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Oxalic acid overproduction by copper-tolerant brown-rot basidiomycetes on southern yellow pine treated with copper-based preservatives ☆,★★

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Abstract

Accumulation of oxalic acid (OA) by brown-rot fungi and precipitation of copper oxalate crystals in wood decayed by copper-tolerant decay fungi has implicated OA in the mechanism of copper tolerance. Understanding the role of OA in copper tolerance is important due to an increasing reliance on copper-based wood preservatives. In this study, four copper-tolerant brown-rot fungi were evaluated for decay capacity and OA production in early stages of exposure to four waterborne copper-based wood preservatives (ammonical copper quat type B and D, ammonical copper citrate, and chromated copper arsenate, type C) and one oilborne copper-based wood preservative (oxine copper) in southern yellow pine blocks. Weight losses were less than 14% during the 4-week incubation. The presence of copper in waterborne preservatives uniformly stimulated OA production by the test fungi within 2 weeks of exposure of the treated blocks to test fungi; 66% to 93% more OA was produced in treated blocks than untreated controls. Oxine copper, a nickel-containing oilborne preservative, prevented both weight loss and OA production in all fungi tested.
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Keywords: Oxalic acid; Copper tolerance; Ammoniacal copper quat; Ammoniacal copper citrate; Chromated copper arsenate; Oxine copper (copper-8-quinolinolate); Copper-based preservative

1. Introduction

The number of copper-based wood preservatives has increased in recent years in response to public concern about the effects of arsenicals on the environment. Copper generally exhibits good biocidal activity (Nicholas and Schultz, 1997), although fungi can be extremely tolerant of toxic metals (Gadd, 1993; Schmidt and Ziemer, 1976). Efficacy against copper-tolerant fungi is a major requirement of any formulation of copper-based wood preservative. Early reviews of preservative tolerance by Zabel (1954) and Cowling (1957) compared tolerances of economically important decay fungi to then-current preservatives. Cowling (1957) reported that up to 40 times the amount of preservative needed to control the most susceptible fungi was required to prevent decay by the most tolerant fungi in 14 of the 18 fungi tested. Brown-rot wood decay fungi in the genus *Postia* (Fr.) and other genera related to *Postia*, such as *Serpula* Murrill, *Antrodia* P. Karst., and *Wolfiporia* Ryv. & Glb., are known to be copper tolerant (Da Costa and Kerruish, 1964; Davidson and Campbell, 1954; Collet, 1992; Stephan et al., 1995; Schmidt and Moreth, 1996; Tsunoda et al., 1997).

Rabanus (1933) and Shimazono and Takubo (1952) suggested that copper tolerance of brown-rot fungi is linked to oxalic acid (OA) 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 OA had more to do with copper tolerance than low solubility of copper oxalate. In support of this hypothesis, Young (1961) 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. Clausen et al. (2000),

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however, found that low pH did not have an influence on the copper tolerance of *W. cocos* by evaluating the effects of citric acid. Mycelial weight of nine *W. cocos* isolates increased in liquid cultures supplemented with copper citrate (CC) (pH 9.7) compared with a citric acid supplement (pH 2.5), suggesting it was the copper and not the citrate (that is, pH) influencing growth (Clausen et al., 2000).

A relationship between OA and copper tolerance has been implicated due to copper oxalate crystal formation in decayed wood (Murphy and Levy, 1983). Tsunoda et al. (1997) and Sutter et al. (1983, 1984) closely examined copper tolerance in wood decay fungi. Both studies, involving copper (II) sulfate and copper naphthenate, concluded that copper tolerance in *Postia placenta, Antrodia vaillantii, T. palustris, Meruliporia incrassata,* and *W. cocos* is a function of the precipitation of copper oxalate. These studies examined decay capacity and microscopic copper oxalate crystal formation but did not correlate OA production with an increase in decay capacity. Green and Clausen (2001) linked rapid OA production and the ability of copper-tolerant fungi to maintain a level of 400 mM OA in the presence of CC with an increased decay capacity of CC-treated wood.

