxalic acid production bi-weekly in southern yellow pine (SYP) blocks treated with 1.2% ammoniacal copper citrate (CC). Eleven fungi were designated copper-tolerant based upon weight loss in CC-treated blocks. After 2 weeks, these fungi produced 2–17 times more oxalic acid in CC-treated blocks than in untreated blocks. After 10 weeks, weight loss ranged from 32% to 57% in CC-treated SYP. Four fungi were copper sensitive, producing low levels of oxalic acid and minimal weight loss in CC-treated blocks. Rapid induction of oxalic acid appeared to correlate closely with copper tolerance. We conclude that the brown-rot fungi tested that were able to exceed and maintain an oxalic acid concentration of $\geq 600 \mu\text{mol/g}$ effectively decayed SYP treated with CC.

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1. Introduction

Known fungal species that are particularly tolerant to preservatives have been defined in the ASTM standards for preservative testing (ASTM, 1996). Tolerance can be defined as the relative ability of an organism to grow or thrive when subjected to an unfavorable environmental factor or toxin. Tolerance to the lethal action of an otherwise biocidal agent or chemical can also be acquired through its continued use or repeated exposure (Tenenbaum and Kaplan, 1982).

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 40 times the amount of preservative needed to control the most susceptible fungus was required to prevent decay by the most tolerant fungi.

Brown-rot wood decay fungi in the genus *Postia* Fr. and other genera related to *Postia*, such as *Meruliporia* Murrill, *Antrodia* P. Karst, and *Wolfiporia* Ryv. & Gilb, are known to be copper tolerant (Davidson and Campbell, 1954; Collet, 1992; Stephan et al., 1995; Schmidt and Moreth, 1996; Tsunoda et al., 1997). Duncan (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 *W. cocos* isolates in samples of copper-treated wood was greater than that in untreated controls, but considerable variation among individual isolates was noted. A subsequent study by Clausen et al. (2000) confirmed the variation within *W. cocos*, but failed to confirm a statistical

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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. This author noted that variation in preservative tolerance among isolates of individual fungal species had not been adequately investigated.

Oxalic acid production has been implicated in copper tolerance; copper oxalate crystals are formed in decayed wood (Murphy and Levy, 1983; Sutter et al., 1984). Rabanus (1931) and Shimazono and Takubo (1952) hypothesized that tolerance of brown-rot fungi may be linked to oxalic acid production, which precipitates copper into the insoluble form of the oxalate, rendering the copper metabolite inert. However, both groups concluded that lowering of the pH by oxalic acid contributes more to copper tolerance than does the low solubility of copper oxalate. In support of this hypothesis, Young (1961) studied *W. cocos* isolated from failed fence posts that had been treated with copper naphthenate. He demonstrated a striking increase in tolerance to copper when the pH of agar medium supplemented with copper sulfate was lowered from 6 to 2. However, Clausen et al. (2000) did not find increased mycelial weights in copper-containing medium with citric acid (pH 2.5).

Chou (1971) reported that copper oxalate crystals could not be detected in wood infested with *Fibroporia vaillantii* (D.C.) Cke. (syn. *Antrodia vaillantii*), reportedly one of the most copper-tolerant *Postia* species. He concluded that fungal metabolism in wood may differ from that in artificial medium. Clausen et al. (1994) also noted fungal physiological differences in wood compared with an artificial medium for *Postia placenta*. More recent studies by Tsunoda et al. (1997) and Sutter et al. (1983, 1984) closely examined copper tolerance in wood decay fungi. Both studies, which involved copper (II) sulfate and copper naphthenate, concluded that copper tolerance in *Postia* species is a function of copper oxalate precipitation. These studies examined decay capacity and microscopic copper oxalate crystal formation, but they did not correlate oxalic acid production with an increase in decay capacity.

In a previous study, we examined the relationship of oxalic acid production with the decay capacity of 19 isolates of copper-tolerant *W. cocos* in wood treated with ammoniacal copper citrate (CC) after 2 weeks incubation. In the study reported here, our objective was to evaluate the relationship of oxalic acid production with the decay capacity of 15 known copper-tolerant and copper-sensitive brown-rot fungi at 2-week intervals in wood treated with CC.

2. Materials and methods

2.1. 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.

