Defeating Copper Tolerance: An Example of How "Omics" Research Can Accelerate Discovery of New Wood Protection Compounds

Juliet D. Tang
Tina Ciaramitaro
USDA Forest Service Forest Products Laboratory
Starkville, Mississippi

Darrel D. Nicholas
Maria Tomaso-Peterson
Susan V. Diehl
Mississippi State University
Starkville, Mississippi

ABSTRACT

Imagine if you could measure all the genes being expressed at any one time in an organism and you knew what all the genes did in a cell. The power of this knowledge would allow you to determine how organisms regulate gene activity to survive. This is the essence of how "omics" science accelerates biological understanding. From a wood protection standpoint, understanding which genes regulate copper tolerance effectively identifies metabolic chokepoints that would have the highest likelihood of interfering with copper tolerance. Thus, the search for copper co-biocides is no longer random, but instead, follows a rational process, guided by knowledge of which steps in metabolism are being differentially regulated for survival on substrates containing copper. We used this rational process to identify two potential inhibitors that might defeat copper tolerance: pyraclostrobin (a member of the strobilurin family of fungicides) and salicylhydroxamic acid (SHAM). The strobilurins and SHAM disrupt different steps in mitochondrial respiration, a metabolic pathway we found to be up-regulated with the biosynthesis of oxalate in our omics analysis. Oxalate is the molecule that has a direct role in removing copper toxicity by trapping the copper in an insoluble copper oxalate crystal. In accordance with our hypothesis, the triple combination of 5000 µg/mL copper sulfate, 4 or 8 µg/mL pyraclostrobin, and 100 µg/mL SHAM completely inhibited growth compared to compounds tested alone over a 20-day observation period in agar plate studies with the copper-tolerant fungus, *Fibroporia radiculosa*. Results from AWPA E22 soil block tests, which uses percent compression strength loss as a measure of decay, were also promising when tested against *F. radiculosa* and another copper-tolerant species, *Fomitopsis palustris*. Using above ground retentions for copper ethanolamine, the triple combination wood treatment (copper, pyraclostrobin, and SHAM) performed much better than copper ethanolamine alone, and performed as well or better than wood treated with our reference, the above ground retention for ACQ-D. With over 900 potential targets or genes that showed differential regulation for survival on copper, it is our hope that co-biocide discovery will no longer be the bottleneck in wood preservative development.

Keywords: copper tolerance, co-biocide, strobilurin, decay fungi, salicylhydroxamic acid

OBJECTIVES

1. Identify the glyoxylate (GLOX), tricarboxylic acid (TCA), and adenosine triphosphate (ATP) cycles as potential metabolic chokepoint for copper tolerance.
2. Identify the strobilurins and salicylhydroxamic acid (SHAM) as potential inhibitors of the ATP cycle.
3. Test the activity of one strobilurin (namely pyraclostrobin) and SHAM for growth inhibition of one copper-tolerant fungus.
4. Test activity of copper, pyraclostrobin, and SHAM for protecting wood from decay by two copper-tolerant fungi.

INTRODUCTION

Many brown-rot decay fungi are copper-tolerant (Cowling 1957; Young 1961) and cannot be controlled by copper alone (Figure 1). The mechanism appears to involve fungus-produced oxalate, which precipitates copper to form an insoluble copper oxalate crystal that is no longer toxic (Murphy and Levy 1983; Clausen and Green 2003; Green and Clausen 2003; Jin et al. 2014). As a result, most copper-based wood preservatives incorporate one or more co-biocides that help to limit growth of these destructive copper-tolerant fungi. Some of these co-biocides, like inorganic arsenic and chromium, can be acutely toxic and are registered by EPA as Restricted Use Products (*e.g.* chromated copper arsenate and ammoniacal copper zinc arsenate) (EPA 2016). Growing environmental concerns and the push for green products, however, have motivated industry

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to develop organic and more eco-friendly alternatives like alkaline copper quaternary (ACQ), micronized copper quaternary (MCQ), and copper azole (CA). ACQ and MCQ combine copper with quaternary ammonium compounds, while CA combines copper with azoles. None of these co-biocides, however, specifically target the biological mechanisms underlying copper tolerance.

