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Activity of Two Strobilurin Fungicides Against Three Species of Decay Fungi in Agar Plate Tests

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ABSTRACT

The objective of this study was to examine the toxicity of strobilurin fungicides against wood decay fungi in order to assess their potential to act as a co-biocide for copper-based wood protection. Two strobilurin fungicides, Heritage (50% azoxystrobin active ingredient) and Insignia (20% pyraclostrobin active ingredients), and copper sulfate pentahydrate were tested against one white rot fungus (*Trametes versicolor*) and two brown rot fungi (*Gloeophyllum trabeum* and *Fibroporia radiculosa*). SHAM (salicylhydroxamic acid) was also included in the study because it is a known potentiator of strobilurin activity. All treatments were incorporated in an agar-based media and evaluated for their effects on mycelial growth rate. Based on minimum inhibitory concentration values found for *F. radiculosa* (a copper-tolerant fungus), Insignia was 5.5x more toxic than Heritage. SHAM at 100 ppm, increased toxicity 9x for Heritage and 1.2x for Insignia. In a 20-day toxicity screening study, the four one-compound treatments tested were: copper sulfate (5000 ppm), Heritage (20 ppm), Insignia (20 ppm) and SHAM (100 ppm). The two two-compound treatments were: Heritage + SHAM and Insignia + SHAM. The two three-compound treatments were: copper sulfate + Heritage + SHAM and copper sulfate + Insignia + SHAM. For the two copper-susceptible fungi (*T. versicolor* and *G. trabeum*), the treatments that caused complete growth inhibition were copper sulfate alone and the two three-compound treatments (copper sulfate + Heritage + SHAM and copper sulfate + Insignia + SHAM). For *F. radiculosa*, the two- and three-compound treatments were the most toxic, with maximum daily average growth rates statistically similar to the copper sulfate treatment. A key result, however, was that only the three-compound treatment of copper sulfate + Insignia + SHAM completely inhibited growth of the copper-tolerant fungus. Thus, it appears that pyraclostrobin, which is the active ingredient in Insignia, has greater potential than azoxystrobin to act as a co-biocide for completely inhibiting growth of a copper-tolerant fungus.

Keywords: wood protection, decay fungi, copper tolerance, strobilurin

1. INTRODUCTION

Strobilurins are one of the newest classes of organic fungicides for the control of pathogenic fungi attacking agricultural crops, turfgrass, and other economically important plants. First released in 1996, there are now ten major strobilurin fungicides on the market, which account for 23-25% of the global fungicide sales (Anonymous 2016). Their benefits include broad spectrum activity against most disease-causing fungi, acute toxicity against germinating fungal spores, and relatively low toxicity for terrestrial animals. Some of the major drawbacks of strobilurins are

their toxicity to aquatic organisms and the number of fungal pathogens that are showing signs of resistance (Young *et al.* 2010; Standish *et al.* 2015).

The original strobilurin was isolated from *Strobilurus tenacellus* (Pers.) Singer (Anke *et al.* 1977), a gilled mushroom known as the pinecone cap. This small brown mushroom is native to Europe and Asia, where it decays fallen pine and spruce cones. In nature, strobilurins most likely function as a defensive secondary metabolite, protecting valuable food resources by preventing spore germination of competing saprobes (Anke 1995). Chemical modifications of the original strobilurin by pesticide manufacturers, such as Syngenta, BASF, and Bayer, have produced synthetic strobilurins like azoxystrobin, picoxystrobin, pyraclostrobin, and trifloxystrobin. Most of these synthesized compounds share the β-methoxyacrylate moiety found in the natural strobilurins (Fig. 1). The synthetics, however, exhibit higher toxicity and improved physical characteristics, such as low volatility and increased resistance to UV breakdown (Bartlett 2002).

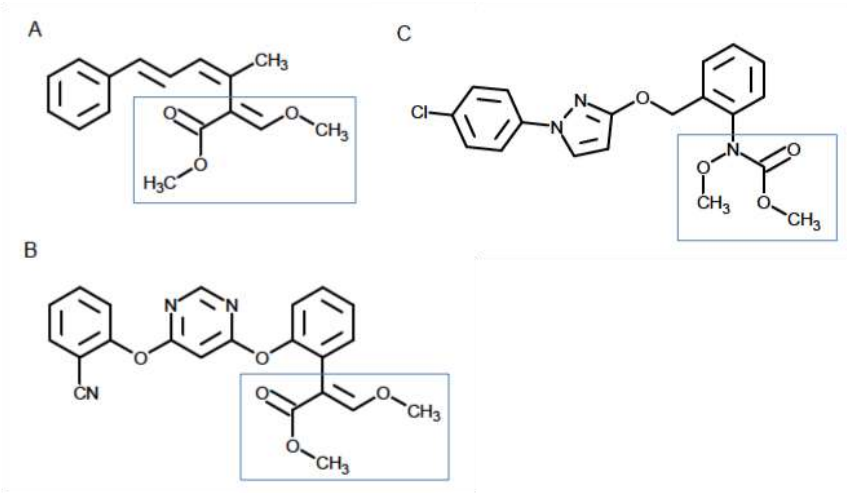

Figure 1. Chemical structures of three strobilurins. A. Strobilurin A, a natural product was isolated from the litter decay fungus, *Strobilurus tenacellus*. B. Azoxystrobin, a synthetic developed by Syngenta. C. Pyraclostrobin, an invention of BASF Corporation. The boxed area shows the toxophore: methyl *β*-methoxyacrylate in A and B and methyl *N*-methoxycarbamate in C. Reprinted with permission from Tang *et al.* (2016).