DeGroot and Woodward (1999) studied the decay capacity of a group of *W. cocos* isolates in CC-treated wood. They showed that certain isolates demonstrated an increased decay capacity in CC-treated wood compared with untreated controls but also noted considerable variability among isolates. In a subsequent study by Clausen et al. (2000), OA production in the same group of *W. cocos* isolates did not correlate statistically with decay capacity in CC-treated wood. Green and Clausen (2001) then evaluated OA production in representatives of nine genera of brown-rot fungi in CC-treated wood over time. The results showed that copper-tolerant brown-rot fungi were able to produce and maintain a high level of soluble OA for 10 weeks, regardless of precipitation of insoluble calcium oxalate and copper oxalate.

CC has been used as a preservative model to study copper-tolerant fungi in wood because it lacks co-biocides that are found in most copper-based preservatives. Inorganic salts of metals, which are toxic even at low concentrations, are often combined as co-biocides to impart broad-based efficacy in wood preservative formulations. For the purpose of studying copper tolerance, the presence of co-biocides, such as chromium and arsenic, could confound results (DeGroot and Woodward, 1998). Thus, OA production by copper-tolerant brown-rot fungi in wood treated with various formulations of copper-based wood preservatives has not been extensively studied. Our objective in this study was to evaluate OA production of four copper-tolerant brown-rot fungi during the first 4 weeks of colonization in southern yellow pine treated with five copper-based wood preservative formulations.

Table 1
Copper-based wood preservatives and retention

Preservative treatment	Copper composition	Active ingredient (w/w%)	Calculated retention (kg/m^3)
Waterborne			
Ammoniacal copper quat B	66.7% CuO	0.6	4.0
Ammoniacal copper quat D	66.7% CuO	0.6	4.0
Chromated copper arsenate-C	18.5% CuO	0.3	2.0
Ammoniacal copper citrate	62.3% CuO	2.4	17.0
Oilborne			
Oxine copper	10% copper-8-quinolinolate	0.075	0.33

2. Materials and methods

2.1. Fungal cultures

Four copper-tolerant brown-rot fungi, *P. placenta* (Fr.) M. Lars. & Lomb. (MAD 698), *M. incrassata* (Burk. & Curt.) Murr. (TFFH 294), *W. cocos* (F.A. Wolf) Ryv. & Gilb. (MD 106R), and *A. vaillantii* (D.C.:Fr.) Ryv. (FP 90877R) were maintained on 2% malt extract agar (Difco Laboratories, Detroit, MI).

2.2. Preservative treatment and decay test

Southern yellow pine sapwood blocks ($10 \times 10 \times 10\ mm^3$) were conditioned to 6% equilibrium moisture content and weighed. The blocks were then vacuum-treated with four water-based copper preservatives (ammoniacal copper quat (same as copper quaternary ammonia)-type B (ACQB) and D (ACQD), chromated copper arsenate (CCA)-C, and ammoniacal CC) and one oil-based copper preservative (oxine copper) to give standard American Wood Preservers' Association (AWPA) retentions (Table 1). Preweighed blocks were submerged in respective treating solutions and subjected to a vacuum of – 165.5 kPa (24 lb/in^2) gage pressure twice for 20 min each. Blocks were dried at 60°C, reconditioned, and reweighed to calculate preservative retentions. Oxine copper was diluted with toluene to achieve the desired solution concentration before treatment. Working retentions were selected based on minimum inhibitory concentrations determined previously by Woodward and DeGroot (1999). Sublethal preservative retentions were selected to prevent complete inhibition of fungal growth. Untreated blocks served as controls for waterborne preservatives, and toluene-treated blocks served as controls for oxine copper-treated blocks. The blocks were then subjected to the test fungi in a soil-block test (ASTM, 1998) following the guidelines of AWPA Standard E-IO (AWPA, 1997). Soil bottle cultures were incubated at 27°C and 70% relative humidity for 4 weeks. Six replicates of treated and untreated blocks for each fungus were tested after incubation for 1–4 weeks. Following incubation, blocks were

Table 2
Weight loss caused by four copper-tolerant brown-rot fungi in southern yellow pine blocks treated with five copper-based wood preservatives[a]

