Proceedings IRG Annual Meeting (ISSN 2000-8953)

IRG/WP 16-10859

THE INTERNATIONAL RESEARCH GROUP ON WOOD PROTECTION

Section 1 **Biology**

The copper-transporting ATPase pump and its potential role in copper-tolerance

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Paper prepared for the 47th IRG Annual Meeting
Lisbon, Portugal
15-19 May 2016

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ABSTRACT

Copper-tolerant brown-rot decay fungi exploit intricate mechanisms to neutralize the efficacy of copper-containing preservative formulations. The production and accumulation of oxalate is the most widely recognized theory regarding the mechanism of copper-tolerance in these fungi. The role of oxalate, however, may be only one part of a series of necessary components required for this complex mechanism. Annotation of the *Fibroporia radiculosa* genes involved in copper-tolerance characterized a subset of proteins, three copper-transporting ATPase pumps, which regulate copper concentrations inside the fungal cell by exporting excess copper ions. The goal of this study was to determine the relevance of copper-transporting ATPase pumps in the mechanism of *F. radiculosa* copper-tolerance. Southern pine test blocks were pressure-treated with 0.6%, 1.2%, and 2.4% ammoniacal copper citrate and subjected to a copper-tolerant strain of *F. radiculosa* and a copper-sensitive strain of *Gloeophyllum trabeum* in decay tests over a four week period. Untreated Southern pine test blocks subjected to both test fungi served as controls. Expression levels of three copper-transporting ATPase pumps were evaluated each week by qRT-PCR. *F. radiculosa* showed up-regulation of all three ATPase pumps when exposed to the copper treatments over the course of this study. *G. trabeum* showed down-regulation of ATPase1 and ATPase2 and no expression of ATPase3 when exposed to the copper treatments over the course of this study. Up-regulation of the three ATPase pumps can be correlated to the ability of *F. radiculosa* to decay copper-treated wood (12% weight loss at week 4). Down-regulation of ATPase1 and ATPase2 and lack of ATPase3 expression can be correlated to the inability of *G. trabeum* to decay copper-treated wood (1% weight loss at week 4). Preliminary results indicate these three ATPase pumps function as an essential component of the complex mechanism of copper-tolerance utilized by *F. radiculosa*.

Keywords: brown-rot decay, copper-tolerance, copper-transporting ATPase pump, *Fibroporia radiculosa*, gene expression

1. INTRODUCTION

Certain wood decay fungi have evolved the relative ability to proliferate when subjected to an unfavorable environment like chemically treated wood (DeGroot and Woodward 1999; Hall 2002; Green and Clausen 2003). Tolerance can be quite variable depending on preservative formulations, fungal species, and sometimes even fungal isolate (Hastrup *et al*. 2005; Freeman and McIntyre 2008). A copper-tolerant fungus has the capability to survive and usually even thrive in the presence of copper ions, and these fungi have even been documented to decay copper-treated wood containing concentrations higher than 1.6 mM/l (Gadd 1993; Hastrup *et al*. 2005). Copper-tolerant fungi thrive in these environments due to their ability to detoxify the copper ions found in copper-treated wood (Hastrup *et al*. 2005).

Copper-tolerant fungi present a major hurdle to successful protection of wood, causing premature failure and decreasing service life of treated wood products.

The mechanism of copper-tolerance has often been connected to production and subsequent accumulation of oxalate (Murphy and Levy 1983; Sutter et al. 1983; Daniel 1994; Leithoff et al. 1995; Pohleven et al. 2002; Green and Clausen 2003; 2005; Hastrup *et al*. 2006; Freeman and McIntyre 2008; Arango *et al*. 2009; Schilling and Inda 2011). It has been theorized that high extracellular accumulation of oxalate initiates the precipitation of copper into insoluble copper-oxalate, forms crystals, and ultimately renders the copper ion inactive (Pohleven et al. 1999; De Groot and Woodward 1999; Humar et al. 2002; Green and Clausen 2003). It has been demonstrated that decayed wood treated with copper-based preservatives contain not only copper-oxalate crystals, but calcium-oxalate crystals as well (Freeman and McIntyre 2008). Oxalate regulates the critically low pH environment needed by brown-rot fungi, and chelates excess calcium and copper ions in the immediate decay environment (Humar et al. 2001; Goodell 2003). It is widely accepted that both oxalate accumulation and precipitation of copper-oxalate play a critical role in the mechanism of copper-tolerance; however, oxalate may not be the most significant factor in this complex mechanism.