2.2. Preservative treatment and decay test

Southern yellow pine (SYP) sapwood blocks ($10 \times 10 \times 10 \text{ mm}^3$) were conditioned to 6% equilibrium moisture content and weighed. The blocks were then treated with a 1.2% solution of CC (pH 9.0), giving an average in-service retention of approximately 8.5 kg/m^3 ($5.7 \text{ kg/m}^3 \text{ CuO}$). (Note: CC is approximately 67% CuO and 33% citric acid.) After treatment, blocks were conditioned for 4 weeks, reweighed, and steam sterilized. Untreated (control) and treated blocks were subjected to 15 brown-rot fungi in a soil-block test (ASTM, 1996) following the guidelines of AWPAS Standard E-10 (AWPA, 1997). Soil-block bottles were incubated at $27^\circ\text{C}/70\%$ relative humidity for 10 weeks. Six replicate blocks were removed at 2-week intervals, dried to constant weight at 60°C , reconditioned, and reweighed before testing. Percentage of weight loss was calculated for each group of six blocks.

2.3. Oxalic acid production

Each block was extracted in 3 ml 0.1 M phosphate buffer, pH 7.0, for 2 h with mixing. For each extracted sample, oxalic acid was determined by microassay using a commercial test kit (Sigma, St. Louis, MO). Units were expressed as micromoles oxalic acid per gram of final dry weight of wood.

3. Results and discussion

3.1. Decay test

The decay capacity of 15 brown-rot fungi in untreated SYP was compared to that in CC-treated SYP. Eleven fungi were copper-tolerant (Table 1). Mean percentage of weight loss in CC-treated SYP ($n = 6$) ranged from 32% to 57% for copper-tolerant fungi. Ammoniacal CC treatment of the wood stimulated decay capacity (as determined by maximum weight loss) in four copper-tolerant fungi: *A. radiculosa* FP90848T (26–49%), *T. palustris* TYP-6137 (42–51%), and *M. incrassata* TFFH-294 (37–45%), and *T. palustris* L15755sp (35–39%). For these isolates, treated wood had an 11–8% increase in maximum weight loss compared with untreated wood. A similar increase was observed for 7 copper-tolerant isolates of *W. cocos* (Clausen et al., 2000).

3.2. Oxalic acid production

Table 1 shows the mean maximum values of oxalic acid produced for each fungus and weight loss of control and CC-treated SYP. These values represent maximum oxalic

Table 1

Decay capacity and oxalic acid production of test fungi in copper citrate (CC) treated and untreated southern yellow pine^a

Fungus	CC-treated ^b		Copper tolerance	control ^b	
	Oxalic acid (mM)	Weight loss (%)		Oxalic acid (mM)	Weight loss (%)
<i>Antrodia vaillantii</i> FP90877	624 ± 16	32 ± 5	+	380 ± 31	38 ± 4
<i>Antrodia radiculosa</i> FP90848T	580 ± 19	49 ± 9	+	490 ± 7	26 ± 3
<i>Laetiporus sulphureus</i> Boat 206	518 ± 66	32 ± 5	+	438 ± 26	54 ± 4
<i>Meruliporia incrassata</i> TFFH-294	486 ± 20	45 ± 4	+	426 ± 24	37 ± 5
<i>Wolfiporia cocos</i> MD106R	474 ± 115	57 ± 10	+	126 ± 8	61 ± 6
<i>Postia placenta</i> MAD 698	468 ± 21	39 ± 7	+	403 ± 22	65 ± 3
<i>Meruliporia incrassata</i> Mad 563	446 ± 46	51 ± 11	+	148 ± 62	59 ± 6
<i>Tyromyces palustris</i> Typ 6137	442 ± 44	51 ± 4	+	581 ± 25	42 ± 3
<i>Postia placenta</i> TFU2556	429 ± 14	55 ± 3	+	386 ± 27	63 ± 3
<i>Wolfiporia cocos</i> FP97438sp	405 ± 40	32 ± 11	+	99 ± 61	50 ± 7
<i>Tyromyces palustris</i> L15755sp	373 ± 57	39 ± 5	+	45 ± 8	35 ± 3
<i>Coniophora puteana</i> MAD 515	239 ± 150	6 ± 5	–	291 ± 43	34 ± 3
<i>Serpula lacrymans</i> Bam Ebers 315	178 ± 67	2 ± 1	–	169 ± 22	53 ± 4
<i>Gloeophyllum trabeum</i> MAD 617	73 ± 15	0 ± 0	–	36 ± 6	50 ± 3
<i>Serpula lacrymans</i> Harm 888-R	49 ± 16	5 ± 1	–	27 ± 3	15 ± 2

^aAll data are maximum values.^bMean of six replications plus/minus standard deviation.

acid and weight loss achieved for each fungus over the 2–10-week evaluation period. In general, maximum weight loss was achieved at 10 weeks; in certain cases, maximum oxalic acid was achieved earlier in the evaluation period.