Figure 1. *Fibroporia radiculosa*, a brown-rot fungus, attacking wood treated with a copper-based wood preservative.

Whether a co-biocide that targets copper tolerance can perform better than one that does not is unknown because there has been little research elucidating the metabolic processes copper-tolerant fungi use to survive on copper. To address this knowledge gap, Tang et al. (2013) used transcriptomics, a high-throughput DNA sequencing technology, to comprehensively analyze activity of all the genes that were being regulated for survival on wood treated with a copper-based preservative. They found up-regulation of genes in the GLOX cycle, thereby providing the first genetic evidence that copper-tolerant fungi were controlling rates of oxalate production to remove copper toxicity (oxalate is an end product of the GLOX cycle). Moreover, co-regulation of genes in the TCA and ATP or energy production cycles of the mitochondria gave insight that these biological processes were also critical for survival on copper. Metabolically, the TCA and GLOX cycles can be linked through their shared intermediates, isocitrate and succinate (Munir et al. 2001), while the connection between the TCA and ATP cycles exists because the TCA cycle provides the electrons that drive ATP production (Nelson and Cox 2004). The data from Tang et al. (2013) suggested that copper removal from treated wood was an energy-intensive process with these three cycles functioning as one metabolic network. By switching into high gear, enough oxalate and energy could be produced for survival on copper (Figure 2).

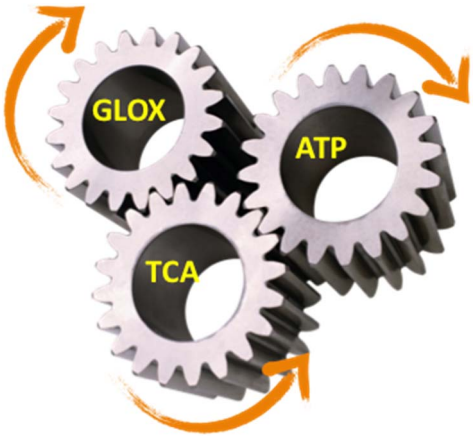

Figure 2. Potential metabolic chokepoints for copper tolerance. Inhibition of any one of these three linked cycles (GLOX, glyoxylate; TCA, tricarboxylic acid; ATP, energy production) could theoretically prevent growth of copper-tolerant fungi in the presence of copper.

From the standpoint of wood protection, we propose that these three cycles could act as metabolic targets or chokepoints that could be inhibited to defeat copper tolerance (Figure 2). This approach replaces the more traditional, random search for co-biocides with a rational process that begins with an understanding of how fungal genes are being regulated for survival in a changing environment. Theoretically, paralysis of just one of these three cycles could knock out the entire network and inhibit growth of copper-tolerant fungi. Ecologically speaking, an advantage of this approach is that inhibitors that target copper tolerance are less likely to have an effect on non-target organisms. A co-biocide that potentiates or enhances the effect of copper could also benefit producers because it could reduce the amount of copper and co-biocide needed to obtain the same level of protection in ground contact and above ground applications. Reduced retentions, in turn, translate to reduced environmental impact, lower costs for the producer, and more savings for the consumer.

MATERIALS AND METHODS

Fungi

Two strains of brown-rot fungi were used: *Fibroporia radiculosa* (Peck) Parmasto TFFH 294 and *Fomitopsis palustris* (Berk. & M.A. Curtis) Gilb. & Ryvarden ATCC 62978. The copper tolerance is well known for *F. radiculosa* TFFH 294 (Green and Clausen 2003; Clausen and Jenkins 2011), but not for *F. palustris* ATCC 62978, although tolerance has been described for other strains of this fungus (Green and Clausen 2003).