Mode of action studies have demonstrated that strobilurins exert their toxicity by paralyzing adenosine triphosphate (ATP) production (Von Jagow *et al.* 1986; Bartlett *et al.* 2002). Mitochondrial respiration is how organisms convert carbohydrates and fats into ATP, the universal currency of energy in cells. In the mitochondria, cofactors produced by the tricarboxylic acid (TCA) cycle, namely NADH (reduced nicotinamide adenine dinucleotide) and $FADH_2$ (reduced flavin adenine dinucleotide) are oxidized by three large enzymes called Complexes I, II, III, and IV. As electrons are passed from one enzyme complex to the next, an electrochemical gradient of hydrogen ions (H^+) is formed that powers ATP production (**Fig. 2)**. By binding tightly and specifically to a site known as the quinone-oxidizing pocket of Complex III, strobilurins halt the flow of electrons and prevent the reduction of ubiquinone (Q) to ubiquinol. Thus, another name for strobilurins is quinone outside (Qo) inhibitors. The interrupted electron flow results in diminished ATP production. The final step in electron transport, which is the reduction of O_2 to H_2O, also ceases to occur.

When mitochondrial respiration is inhibited at Complex III or IV, plants and fungi can survive the resulting oxidative stress because they have a bypass pathway through an enzyme called alternative oxidase (Wood and Hollomon 2003). In this bypass pathway, electron transport is essentially grounded via alternative oxidase, which transfers the electrons from ubiquinol to O_2 directly (Fig. 2). The trade-off, though, is reduced ATP production. One of the known inhibitors of alternative oxidase is salicylhydroxamic acid (SHAM), which is often used in combination with strobilurins to achieve more complete inhibition of fungal growth. Because SHAM targets the bypass pathway, it is generally non-toxic, by itself. Therefore, it is known as a potentiator of strobilurin activity, as opposed to a synergist, which would have inherent toxicity.