Tang *et al*. (2012) analyzed the genes related to copper-tolerance for *Fibroporia radiculosa* isolate TFFH 294. In this analysis, specific proteins with functions related to regulating copper concentrations were characterized, and it was found that three copper-transporting ATPase pumps and one copper homeostasis (CutC) gene were up-regulated during the early stages of decay. The copper-transporting ATPase pumps are largely responsible for preventing intracellular copper concentrations from becoming toxic in non-wood decaying fungi (Weissman *et al*. 2000). These three ATPase pumps were annotated for *F. radiculosa* and will be represented as: ATPase1 (XP_003046433), ATPase2 (XP_002475568), and ATPase3 (XP_002473512). It was also determined that of these three pumps, only one (ATPase2) encoded a signal peptide, and only one (ATPase1) had no comparable homolog in another copper-tolerant fungus, *Serpula lacrymans*. It was hypothesized that this ATPase1 homolog could be indicative of the higher copper-tolerance normally observed in *F. radiculosa* (Tang *et al*. 2012).

Ohno *et al*. (2015) measured expression of one of these ATPase pumps in four *F. radiculosa* isolates on wood treated with 1.2% ammoniacal copper-citrate over an 8-week period. It was determined that ATPase expression was greater in the presence of copper (compared to controls) for every sampling point. The present study evaluated all three characterized ATPase genes in a copper-tolerant fungus and a copper-sensitive fungus for expression when subjected to varying concentrations of copper. In studying all three ATPase pumps, this experiment has the potential to produce an accurate representation of how each of these pumps function in the mechanism of copper-tolerance.

2. METHODS

2.1 Fungal isolates

Fibroporia radiculosa (Peck) Gilb & Ryvarden isolate FP-90848-T was the copper-tolerant fungus used in this study. *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. isolate MAD 617 was the copper-sensitive fungus used in this study. Cultures were maintained on malt extract agar (BD, Fisher Scientific) at 27°C and 70% relative humidity (RH).

2.2 Preservative treatment and test block preparation
Southern pine (SP) test blocks (19mm^3) were vacuumed-treated with 0.6%, 1.2%, and 2.4% ammoniacal copper citrate according to the AWPA E10-15 Standard (AWPA 2015). Untreated SP test blocks (19mm^3) were included as controls. All test blocks were conditioned to 27°C and 30% RH for two weeks prior to being steam-sterilized for 20 minutes (122°C) and introduced to the two test fungi via mycelial plugs.

2.3 Decay test and sample preparation
Both fungi were subjected to the AWPA E10-15 Standard (2015) decay test at 27°C and 70% RH for two weeks to allow initial colonization. Following initial colonization, two test blocks were introduced to each glass jar for each fungus and incubated at 27°C and 70% RH up to four weeks. Sampling occurred weekly (n=3). Sawdust-like material was extracted from each test block using a sterilized drill bit. Approximately 0.2g of test material was placed into tubes and immediately extracted for RNA. The second test block (from the same glass jar) was used to calculate percent weight loss. These test blocks were oven-dried overnight (60°C) and reconditioned (27°C and 30% RH) for two weeks prior to calculating weight loss.

2.4 RNA extraction
RNA was isolated using the Ambion® RNAqueous™ Kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's specifications with an added DNaseI (Promega, Madison, WI) digestion to remove unwanted genomic DNA contaminants. DNase I digestion was prepared by adding 40 µl DNase-free water, 5 µl DNase I 10x Buffer, and 5 µl DNase I enzyme. This step was included between Wash Solution I and Wash Solution II step of the RNAqueous™ Kit. Following elution, RNA was kept on ice at all times. RNA was quantified via NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). All RNA was stored at -80°C.

2.5 cDNA synthesis
RNA was synthesized to first strand cDNA using the SuperScript™ II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) following the manufacturer's specifications. A concentration of 18ng/rxn was used for all samples. cDNA synthesis was carried out with the following settings: incubation at 42ºC for 50 min and inactivation at 70ºC for 15 min. Both non-template controls and reverse transcriptase-free controls were included. All cDNA was stored at -20°C.

2.6 Primer design
Gene specific primers were designed using gene and coding sequences (CDS) of *F. radiculosa* previously characterized (Tang 2011). Target regions spanning at least one intron were determined by Lasergene MegAlign (DNASTAR, Madison, WI). Forward and reverse primer pairs were designed for these target regions to have at least 50% GC content, no 3' complementarity, and to be uniquely specific for the desired region. Sequences used to measure expression levels for three ATPase pump genes (ATPase) are listed in Table 1. β-Actin was used as the housekeeping gene (HK).