Growth Studies in Agar

Copper (II) sulfate pentahydrate (abbreviated as $CuSO_4$ henceforth) ($\geq$ 98% purity, Sigma-Aldrich, St. Louis, MO) crystals were weighed and used directly (w/v). Stock solutions of pyraclostrobin (99.9% purity, Sigma-Aldrich, St. Louis, MO) and salicylhydroxamic acid or SHAM (99% purity, Acros Organics, Pittsburgh, PA) were prepared (w/v) in acetone and ethanol, respectively. After sterilizing the nutrient-rich MYA media (2% malt extract, 0.2% yeast extract, 1.5% agar) by autoclaving for 20 min, the media was aliquoted (25 mL) into polypropylene tubes, and allowed to equilibrate at 52°C in a hot water bath. Chemicals from stock solutions (62.5 µL), weighed $CuSO_4$ crystal, or solvent controls (62.5 µL) were added to the molten MYA and hand-vortexed (10 s). After chemical additions were done, each tube was poured into one petri dish (100 mm x 15 mm), covered, and allowed to cure 24 hr at room temperature. Each plate was then centrally inoculated with a plug (6 mm diam.) cored from the leading edge of thick mycelial growth. Fungi were grown in the dark at 27 $\pm$ 1°C and 70 $\pm$ 1% RH. All fungal procedures were performed aseptically in a Class II biological safety cabinet. One experiment was comprised of the complete set of treatments replicated on three different days (n = 3), where each day replicate of treatments was prepared from a separate set of stock solutions and fungal master plates. Concentrations of chemicals in agar were reported in µg/mL. The distance of radial growth from the plug at 5, 10, 15, and 20 days after inoculation (DAI) was recorded and used to calculate the average daily growth rate (mm/day) within each five-day interval. The maximum average daily growth rate was then used for treatment comparisons in the statistical analysis. Readings for a plate were terminated if growth reached the outside edge of the petri dish.

Decay Studies in Wood

The copper ethanolamine (CuE) and dimethyldidecyl ammonium carbonate (DDAC) stock solutions were generously donated by Viance LLC (Charlotte, NC) and were 11.2% copper as CuO and 47.8% DDAC, respectively. As prescribed by the AWPA Standard P5-15 for waterborne preservatives, the treating solution for ACQ-D was a 2:1 (w:w) formulation of CuE:DDAC (AWPA 2015b). ACQ-D was used as the reference treatment at a retention of 0.15 pcf, representing UC3A for sawn products in above ground exterior applications (AWPA 2015c). Treating solutions with pyraclostrobin (w/w) were obtained by adding the appropriate weight of fungicide Insignia WG (20% active ingredient [AI] as pyraclostrobin, BASF Corporation, Research Triangle Park, NC), then adding toluene to the desired final weight. Extraction efficiency of the fungicide was assumed to be 100%. Treating solutions of the pyraclostrobin and SHAM (w/w) combination were prepared in a solution of 10% ethanol (w/w) in toluene. The SHAM was dissolved first in the appropriate weight of ethanol, then the toluene and fungicide were added to the desired weights. The retention of SHAM and the highest retention of pyraclostrobin AI were chosen to mimic the 0.05 pcf retention of the DDAC component in ACQ-D.

An accelerated soil block test was set up according to the AWPA Standard E22-15 (AWPA 2015a) with the following minor modifications. Wafers were treated by the vacuum soak method (15 min at -27 mm Hg followed by a 30 min soak). For CuE combination treatments, wafers were treated first with CuE, dried overnight at room temperature in the fume hood, then placed in an oven at 100°C for 48 h to reduce the pH before they were treated with SHAM, fungicide, or SHAM and fungicide. The feeder strips were inoculated with the fungus (12 x 5 mm diam. plugs per container, evenly spaced around the long sides of each feeder strip) and allowed to grow at 28 $\pm$ 1°C and 70$\pm$ 2% RH until the strip was fully colonized (about 2.5 weeks). Each block replicate (10 wafers, each receiving a different treatment) was split into two test containers (3 wafers on one feeder and 2 on the other feeder) such that one set of containers had the wafers without SHAM treatment and the other held the treatments with SHAM plus the ACQ-D reference. Retentions in wood were reported as pcf and compression

strength loss was the maximum value recorded during the period the wafer was crushed a distance equal to 5% of its radial dimension. Percent compression strength loss for each wafer was calculated relative to the average of the unexposed water-treated controls.