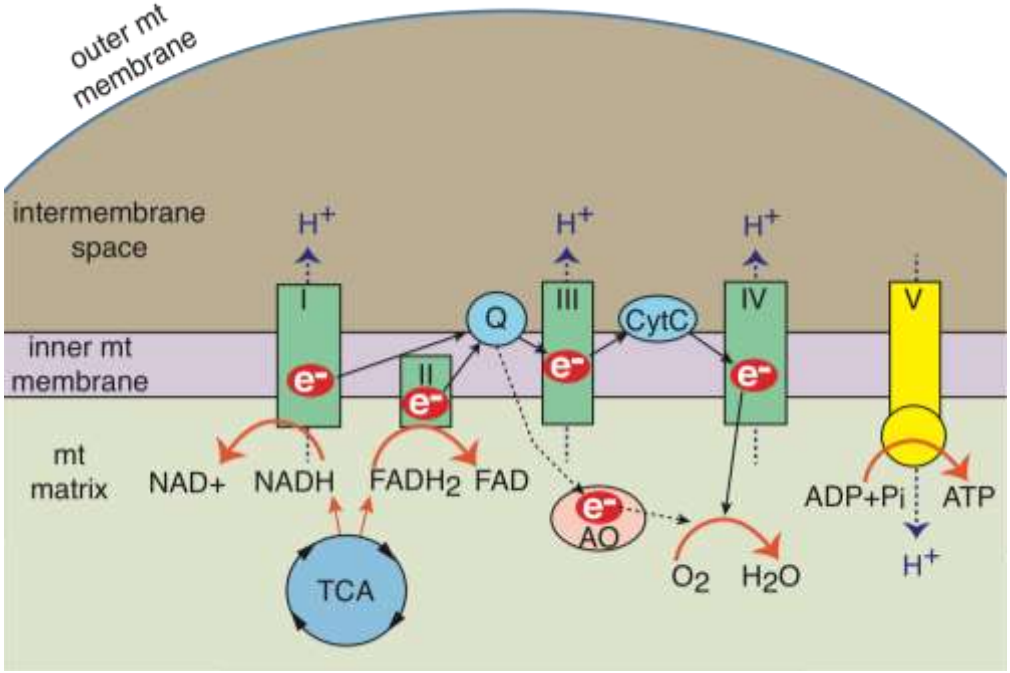

Figure 2. Mitochondrial (mt) respiration in plants and fungi. Complexes I-V, ubiquinone (Q) and cytochrome C sit on the inner mitochondrial membrane. Complexes I-IV are proton pumps that use electrons (e^-) derived from NADH (reduced nicotinamide adenine dinucleotide) or $FADH_2$ (reduced flavin adenine dinucleotide) to move hydrogen ions (H^+) from the mitochondrial matrix into the space between the inner and outer mitochondrial membranes. The resulting proton gradient drives ATP (adenosine triphosphate) synthesis by the action of ATP synthetase (complex V). O_2 acts as the final electron acceptor and is reduced to H_20 by complex IV or by alternative oxidase (AO) in the bypass pathway. Strobilurins interfere with electron transport by binding at the quinone-oxidizing pocket of Complex III. Salicyl hydroxamic acid or SHAM inhibits the bypass pathway by binding alternative oxidase. Other abbreviations: TCA, tricarboxylic acid cycle; *Pi*, inorganic phosphate; ADP, adenosine diphosphate.

Interpretation of our prior gene expression studies (Tang *et al.* 2013) with *Fibroporia radiculosa* (Peck) Parmasto suggested that strobilurins might act as an effective co-biocide for copper-based wood protection. *F. radiculosa,* like many other brown rot fungi, grows at copper concentrations that are toxic to most wood-destroying organisms (Green and Clausen 2003; Clausen and Jenkins 2011). The generally accepted mechanism is solubilization then biotransformation of extracellular copper into insoluble copper-oxalate crystals by fungal-produced oxalic acid. Increased oxalic acid and copper-oxalate crystal production have been observed for copper-tolerant wood decay fungi (Clausen *et al.* 2000; Green and Clausen 2003; Tang *et al.* 2013) and

non-wood decay fungi (Fomina *et al.* 2005). Furthermore, single gene expression analysis of *F. radiculosa* showed that exposure to wood treated with a copper-based preservative induced higher than normal expression of genes with putative functions in oxalic acid biosynthesis (Tang *et al.* 2013; Ohno *et al.* 2015). In addition, transcriptomics (whole genome expression analysis) showed that there were concurrent increases in expression of other genes besides those involved in oxalic acid biosynthesis. These genes had roles in the TCA cycle, quinone biosynthesis, and ATP production (Tang *et al.* 2013). Co-regulation of these genes suggested that these distinct metabolic pathways were operating together as a single metabolic network for survival on wood treated with copper-based preservatives. Given that strobilurins interfere with ATP production, it was conceivable that they might serve as a co-biocide for copper to defeat copper-tolerance. However, there was no data describing the effects of strobilurin, SHAM, or combinations of copper, strobilurin, and SHAM on wood-destroying fungi.

Therefore, the first part of this study was designed to determine the minimum inhibitory concentration (MIC) and concentration that inhibits 50% growth (IC_{50}) of two strobilurin fungicides when tested alone and when tested in combination with SHAM against one species of copper-tolerant brown rot fungus. The second part screened toxicity of the strobilurin fungicides when tested alone, in a two-compound mix that consisted of one strobilurin fungicide plus SHAM, and in a three-compound mix that consisted of copper sulfate, one strobilurin fungicide, and SHAM. The toxicity screening study was performed against three species of wood destroying fungi that included a white rot fungus, a copper-tolerant brown rot fungus, and a copper-susceptible brown rot fungus. All fungal tests were performed in agar-based media.

2. EXPERIMENTAL METHODS

2.1 Chemicals

Commercially sold strobilurin fungicides marketed under the names Heritage® 50 WG (50% azoxystrobin active ingredient, Syngenta Professional Products, Greensboro, NC) and Insignia® 20 WG (20% pyraclostrobin active ingredient, BASF Corporation, Raleigh, NC) were purchased from local vendors. SHAM and copper sulfate ($CuSO_4 \cdot 5H_20$) were obtained from Sigma-Aldrich (St. Louis, MO). To obtain the final desired chemical concentration in agar, a 10-fold dilution series was prepared from individual stock solutions. The stock solution solvents and concentrations were: 8 mg Heritage per ml acetone, 8 mg Insignia per ml acetone, 40 mg SHAM per ml ethanol, and 0.08 M copper sulfate. Final concentrations were reported as weight per volume of agar media and expressed as parts per million (ppm). The exceptions were copper sulfate tested at concentrations equal to or above 500 ppm. In these cases, the concentration of the stock would have exceeded its solubility. Instead, copper sulfate crystals were weighed and added directly to the agar media to obtain the final desired concentration (0.125 g per 25 ml for 5000 ppm and 0.0125 g per 25 ml for 500 ppm).