Fungus	Time	Weight loss (%)						
	weeks	ACQB	ACQD	CC	CCA-C	Untreated control	OC	Toluene control
A. vaillantii	1	2.2	2.9	1.0	0.8	1.7	6.2	9.2
	2	1.9	2.6	3.3	3.3	1.6	7.0	12.3
	3	1.9	2.1	6.4	13.1	5.6	7.4	13.6
	4	0.4	1.2	11.2	14.5	4.8	4.4	12.4
P. placenta	1	2.7	2.7	1.9	1.2	1.6	7.7	9.8
	2	2.3	3.2	2.7	1.9	9.9	7.9	15.4
	3	2.0	1.3	2.7	2.0	17.2	7.0	19.4
	4	1.3	3.3	1.0	0.0	23.7	3.8	21.2
W. cocos	1	2.2	2.1	0.9	1.1	3.7	4.5	11.0
	2	1.3	3.2	3.4	1.9	12.9	6.6	16.0
	3	1.6	1.9	2.8	0.4	18.2	6.7	22.1
	4	0.4	0.9	0.3	0.0	27.0	4.5	24.6
M. incrassata	1	4.3	2.0	1.6	0.6	0.0	6.3	9.1
	2	2.6	1.9	3.5	1.3	4.9	7.0	11.6
	3	1.0	1.6	3.6	6.2	10.2	6.8	12.7
	4	1.3	0.7	8.0	8.1	17.2	6.5	12.4

[a]ACQB, ammoniacal copper quat B; ACQD, ammoniacal copper quat D; CC, ammoniacal copper citrate; CCA-C, chromated copper arsenate-C; OC, oxine copper.

removed from bottles, brushed free of mycelium, ovendried at 60°C, conditioned to 6% equilibrium moisture content, and reweighed. Percentage of weight loss was calculated from the treated weights before and after decay testing.

2.3. Oxalic acid assay

Each block was extracted in 3 ml 0.1 M phosphate buffer, pH 7.0, for 2 h with mixing on a rotating platform at 100 revolutions min^{-1}. Oxalic acid was determined for each extracted solution by microassay using a commercial test kit and standards (Sigma, St. Louis, MO).

3. Results and discussion

3.1. Decay capacity

Weight losses were expected to be low, both because of the selected preservative retention and the short duration of the study. The objective was to evaluate weight loss and OA production during early stages of decay by known copper-tolerant brown-rot fungi (Clausen et al., 2000; Green and Clausen, 2001). Decay capacity, measured as weight loss, was less than 10% for **P.** *placenta* MAD 698, *M. incrassata* TFFH 294, and *W. cocos* MD 106R in all preservative treatment groups during the 4 weeks of this study. Weight losses for control blocks ranged from 4.8% for *A. vaillantii* to 27% for *W. cocos* after 4 weeks (Table 2). In both untreated blocks and blocks treated with ACQB and ACQD, *A. vaillantii* FP 90877R showed less than 6% weight loss. Weight losses in CC- and CCA (11% and 14%, respectively) exceeded weight losses in untreated controls for *A. vaillantii* after the 4-week incubation period. *A. vaillantii* is known to be tolerant of CC (Green and Clausen, 2001) and has been reported to cause significant weight loss in CCA-treated blocks (Da Costa and Kerruish, 1964). Decay capacity was uniformly inhibited by oxine copper for all fungi tested and at each test time compared with toluene controls. Toluene controls demonstrated that the preservative carrier alone did not inhibit decay fungi; toluene is the carrier for oxine copper. Except for *A. vaillantii*, which grew slower than expected in untreated controls, toluene control weight losses closely resembled untreated control weight losses (Table 2). Decay in OC-treated blocks was insignificant (< 10%) for all fungi after 4 weeks incubation. The retention selected (0.33 kg/m^3) for OC was not lethal for the fungi and allowed evaluation of OA production under conditions of inhibited fungal growth in the presence of the preservative.