Statistical Analysis

Statistical analyses to test for significance ($p \leq 0.05$) were conducted in R (R Core Team 2013) using the RStudio graphical user interface (RStudio Team 2015). Shapiro-Wilk and Bartlett tests were used to test for normality and homogeneity of variance of the data. If both normality tests failed after logarithmic and inverse transformations, then Dunnett's non-parametric multiple contrast test (and simultaneous confidence intervals) was used to make pair-wise comparisons between each treatment and the indicated combination treatment. A multiple contrast test was performed because our hypothesis was that the indicated combination treatment would exhibit less growth and less percent compression strength loss than any of the other treatments. The arguments for the mctp function used to run the multiple contrast test in the nparcomp package were: type = Dunnett, alternative = greater, control = indicated combo treatment, asy.method = fisher, and effect = unweighted.

RESULTS AND DISCUSSION

Metabolic Chokepoint Inhibitors

A literature search identified several potential inhibitors of the three metabolic chokepoints (GLOX, TCA, and ATP cycles). Of those identified, the most readily available chemicals were those that bind and inhibit different steps in the ATP cycle, specifically the strobilurins and SHAM. Strobilurins belong to a family of anti-fungal organic compounds (Figure 3) that exert their toxicity by binding Complex III, thereby interfering with electron transport and ATP production in the mitochondria (Bartlett et al. 2002). Since 1996, strobilurins have seen widespread use for disease prevention in agriculture with registrations on over 84 different crops world-wide (Bartlett et al. 2002). For this investigation, we focused on one strobilurin, namely pyraclostrobin (Figure 3C).

SHAM, although non-toxic by itself, is known to potentiate the activity of some strobilurins because it binds and inhibits the enzyme alternative oxidase (Wood and Hollomon 2003). This enzyme also occurs in the mitochondria and has been attributed to two different roles: (1) it can act like a pressure-relief valve, preventing oxidative stress when energy production demands on the mitochondria are high (Wood and Hollomon 2003), and (2) it can help stabilize the redox state by regenerating oxidized nicotinamide adenine dinucleotide (NAD+) when demands for glucose-derived biosynthetic intermediates are high (Vanlerberghe 2013). Their effects on wood decay fungi and their potential to act as co-biocides for copper-based wood protection, however, are unknown.

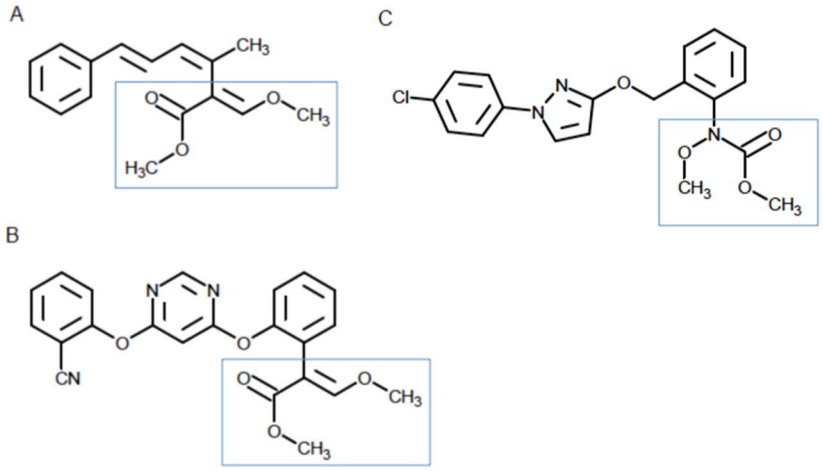

Figure 3. Chemical structures of some strobilurins. A. Strobilurin A, a natural product was isolated from the litter decay fungus, *Strobilurus tenacellus* (Pers.) Singer. B. Azoxystrobin, a synthetic developed by Syngenta. C. Pyraclostrobin, an invention of BASF Corporation. The boxed area shows the toxophore: methyl *β*-methoxyacrylate in A and B and methyl *N*-methoxycarbamate in C.

Growth Studies in Agar

The results of the *F. radiculosa* growth studies in agar are shown in Table 1. SHAM showed no toxicity and maximum average mycelial growth rate was generally similar to that observed in the controls. This confirmed our expectations since a review of the literature described lack of toxicity for SHAM against various agricultural pathogens (Wood and Hollomon 2003).