2.2 Fungi

The strains and decay strategies of the three species of wood decay fungi selected for this study are listed in Table 1. Of the two brown rot species, *Gloeophyllum trabeum* (Pers.) Murrill was copper-susceptible (Green and Clausen 2003) and *F. radiculosa* was copper-tolerant (Green and Clausen 2003; Clausen and Jenkins 2011). Although white rot fungi are generally copper-susceptible (Cowling 1957; Young 1961), *Trametes versicolor* (L.) Lloyd was included in order to understand how white rot fungi respond to the strobilurins. Fungi were maintained on sterile malt yeast agar (MYA) plates (2% malt extract, 0.2% yeast extract, 1.5% agar) in the dark at 27 $\pm$ 1°C and 70 $\pm$ 5% RH. Isolates were purchased from ATCC or acquired from the Center for Forest Mycology Research (USDA Forest Service, Madison, WI).

Table 1. Strains of brown rot (BR) and white rot (WR) fungi used

Species	Decay	Strain
Fibroporia radiculosa	BR	TFFH 294
Gloeophyllum trabeum	BR	Mad-617-R
Trametes versicolor	WR	FP-101664-Sp

2.3 Growth Inhibition in MYA Plates

After sterilizing the MYA media by steam autoclaving for 20 min at 121ºC and 1.05 kg per cm^2, the media was aliquoted (25 ml) into polypropylene tubes and allowed to equilibrate at 52°C in a hot water bath. Strobilurin fungicide extracts (62.5 µl) and/or weighed copper sulfate crystal and/or solvent control solutions (62.5 µl) were added to the molten MYA and hand-mixed for 10 s to obtain the desired treatment concentrations. Once the chemicals were incorporated, the tubes were removed from the hot water bath, and poured into individual petri dishes (100 mm x 15 mm). Plates were cured for 24 hr at room temperature, then centrally inoculated with a plug (6 mm diam) cored from culture plates at the leading edge of mycelial growth. Plates with fungi were incubated in the dark at 27 $\pm$ 1°C and 70 $\pm$ 1% RH. All procedures were performed aseptically in a Class II biological safety cabinet.

MIC and IC_{50} determinations for the two strobilurin fungicides (with or without SHAM at 100 ppm) and copper sulfate (alone) were performed with *F. radiculosa* only. The strobilurin fungicide concentrations tested were: 20, 2, 0.2, 0.02, and 0.002 ppm plus a solvent control. Copper sulfate was tested at 5000, 500, 50, 5, and 0.5 ppm plus a solvent control. Each concentration series was replicated on three different days with separate stock solutions prepared each day. The distance of radial growth from the plug at 5, 10, 15, and 20 days after inoculation was recorded and used to calculate the average daily growth rate in mm per day for each five-day interval. Because the lag time appeared to vary with treatment, the maximum average daily growth rate was used as the response variable in the statistical analysis. Readings for a plate were terminated when growth reached the dish edge.

Toxicity screening studies in MYA were conducted against each of the three species of fungi listed in Table 1. For each test, a series of one-, two-, and three-compound treatments were run plus the controls (water, acetone, and ethanol). The one-compound treatments were: 5000 ppm copper sulfate, 20 ppm Heritage, 20 ppm Insignia, and 100 ppm SHAM. The two-compound treatments consisted of the addition of 100 ppm SHAM to the strobilurin fungicide treatment. The three-compound treatments included 5000 ppm copper sulfate with the two-compound combinations. Tests were replicated three times for each species with separate stock solutions prepared each time. Judgments of relative toxicity were based on the observed differences in the maximum average daily growth rate between the treatments and the controls. Relative growth was calculated as a percentage of the control treatment that exhibited the largest maximum average daily growth rate.

2.4 Statistical Analysis

Graphing and statistical analyses for MIC and IC_{50} determinations were performed in R (R Core Development Team 2013). Both growth parameter determinations were made by estimating a simple linear regression model between the dependent variable y or maximum average daily growth rate, and the log(x + 1), where log was the natural logarithm computed by R and x was concentration of the fungicide. The value where the line crossed the x-axis was used to solve the equation for the predicted MIC. IC_{50} was calculated from the regression at the point where maximum average daily growth rate was half the growth when log(x + 1) equaled zero. The coefficient of determination (R^2), which is the proportion of variance in y explained by x, was also reported.

Graphing and statistical analyses for the one-, two-, and three-compound treatment effects on the maximum average daily fungal growth rate in the toxicity screening studies were performed in R (R Core Development Team 2013) and SAS (SAS Institute Inc 2013). For all species examined, the Shapiro-Wilk test showed that the maximum average daily growth rate data did not follow a normal distribution but was best described by a right-skewed distribution that was not improved by transformation. However, the Levene's test statistic (Brown and Forsythe 1974) indicated equal variance among the treatment groups when absolute deviations from the median were used in the calculation. Since analysis of variance can tolerate skewed distributions with only a small effect on the Type I error rate (as long as the design is balanced), PROC ANOVA followed by the Tukey-Kramer mean separation test was deemed appropriate.

3. RESULTS AND DISCUSSION

3.1 MIC Determinations

Growth curves for the brown rot fungus, *F. radiculosa*, growing on MYA media containing different concentrations of the strobilurin fungicide extracts tested alone or in combination with 100 ppm SHAM are plotted in Fig. 3.