Table 1: Target gene designation, nucleotide sequence, and reference gene accession number for the four oligonucleotide primers used in this study.

Target Gene	Primer Sequence	Accession Number
HK	Forward 5'-GTGATGGTTGGTATGGGTCAGAAGG Reverse 5'-GAAGCTCGTTGTAGAAAGTGTGATGC	XP_003026150
ATPase1	Forward 5'-CACTTCTCACAACCTCGTTACT Reverse 5'-CCCAGCATCGTTAGCACATA	XP_003046433
ATPase2	Forward 5'-CGGTCTTATCGAGTGCCTTTAG Reverse 5'-GGCATCAAAGCCCATTTCTTC	XP_002475568
ATPase3	Forward 5'-TTGAGGACAGCAAGGGAAAG Reverse 5'-CTGATGATGAACGTCGGGATAG	XP_002473512

2.7 qRT-PCR analysis

To determine gene expression, Real Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) was monitored in real time for the four genes in question. RT-qPCR reactions were carried out in 96 well PCR plates with Microseal® 'B' Film using the MyiQ™ 5 Real-Time PCR Detection System (Bio-Rad, Carlsbad, CA). All genes of interest were analyzed in duplicate for each sampling series (n=3). The 20 µl reactions contained 10 µl 2x iQ™ SYBR® Green Supermix (Bio-Rad, Carlsbad, CA), 7 µl sterile molecular grade water, 0.5 µl of both forward and reverse primers (10 µM), 1 µl BSA, and 1 µl cDNA template. The reaction protocol included an initial denaturation at 95°C for 3 min, followed by 40 cycles of a 15 sec 95°C denaturation, 30 sec annealing at 60°C, and a 30 sec 72°C extension. For ATPase3, annealing was carried out at 55ºC. A melt curve analysis of the resulting PCR product was performed immediately following the amplification and consisted of a 1 min incubation at 95°C followed by a 1 min annealing at 55°C prior to 81 cycles of 10 sec with temperatures increase 0.5°C each cycle. Using the actin (HK) mean C_T, average replicate C_T values were normalized. Normalized C_T values were used to calculate the mean fold change ($2^{-\Delta\Delta C_T}$) in expression of the five genes using the comparative C_T method (Livak and Schmittgen 2001). Mean fold change (expression ratio) was then converted to a $\log_2$ scale to equally represent up-regulation and down-regulation.

3. RESULTS

The overall goal of this work was to assess the activity of the three copper-transporting ATPase proteins in copper-tolerant *F. radiculosa* compared to copper-sensitive *G. trabeum* when exposed to varying concentrations of copper. In addition, evaluating differences in decay could indicate fungal efficiency to breakdown the copper treatments used in this study. Correlation of decay rates and expression levels could offer valuable insight into the mechanism of copper-tolerance.

3.1 Primer specificity

Prior to evaluating gene expression, primer specificity for the four genes (HK, ATPase1, ATPase2, and ATPase3) was examined for the two test fungi using gradient PCR. With this method, suitable annealing temperatures for each primer were determined. For HK, ATPase1 and ATPase2, annealing was carried out at 60°C. ATPase3 annealing was carried out at 55°C. In addition, visualization using gel electrophoresis was conducted to determine if there was gene amplification in *G. trabeum*, since the primer sequences were designed from *F. radiculosa* CDS. Figure 1 indicates good primer specificity of all four genes in *G. trabeum*.

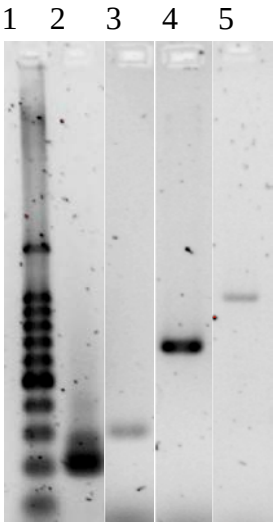

Figure 1: PCR gel analysis detailing ATPase primer specificity in copper-sensitive *G. trabeum* MAD 617. Lane 1: molecular weight marker, Lane 2: HK gene (270 bp), Lane 3: ATPase1 (299 bp), Lane 4: ATPase2 (649 bp), and Lane 5: ATPase3 (1019 bp).