Pyraclostrobin acted in a dose-dependent manner with higher concentrations being more toxic. The maximum average growth rates observed for the acetone control, 4 µg/mL, and 8 µg/mL concentrations were 3.0, 1.22, and 0.47 mm/day, respectively (Table 1). It should be noted that strobilurins are generally not acutely toxic to established mycelia (Bartlett et al. 2002). Instead, they exert their most toxic effects during spore germination and zoospore motility, which are energy intensive developmental and physiological stages (Bartlett et al. 2002).

Table 1 also shows that the addition of SHAM increased toxicity of pyraclostrobin at the 4 µg/mL concentration (1.22 and 0.4 mm/day without and with SHAM, respectively), but less so at the 8 µg/mL (0.47 and 0.4 mm/day without and with SHAM, respectively). Apparently, the potentiation effect of SHAM was more obvious at the low concentration of pyraclostrobin, although it was also associated with more variation (SD = 0.3). The term potentiation rather than synergism is used when the additive is non-toxic by itself (Wood and Hollomon 2003). One possible interpretation of this conditional potentiation is that alternative oxidase has a greater role in providing biosynthetic intermediates than preventing oxidative damage. Consequently, potentiation was more evident at concentrations of pyraclostrobin that were less inhibitive for growth.

The magnitude of copper tolerance of *F. radiculosa* was demonstrated by the fact that growth continued, albeit more slowly (0.12 mm/day), at the high concentration of 5000 µg/mL $CuSO_4$ (Table 1). In the Dunnett's non-parametric multiple contrast statistical analysis (Table 1), the 4 Combo treatment (4 µg/mL pyraclostrobin + $CuSO_4$ + SHAM) exhibited a significantly lower growth rate compared to $CuSO_4$ or to any of the other single or two compound treatments tested, but was not significantly different from the 8 Combo treatment (8 µg/mL pyraclostrobin + $CuSO_4$ + SHAM). The only treatments that showed complete growth inhibition were the Combo treatments that combined pyraclostrobin with $CuSO_4$ and SHAM. This result was promising because it provided the first preliminary evidence for our hypothesis that copper tolerance could be defeated by inhibiting one of the three metabolic chokepoints.

Table 1. Maximum average growth rate of *F. radiculosa* on agar amended with different additives

Treatment (µg/mL)	Maximum Average Growth Rate (mm/day) $\bar{x} \pm$ SD
CTL Acetone	3.00* ± 0.00
CTL Ethanol	2.83* ± 0.12
CTL Water	3.00* ± 0.00
100 SHAM	2.87* ± 0.06
4 PS	1.22* ± 0.06
8 PS	0.47* ± 0.06
4 PS + 100 SHAM	0.40* ± 0.30
8 PS + 100 SHAM	0.40* ± 0.00
5000 $CuSO_4$	0.12* ± 0.03
4 Combo	0.00 ± 0.00
8 Combo	0.00 ± 0.00

***** Denotes a significant difference between paired comparisons with the 4 PS Combo treatment by Dunnett's non-parametric multiple contrast test (n = 3). Abbreviations: CTL, control; PS, pyraclostrobin; SHAM, salicylhydroxamic acid; 4 Combo, combination of 4 µg/mL PS + 100 µg/mL SHAM and 5000 µg/mL $CuSO_4$; 8 Combo, combination of 8 µg/mL PS + 100 µg/mL SHAM and 5000 µg/mL $CuSO_4$

Decay Studies in Wood

Tables 2 and 3 show the percent compression strength loss of wood for *F. radiculosa* and *F. palustris*, respectively, in the E22 decay studies. As expected, both species of fungi exhibited high levels of copper tolerance. Percent compression strength loss of wood treated with water or 0.1 pcf CuE + solvent was among the highest observed (83.5 and 72.6% for *F. radiculosa* and 75.0 and 78.1% for *F. palustris,* respectively), indicating that neither water nor CuE offered much protection from decay. Unlike the agar study, none of the wafer treatments completely inhibited mycelial growth which covered all the

wafers regardless of treatment by the end of the test. We did observe, however, that the lag period for mycelial growth was noticeably longer on wood wafers that exhibited lower percent compression strength loss.