3.2. Oxalic acid production

The presence of copper in waterborne preservatives uniformly and rapidly stimulated OA production by four copper-tolerant fungi despite the presence of various co-biocides. Because of expected low weight losses during the 4-week study, OA results were expressed and evaluated as total free acid and salts produced over time. *A. vaillantii* FP 90877R produced at least 400 mM of OA on ACQB-,

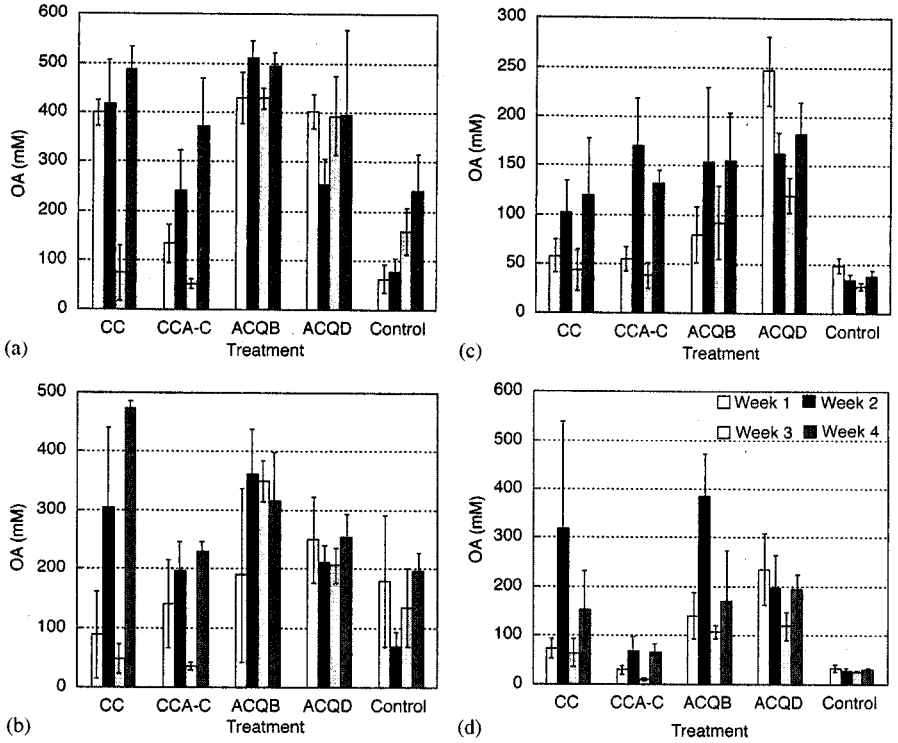

Fig. 1. Oxalic acid (OA) production in southern yellow pine blocks treated with four copper-based wood preservatives: (a) *A. vaillantii,* (b) *M. incrassata, (c) P. placenta,* and (d) *W. cocos* (bars represent standard deviation. $N = 6$).

ACQD-, and CC-treated blocks after 1 week of incubation (Fig. 1a). OA production increased after 2 weeks incubation for three of the four waterborne preservatives. OA production for *M. incrassata* TFFH 294, which was similar to *A. vaillantii,* showed an increase at week 2 for all waterborne preservatives except ACQD (Fig. 1b). Maximum OA production on ACQD-treated blocks occurred after 1 week for three of the 4 fungi evaluated in this study. *P. placenta* MAD 698 produced 55 to 247 mM OA in the first week of incubation on blocks treated with any of the four waterborne wood preservatives. The highest reading (247 mM) occurred on ACQD-treated blocks, with peak amounts of OA (102–171 mM) for the remaining preservative treatments occurring after the second week of incubation (Fig. 1c). *W. cocos* MD 106R showed a similar pattern in ACQB, CC, and CCA-C peak OA production after 2 weeks incubation (Fig. 1d). *W. cocos* produced the greatest amount of OA on ACQD-treated blocks after 1 week (235 mM).

With few exceptions, OA results declined after 3 weeks (Fig. 1-d) with increased levels of OA detected once again at 4 weeks. This might represent binding of copper to oxalate; copper oxalate cannot be detected by our assay method. Presumably, the fungi continue to produce OA at a continuous rate, with precipitation of copper oxalate causing a decline in soluble oxalate after 3 weeks. Acid solubilization of copper oxalate from test blocks would enable determination of the total amount and form of oxalate. However, the presence of copper stimulated production of free oxalic acid and salt despite insoluble copper oxalate formation.

Oxalic acid production in oxine-copper-treated blocks was inhibited (Fig. 2). Since the toluene control blocks did not demonstrate inhibition of OA production, the authors conclude that the nickel-2-ethylhexoate co-biocide in oxine copper has an inhibitory effect on decay capacity and prevented OA metabolism in all four copper-tolerant fungi used in this study.