3.2 Decay test

In the subsequent graphs, decay rates (%) for untreated (UN), 0.6% pressure-treated ammoniacal copper citrate (0.6% CC), 1.2% pressure-treated ammoniacal copper citrate (1.2% CC), and 2.4% pressure-treated ammoniacal copper citrate (2.4% CC) are represented over time for both test fungi. Decay rates represented in the graphs are the average of three biological replicates. Decay results for copper-sensitive *G. trabeum* MAD 617 are depicted in Figure 2. Copper sensitivity is clearly represented in *G. trabeum* when exposed to all three copper treatments at weeks 2, 3, and 4. The slightly higher decay rates of the copper treatments at week 1 could be attributed to moisture.

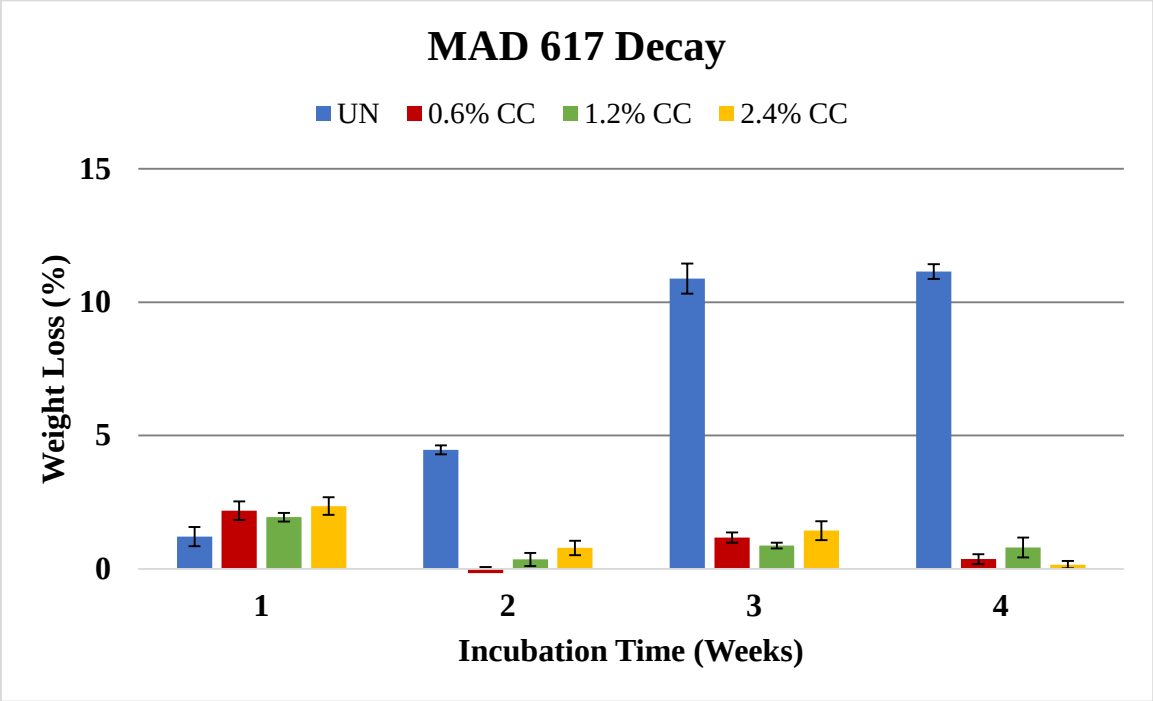

Figure 2: Decay in % weight loss of copper-sensitive *G. trabeum* MAD 617 exposed to untreated (UN) (), 0.6% pressure-treated copper citrate (CC) (), 1.2% pressure-treated CC (), and 2.4% pressure-treated CC () for the four sampling time points. Error bars represent the mean of three biological replicates.

Decay results for copper-tolerant *F. radiculosa* FP-90848-T are depicted in Figure 3. As expected, *F. radiculosa* is not deterred by the three copper treatments. By week 4, decay of the three copper treatments is much higher than the control. Slight differences in the decay rates between the three copper treatments can be seen at week 3; however, by week 4 these differences have diminished.