In the 0.1 pcf CuE and Insignia combination treatments, pyraclostrobin appeared to act in a concentration-dependent manner; increasing the pyraclostrobin AI increased protection from decay. The concentration-dependent effect across retentions, however, appeared to be more consistent for *F. radiculosa* (72.6, 44.3, 29.7, and 23.6% percent compression strength loss for 0, 0.0125, 0.025, and 0.05 pcf pyraclostrobin AI, respectively, Table 2) than *F. palustris* (78.1, 48.7, 47.5, 27.7% percent compression strength loss for 0, 0.0125, 0.025, and 0.05 pcf pyraclostrobin AI, respectively, Table 3).

Table 2. Percent compression strength loss for wafers sequentially treated with CuE in process 1 and Insignia and SHAM (alone or in combination) in process 2 after four weeks of exposure to *F. radiculosa* in an E22 accelerated soil block test

Process 1 (pcf)	Process 2 (pcf active ingredient)	Percent Compression Strength Loss $\bar{x} \pm$ SD
Water	--	83.5* ± 15.1
CuE (0.1)	Solvent	72.6* ± 15.4
CuE (0.1)	SHAM (0.05)	56.0* ± 22.6
CuE (0.1)	Low Insignia (0.0125)	44.3* ± 21.0
CuE (0.1)	Low + SHAM = Low Combo	38.2* ± 23.1
CuE (0.1)	Med Insignia (0.025)	29.7* ± 18.8
CuE (0.1)	Med + SHAM = Med Combo	22.1 ± 15.4
CuE (0.1)	High Insignia (0.05)	23.6 ± 19.2
CuE (0.1)	High + SHAM = High Combo	12.4 ± 9.4
ACQ-D (0.15)	--	19.0 ± 6.9

***** Denotes a significant difference between paired comparisons with the High Combo treatment by Dunnett's non-parametric multiple contrast test (n = 8). Low, Med, and High refers to the specified pyraclostrobin retention as active ingredient in the Insignia fungicide for the Combo treatments. SHAM retention was 0.05 pcf in all cases.

The addition of 0.05 pcf SHAM to the 0.1 pcf CuE and Insignia treatment combinations seemed to consistently improve efficacy for each pyraclostrobin AI retention tested. For *F. radiculosa*, percent compression strength losses without and with SHAM were: 44.3 and 38.2% for CuE + 0.0125 pcf pyraclostrobin AI; 29.7 and 22.1% for CuE + 0.025 pcf pyraclostrobin AI; and 23.6 and 12.4% for CuE + 0.05 pcf pyraclostrobin AI, respectively (Table 2). Similarly, for *F. palustris*, the percent compression strength losses without and with SHAM were: 48.7 and 23.8% for CuE + 0.0125 pcf pyraclostrobin AI; 47.5 and 29.3% for CuE + 0.025 pcf pyraclostrobin AI; and 27.7 and 15.1% for CuE + 0.05 pcf pyraclostrobin AI, respectively (Table 3). In each case, SHAM appeared to potentiate the pyraclostrobin toxicity in the presence of copper.

In the Dunnett's non-parametric multiple contrast statistical analysis for *F. radiculosa* (Table 2), wood treated with the CuE + High Combo treatment (0.05 pcf pyraclostrobin AI + SHAM) exhibited a significantly lower percent compression strength loss compared to water and CuE controls, and CuE combinations with SHAM, Low Insignia, Low Combo, or Med Insignia. The CuE + High Combo treatment, however, was not significantly different from the ACQ-D reference, Med Combo, and High Insignia treatments (Table 2). For *F. palustris,* the results of the non-parametric contrast analysis were slightly different due to a greater potentiation effect of SHAM. The percent compression strength loss of the High Combo treatment was not significantly different from the other Combo treatments, but was significantly less compared to the remaining treatments (water and CuE controls, CuE combinations with SHAM, Low, or Med Insignia, and the ACQ-D reference, Table 2). Thus, the High Combo treatment protected the wood from decay as much as or better than the ACQ-D reference and, depending upon the fungus, comparable protection levels could be achieved with lower retentions of pyraclostrobin, as long as it occurred in combination with CuE and SHAM.