Fig. 1 shows the time course of oxalic acid production for each fungus on a dry weight of wood basis. Overall, higher levels of oxalic acid were achieved in 2 weeks in the CC-treated blocks exposed to copper-tolerant fungi and remained high throughout the 10-week incubation. In copper-tolerant fungi, oxalic acid production reached a minimum of 700 $\mu\text{mol/g}$ over the 10-week incubation. In contrast, in copper-sensitive fungi (*C. puteana* and *S. lacrymans* Bam Ebers 315), oxalic acid production on a dry weight basis was strikingly lower and increased at 4–10 weeks. Clearly, CC induced high levels of oxalic acid early in the decay process. In this study, oxalic acid measurements represent soluble free acid and salts; oxalic acid values do not take into account the water-insoluble precipitates of copper oxalate and/or calcium oxalate. Although oxalic acid production in control SYP lagged behind that in CC-treated SYP, three copper-tolerant fungi accumulated higher levels of oxalic acid in control blocks over the 10-week test period: *L. sulphureus* Boat 206, *P. placenta* MAD 698, and *P. placenta* TRL2556 (Fig. 1).

Early in the exposure period, oxalic acid values of untreated control blocks (Fig. 1, open circles) were generally lower than oxalic acid values of CC-treated SYP (Fig. 1, bars). On a dry weight basis, oxalic acid production of all copper-tolerant fungi exceeded 600 $\mu\text{mol/g}$ for CC-treated wood (Fig. 1). *Antrodia radiculosa*, *A. vaillantii*, *M. incrassata*, and *P. placenta* TRL2556 sustained high levels of oxalic acid (> 1000 $\mu\text{mol/g}$) throughout the experiment. At

6 weeks, *T. palustris* Typ 6137 had the highest average oxalic acid reading (581 mM; Table 1) in the untreated control blocks, exceeding the oxalic acid reading in the CC-treated blocks. In a previous study by Clausen et al. (2000), a group of 19 copper-tolerant *W. cocos* fungi produced 4–40-fold more oxalic acid at 2 weeks in the presence of CC than in untreated wood. In the 7 diverse genera of copper-tolerant brown-rot fungi evaluated in the study reported here, oxalic acid production in CC-treated SYP was 2–17 times greater at 2 weeks than that in untreated SYP. Rapid production of oxalic acid would likely precipitate out the copper and render it unavailable for fungal inhibition.

Oxalic acid production and a rapid lowering of pH by decay fungi are important in the initial stages of brown rot (Bech-Andersen, 1987; Green et al., 1991; Shimada et al., 1994). 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 oxalic acid. In studies of remediation of treated wood, Clausen and Smith (1998) and Clausen (2000) showed that exposure of chromated copper arsenic (CCA) treated wood to oxalic acid is effective at removing 81% of copper. In the study reported here, with the exception of *L. sulphureus* Boat 206, copper-tolerant brown-rot fungi showed rapid production of oxalic acid in CC-treated SYP compared to that in untreated control blocks. This increase was noticeable within 2 weeks of 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