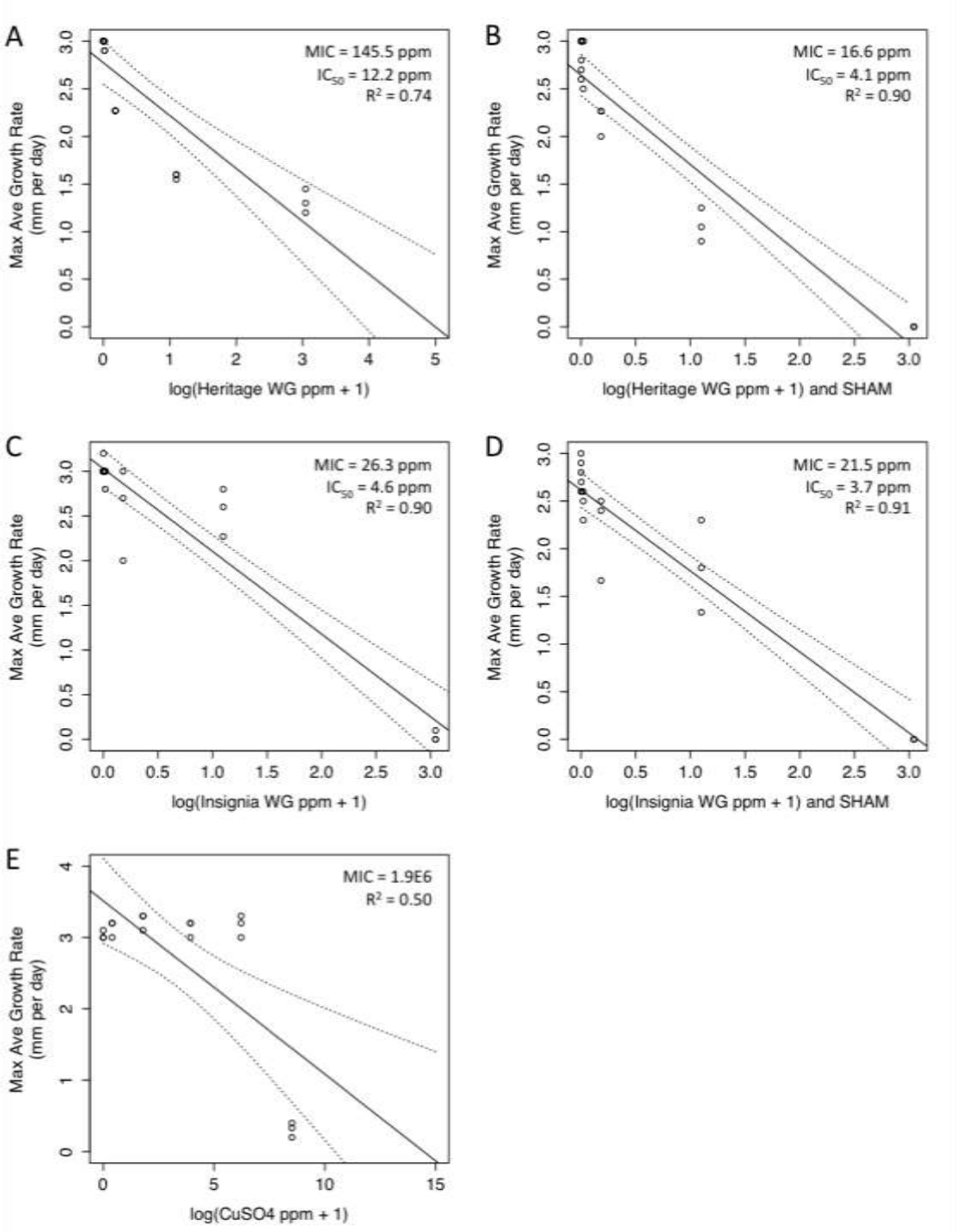

Figure 3. Growth parameter estimates (MIC, IC_{50}, and R^2 coefficient of determination values) for *F. radiculosa*, a copper-tolerant brown rot fungus, when tested on agar media for 20 days. Treatments were: (A) Heritage, (B) Heritage + 100 ppm SHAM, (C) Insignia, (D) Insignia + 100 ppm SHAM, and (E) copper sulfate. The IC_{50} was not estimated for copper sulfate because of the low R^2. Empty circles mark the 18 plotted data values, the solid line is the fitted linear regression, and the two dotted lines define the upper and lower boundaries of the 95% confidence interval for the true mean y values of x. Max ave, maximum average

Concentration effects of Heritage on growth are shown in Fig. 3A. At the highest concentration tested (20 ppm Heritage), mycelial growth was slowed, but not entirely inhibited (1.32 $\pm$ 0.13SD mm maximum average daily growth rate). The predicted MIC and IC_{50} based on the regression model were 145.5 ppm and 12.2 ppm, respectively. The relatively low toxicity contributed to the

low R^2 value (0.74) and the wide 95% confidence intervals. When 100 ppm SHAM was added to each Heritage concentration tested (Fig. 3B), the MIC and IC_{50} values dropped to 16.6 ppm and 4.1 ppm, respectively. The 95% confidence interval for the combination treatment was narrower and the R^2 value for the linear regression was 0.90. The increased toxicity of the combination was also reflected by the nearly complete inhibition of growth (0.0004 $\pm$ 0.0006 SD mm maximum average daily growth rate) for the 20 ppm Heritage + SHAM treatment. Thus, based on the MIC values, SHAM appeared to increase toxicity of Heritage by about 9-fold.