4. Conclusions

It is generally accepted that OA plays a key role in the successful colonization of treated wood by copper-tolerant brown-rot fungi (Rabanus, 1933; Shimazono and Takubo, 1952; Murphy and Levy, 1983; Clausen et al., 2000). It has been hypothesized that extracellular OA accumulated in the hyphal sheath binds to copper and is precipitated around the hyphae (Sutter et al., 1983). Indeed, calcium oxalate crystals are typical in decayed wood (Green et al., 1996) and copper oxalate crystals are commonly observed in decayed wood that has been treated with copper-based preservatives. The OA assay method used in this study measured soluble acid and salts of oxalate but was unable to distinguish between free OA and copper oxalate. It has also been shown that OA effectively solubilizes and removes significant amounts of copper from treated wood (Clausen, 2000). Results from

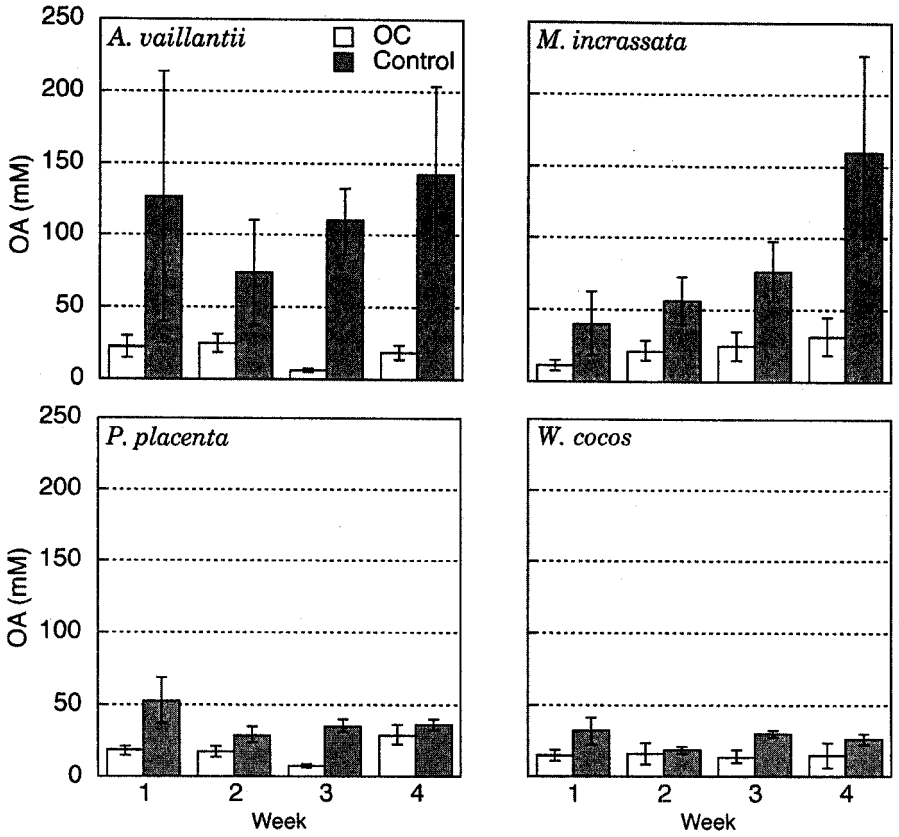

Fig. 2. Oxalic acid (OA) production in southern yellow pine blocks treated with oxine copper (OC) compared with toluene control blocks for the four fungi used in this study (bars represent standard deviation. $N = 6$).

this study revealed that OA production by copper-tolerant brown-rot fungi occurred rapidly in southern yellow pine blocks treated with four waterborne copper-based preservatives. Increases in oxalic acid production were seen after 1 to 2 weeks incubation, depending on the fungus. The presence of copper in the waterborne preservatives uniformly induced OA production by the fungi used in this study compared with OA production in untreated controls. Oxine copper, a nickel-containing oilborne preservative, inhibited both decay capacity and OA metabolism in the copper-tolerant fungi tested (Fig. 2). Decay was not inhibited in the toluene controls (Table 2).

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