Table 3. Percent compression strength loss for wafers sequentially treated with CuE in process 1 and Insignia and SHAM (alone or in combination) in process 2 after four weeks of exposure to *F. palustris* in an E22 accelerated soil block test

Process 1 (pcf)	Process 2 (pcf active ingredient)	Percent Compression Strength Loss $\bar{x} \pm$ SD
Water	--	75.0* ± 10.3
CuE (0.1)	Solvent	78.1* ± 10.7
CuE (0.1)	SHAM (0.05)	60.3* ± 14.6
CuE (0.1)	Low Insignia (0.0125)	48.7* ± 17.1
CuE (0.1)	Low + SHAM = Low Combo	23.8 ± 12.8
CuE (0.1)	Med Insignia (0.025)	47.5* ± 14.6
CuE (0.1)	Med + SHAM = Med Combo	29.3 ± 12.3
CuE (0.1)	High Insignia (0.05)	27.7 ± 10.9
CuE (0.1)	High + SHAM = High Combo	15.1 ± 9.8
ACQ-D (0.15)	--	39.7* ± 9.8

***** Denotes a significant difference between paired comparisons with the High Combo treatment by Dunnett's non-parametric multiple contrast test (n = 8). Low, Med, and High refers to the specified pyraclostrobin retention as active ingredient in the Insignia fungicide for the Combo treatments. SHAM retention was 0.05 pcf in all cases.

An important outcome of this investigation is that it is a "proof of concept" study; we have demonstrated feasibility of using transcriptomics or gene expression data to identify co-biocides with the potential of defeating copper tolerance. There could be many more potent inhibitors, given that half of the 900 or so genes that were differentially expressed for survival on copper were up-regulated when copper was still active enough to prevent strength loss to the wood (Tang et al. 2013). Although our emphasis in this study was to disable a metabolic network, proteins that are not known to act in networks could also be targeted, if their gene expression were up-regulated for survival on copper, like the copper P-type ATPase pump family studied by Ohno et al. (2016). The key, however, is that the inhibitor must be used in combination with the biologically active copper. Otherwise, the effect of the inhibitor will not be as dramatic since the metabolic network must be in high gear before the effects of disabling it can be realized.

CONCLUSIONS

Our previous work examining whole genome expression suggested that there was a co-regulated metabolic network linking the GLOX, TCA, and ATP cycles for survival of copper-tolerant fungi on wood treated with copper-based preservatives. This result meant that paralysis of any one of these three cycles or metabolic chokepoints could theoretically defeat copper tolerance. To test this hypothesis, the toxicity of two mitochondrial inhibitors, pyraclostrobin (a member of the strobilurin family of fungicides that inhibits ATP production) and SHAM (a potentiator of strobilurin toxicity that inhibits the enzyme alternative oxidase), were investigated. In accordance with our hypothesis, only the triple combination of $CuSO_4$, pyraclostrobin, and SHAM was acutely toxic and able to completely inhibit growth of *F. radiculosa* in agar-based media. When pyraclostrobin was tested alone, toxicity was dose-dependent. SHAM was non-toxic by itself, but could potentiate or increase toxicity of pyraclostrobin only at the low concentration levels. Decay studies in wood with *F. radiculosa* and *F. palustris* were also consistent with our hypothesis. The High Combo treatment with CuE, pyraclostrobin, and SHAM (whose retentions were chosen to mimic the above ground retention of ACQ-D) protected wood from decay as much as or better than the ACQ-D reference treatment. The presence of CuE did not appear to interfere with either the concentration-dependent effect of pyraclostrobin or the potentiation effect of SHAM. In fact, by including CuE and SHAM in the treatment, lower retentions of the pyraclostrobin were equally effective at protecting the wood from decay as the High Combo treatment. The results of the toxicity and decay studies presented are encouraging and demonstrate the feasibility of this rational approach for accelerating the discovery of novel copper co-biocides for wood protection.

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