Based on the MIC, Insignia (Fig. 3C) showed 5x greater toxicity than Heritage (Fig. 3A), but toxicity of Insignia was only slightly increased by the addition of 100 ppm SHAM (Fig. 3D). The MIC and IC_{50} for Insignia alone were 26.3 ppm and 4.6 ppm, respectively. For the combination Insignia + SHAM treatments, the MIC and IC_{50} dropped to 21.5 ppm and 3.7 ppm, respectively. The addition of SHAM to the Insignia treatments increased toxicity by only 1.2-fold at the MIC. Although these differences seem small, the effects on observed mycelial growth were more dramatic. At 20 ppm Insignia, the highest concentration tested, the maximum average daily growth rate was 0.03 $\pm$ 0.06 SD mm, compared to 0.0004 $\pm$ 0.0006 SD mm for the combination treatment of 20 ppm Insignia + SHAM.

Observed differences in toxicity of related compounds can be due to numerous factors, the most important of which are structural differences in the inhibitor, structural differences in the molecular target, and interactions of the inhibitor with its target, or for that matter, any other molecule it meets before reaching the target. At present, there are ten major strobilurins sold in the fungicide market. They are: azoxystrobin, kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, famoxadone, fenamidone (Bartlett *et al.* 2002), fluoxastrobin, and dimoxystrobin (Anonymous 2016). Only azoxystsrobin and pyraclostrobin were tested in this paper but future investigations should determine if other strobilurins used in agriculture can be re-purposed for use as a co-biocide in wood protection.

The level of copper tolerance exhibited by *F. radiculosa* is shown in Fig. 3E. Of the 5 copper sulfate concentrations tested, only the highest (5000 ppm copper sulfate) had any effect on slowing growth (0.31 $\pm$ 0.10 mm maximum average daily growth rate). Mycelial growth on the four other concentrations tested was basically no different from the controls ($\geq$ 3.0 mm maximum average daily growth rate). Despite the very low R^2 value (0.50) and broad 95% confidence intervals, the MIC was estimated to be about 1.9×10^6 ppm copper sulfate. These high tolerance levels explain why co-biocide options are needed when wood protection relies on copper-based preservatives.

3.2 Toxicity Screening Against Fungi

The effects of one-, two-, and three-compound treatments on the maximum average daily growth rate of the fungi are shown in Fig. 4. The white rot fungus, *T. versicolor* (Fig. 4A), and the copper-susceptible brown rot fungus, *G. trabeum* (Fig. 4B), showed similar response patterns to the treatments and controls. Copper sulfate alone was highly toxic, causing complete growth inhibition during the 20-day exposure period. None of the one-compound treatments were as toxic as the copper sulfate treatment, but the order from most to least toxic was: Insignia (equivalent to 4 ppm pyraclostrobin), Heritage (equivalent to 10 ppm azoxystrobin), and SHAM. In the two-compound treatments, the addition of SHAM caused a significant increase in toxicity of Heritage, but did not significantly alter toxicity of Insignia. The only treatments that were not significantly different from the copper sulfate treatment were the three-compound treatments that combined copper sulfate and SHAM with Heritage or Insignia. Growth was completely inhibited indicating that the strobilurin and SHAM combination did not interfere with toxicity of the copper sulfate for the copper-susceptible fungi. This was a concern initially because there

was evidence from the literature that SHAM can precipitate metal ions by forming a complex (Fazary 2014), and the effect of this interaction might have reduced copper toxicity.

Decay strategy did not appear to be as important in determining the response pattern in the toxicity screening study as copper-susceptibility was. The difference in decay strategy is that white rot fungi can fully degrade lignin while brown rot fungi cannot. Consequently, the genomes of white rot fungi are particularly rich in oxidases that are absent in brown rot fungi. (Floudas *et al.* 2012). Co-metabolism or the ability of these oxidases to act on substrates with structures similar to lignin is why certain white rot fungi have proved to be useful for bioremediation of sites contaminated with polycyclic aromatic hydrocarbons and other organic pesticides and pollutants (Pointing 2001). The fact that both the white rot fungus and the copper-susceptible brown rot fungus produced similar response patterns, however, suggests that the strobilurins were not being selectively degraded by the white rot fungus.