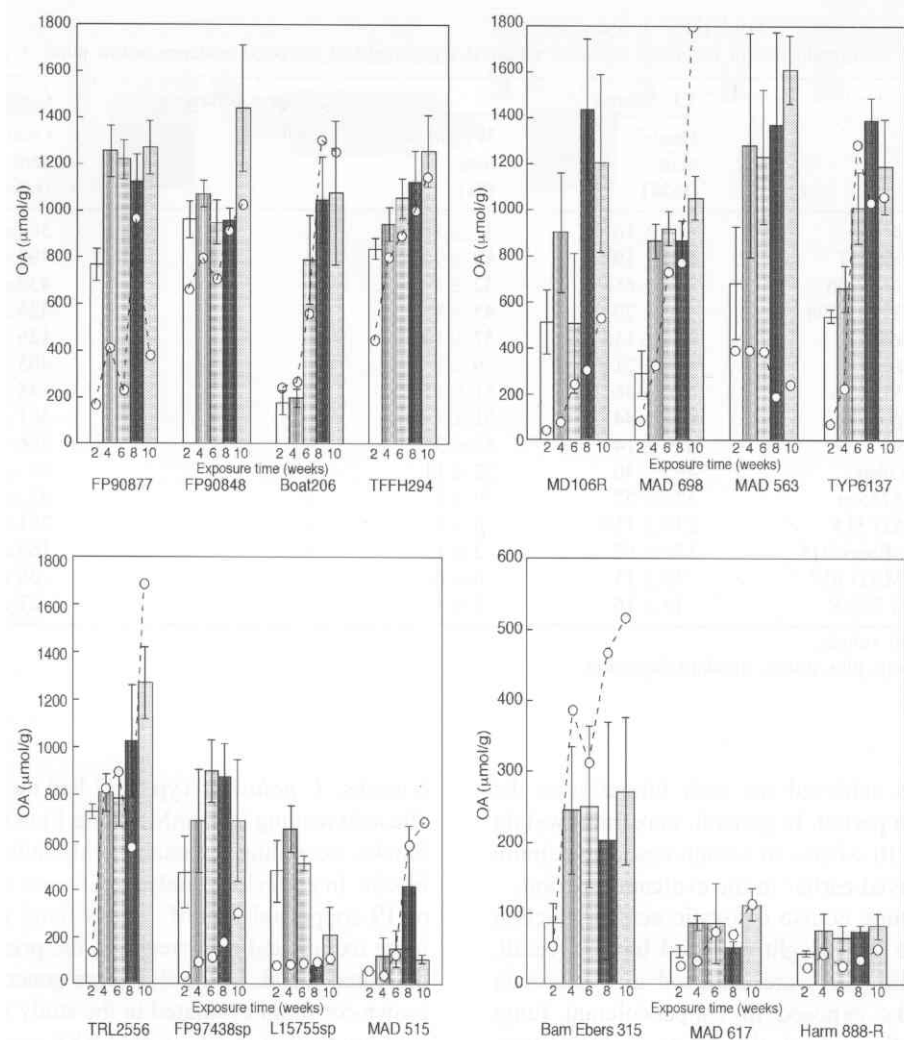


Fig. 1. Mean accumulation of oxalic acid ($\mu\text{mol/g}$) at 2-week intervals over 10-week study period for 15 brown-rot fungi. Open circles represent oxalic acid values for control southern yellow pine (SYP); bars represent oxalic acid values for CC-treated SYP (mean and standard deviation for six samples).

over 10 weeks. The four fungi designated as copper sensitive failed to achieve oxalic acid levels above $600 \mu\text{mol/g}$ in the presence of CC.

Failure to exhibit copper tolerance appears linked to lack of oxalic acid accumulation in *G. trabeum* MAD 617 (Green et al., 1992), even though this species is particularly tolerant to phenolic acid and arsenic compounds (ASTM, 1996). *Coniophora puteana* is purported to produce both oxalic and acetic acid, apparently neutralizing or buffering the oxalic acid effect (Bech-Andersen, 1987). Eight strains of *S. lacrymans* were shown to be resistant to CCA inhibition in agar but sensitive to pentachlorophenol (Thorton, 1991). This author also reported that the German strain was unusually susceptible to copper naphthenate. Sensitivity to environmental conditions and availability of calcium or other divalent cations have been shown to effect oxalic acid production of *S. lacrymans in vitro* (Bech-Andersen, 1987, 1991; Palfreyman et al., 1995). However, in our study, *S. lacrymans* exhibited sensitivity to CC (Table 1).

4. Conclusions

In a previous study on 19 isolates of *Wolfporia cocos*, a known copper-tolerant species of brown-rot fungus, the isolates showed considerable variability in their ability to tolerate ammoniacal CC, ranging from very to barely tolerant. This variability led to the conclusion that there is no direct statistical linear correlation between oxalic acid production and copper tolerance for this group of fungi. In the study reported here, 15 brown-rot fungi from 9 genera were tested for their ability to produce oxalic acid and weight loss of wood in SYP treated with 1.2% CC. Eleven fungi were designated as copper tolerant and four fungi copper-sensitive based upon these criteria. Copper tolerance correlated with the capacity of decay fungi to initiate early and sustained oxalic acid levels over the 10-week incubation period. Although *L. sulphureus* did not initiate oxalic acid production for 6 weeks, oxalic acid values were sustained above $1000 \mu\text{mol/g}$ in weeks 8–10 (Fig. 1). Copper-sensitive fungi

maintained low levels of oxalic acid during the course of study (Fig. 1).

In biological systems, the interaction of a number of diverse factors such as growth rate, pH, oxalic acid production, and decay capacity all contribute to copper tolerance. However, in this study, CC treatment induced rapid oxalic acid production in the copper-tolerant brown-rot fungi. Since oxalic acid has been shown to effectively chelate 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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