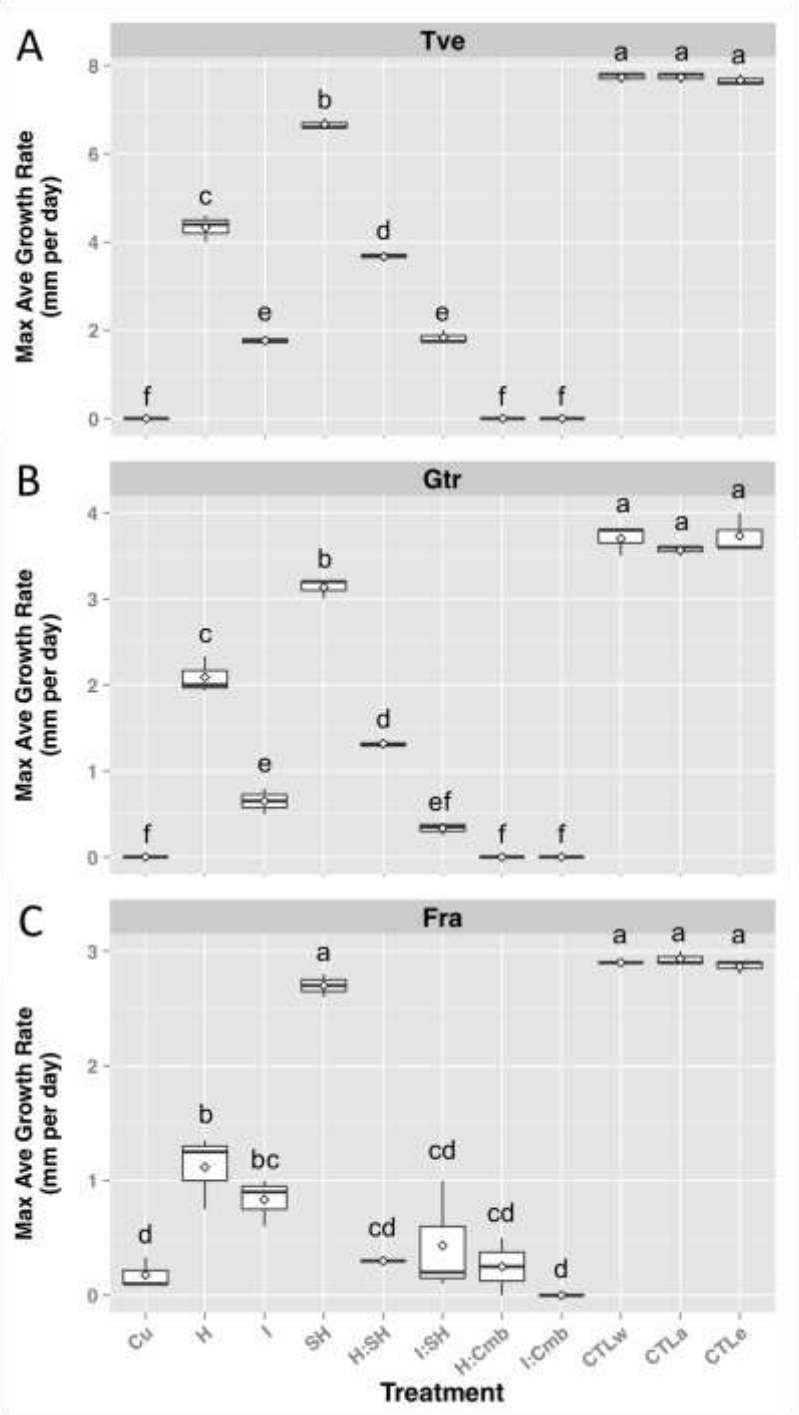

Figure 4. Boxplots of growth rate data for three decay fungi exposed to copper sulfate (Cu, 5000 ppm), Heritage (H, 20 ppm), Insignia (I, 20 ppm), and SHAM (SH, 100 ppm), alone, or in the two (H:SH and I:SH) and three compound combinations (H:Cmb and I:Cmb) that combined copper sulfate and SHAM with Heritage or Insignia. The control (CTL) treatments were water (w), acetone (a), and ethanol (e). A. Tve, *T. versicolor*, B. Gtr, *G. trabeum*, and C. Fra, *F. radiculosa.* Within each species tile, boxes that did not share the same letter were significantly different ($p < 0.05$) by the Tukey-Kramer mean separation test.

A different pattern of treatment responses was observed for the copper-tolerant brown rot fungus, *F. radiculosa* (Fig. 4C). Although the boxplots for *F. radiculosa* showed more variation

in the responses to the treatments compared to the copper-susceptible fungi, significant differences were detectable. Growth was not completely inhibited by the copper sulfate treatment, but it was greatly slowed. When tested alone, the toxicity of Heritage and Insignia was not significantly different from each other, but both were more toxic than SHAM, and less toxic than the copper sulfate treatment. SHAM caused a slight decrease in the maximum average daily growth rate, but it was not significantly different from the controls. The Insignia treatment, which was slightly more toxic than the Heritage treatment, was also not significantly different from the more toxic two-compound treatments that combined strobilurin fungicide with SHAM. The two- and three-compound treatments were the most toxic, with maximum daily average growth rates similar to the copper sulfate alone treatment. A key result, however, was that of all the treatments, only the three-compound treatment of copper sulfate, SHAM, and Insignia completely inhibited growth of the copper-tolerant fungus over the 20-day exposure period.

The results of Fig. 4 are summarized in Table 2, where they are expressed as a percentage of growth relative to the control treatment. The most salient results were that the three fungi tested showed 0% relative growth (i.e. complete growth inhibition) to at least one or more of the copper-based treatments. For the copper-susceptible fungi, zero growth was exhibited by the copper sulfate treatment when it was presented alone or in the three compound combination of copper sulfate, SHAM, and either of the strobilurin fungicides. For the copper-tolerant fungus, *F. radiculosa,* zero growth was observed only in the three-compound treatment that combined copper sulfate, SHAM, and Insignia.

Table 2. Percentage growth from the toxicity screening of the one-, two-, and three-compound treatments*

	Treatment							
		One-Compound			Two-Compound		Three-Compound	
Taxon	Cu	H	I	SH	H:SH	I:SH	H:Cmb	I:Cmb
Tve	0	54	22	83	46	23	0	0
Gtr	0	56	17	84	35	9	0	0
Fra	6	38	28	92	10	15	9	0

**Percentage growth was calculated relative to the control treatment with the highest maximum average daily growth rate. Abbreviations are as in Fig. 4.*

The reproducibility of the strobilurin data can be obtained by comparing relative growth in Table 2 with the predictions made by the linear regressions in Fig. 3 for *F. radiculosa.* The observed percentage growth for the Heritage and Heritage plus SHAM treatments were 38% and 10%, respectively (Table 2). The corresponding predictions (based on the fungicide concentration of 20 ppm) were 39% and 0%, respectively (Fig. 3A and B). The observed percentage growth for the Insignia and Insignia plus SHAM treatments were 28% and 15%, respectively (Table 2). The corresponding predictions were 7% and 1%, respectively (Fig. 3C and D). The observed values were all were within the 95% confidence intervals of the regression lines except for the Insignia treatment, which was just outside the boundary (28% growth exceeded the upper boundary of 21%).

A pressing question is how the strobilurins compare against other co-biocides that are currently used in commercially sold copper-based wood preservative systems. For *Postia placenta,* Nicholas and Schultz (1997) reported 47.5% and 0% growth for DDAC and propiconazole, respectively, when each co-biocide was tested at 20 ppm in an agar plate study. Toxicity of three co-biocides were reported by Archer *et al.* (1995) for two white (*Irpex lacteus* and *T. versicolor*) and two brown rot fungi (*G. trabeum* and *P. placenta*). For isothiazolone, DDAC, IPBC, and

propiconazole (all tested in agar plate studies), the MIC values ranged from 30 to >300 ppm, 100 to >300 ppm, 3 to 100 ppm, and 3 to 30 ppm, respectively. Accounting for the percent active ingredient, which ranged from 90% to 98% for the commercial co-biocides, one can conclude that toxicity of Insignia, which has an MIC of 26.3 ppm (equivalent to 5.3 ppm pyraclostrobin) is at least comparable.

Performance of Insignia extracts in an accelerated laboratory soil block test of wood has been examined for two copper-tolerant species of brown rot fungi (*F. radiculosa* and *Fomitopsis palustris*) (Tang *et al.* 2016). The wood treatments included copper ethanolamine at 0.1 pcf with Insignia extracts at three retentions (0.0125, 0.025, and 0.05 pcf pyraclostrobin) with or without SHAM (0.05 pcf). The experiment was designed to compare percent compression strength loss of the combination treatments with the reference ACQ-D at 0.15 pcf, which is an exterior, above ground retention for sawn wood products (AWPA 2015). After four weeks of fungal exposure, the Insignia treatment with the least decay was the one that combined copper ethanolamine with the highest retention of Insignia, and SHAM. Furthermore, measurements of percent compression strength loss showed that it performed as well as the reference treatment for *F. radiculosa,* and better than the reference for *F. palustris*.

5. CONCLUSIONS

The results of the MIC and toxicity screening studies presented here, along with our previous work using a laboratory soil and wood block test, all provide early evidence that the strobilurin compound, known as pyraclostrobin, has enough toxicity to warrant further investigation into its potential to act as a novel co-biocide for copper-based wood protection. Equally important, this is a "proof of concept" study since it combined knowledge from small molecule databases with biological knowledge gained from "omics" science to initially identify the strobilurins as a potential co-biocide for copper-based wood protection. Although we only examined toxicity of two strobilurins, there are at present, eight other major strobilurins sold in the agricultural fungicide market. Future investigations should reveal if any of these other strobilurins can be re-purposed for use as co-biocides for wood protection.

6. ACKNOWLEDGMENTS

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