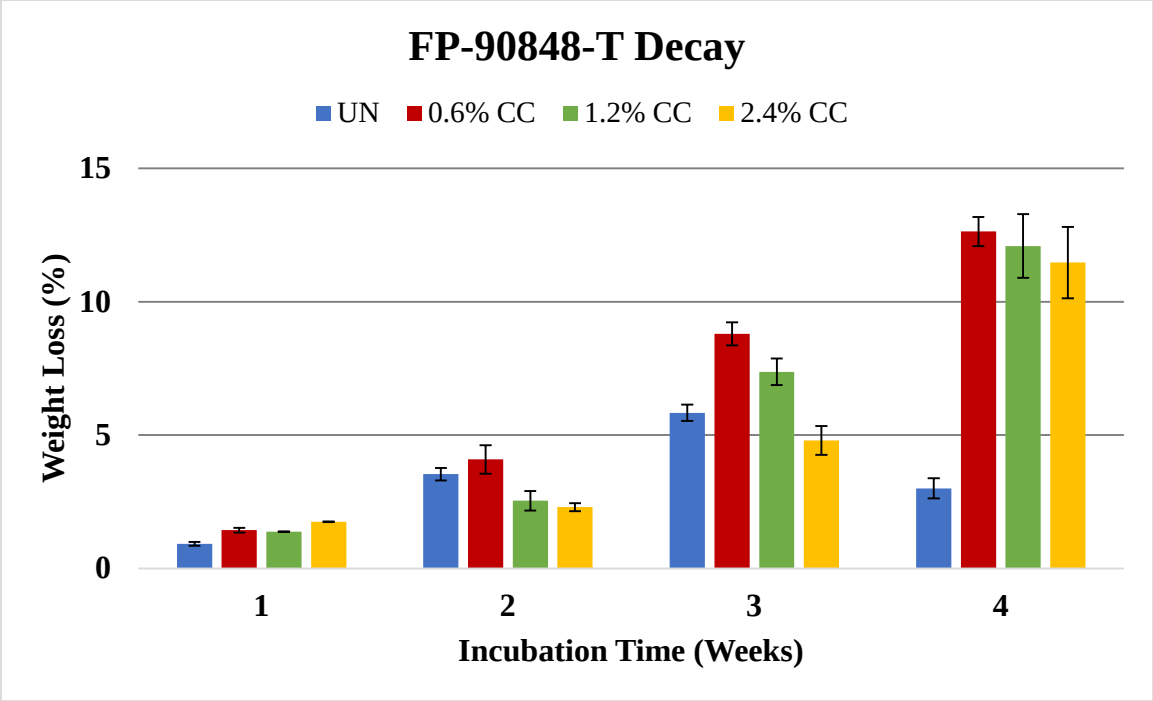

Figure 3: Decay in % weight loss of copper-sensitive *F. radiculosa* FP-90848-T exposed to UN (), 0.6% pressure-treated CC (), 1.2% pressure-treated CC (), and 2.4% pressure-treated CC () for the four sampling time points. Error bars represent the mean of three biological replicates.

3.3 Gene Expression

Expression values (C_T) were determined for HK, ATPase1, ATPase2, and ATPase3 in copper-sensitive *G. trabeum* and copper-tolerant *F. radiculosa* for untreated (UN) and three concentrations of copper-treated test samples via qRT-PCR. These values were normalized using the HK mean C_T for each test sample. For ATPase1, ATPase2, and ATPase3, UN controls for each fungus at week 1 was used as the endogenous control for fold change calculations. Values depicted for each of the following graphs reflect the difference between the UN at week 1 and each treatment in the subsequent weeks. For example in Figure 4 when exposed to UN for 3 weeks, *G. trabeum* ATPase1 was up-regulated 2 times higher than it was at week 1; and when exposed to 0.6% CC for 3 weeks, *G. trabeum* ATPase1 was down-regulated 7 times lower than when it was exposed to UN at week 1. In order to equally represent up-regulation and down-regulation of the three ATPase genes for the test fungi, normalized mean C_T values were converted to a $\log_2$ scale. Values above 1 and below -1, designated by the bold line in each of the graphs indicate significance. In the subsequent graphs, expression ratios ($\log_2$) are depicted for untreated (UN), 0.6% pressure-treated ammoniacal copper citrate (0.6% CC), 1.2% pressure-treated ammoniacal copper citrate (1.2% CC), and 2.4% pressure-treated ammoniacal copper citrate (2.4% CC)

3.3.1 ATPase1 Expression

ATPase1 expression in copper sensitive *G. trabeum* is depicted in Figure 4. ATPase1 was up-regulated in *G. trabeum* when exposed to UN at weeks 2, 3, and 4. When exposed to the three copper treatments, ATPase1 was down-regulated in *G. trabeum*. At week 2, ATPase1 was undetectable when *G. trabeum* was exposed to the three copper treatments. In addition, ATPase1 was undetectable at week 4 when *G. trabeum* was exposed to 1.2% CC.

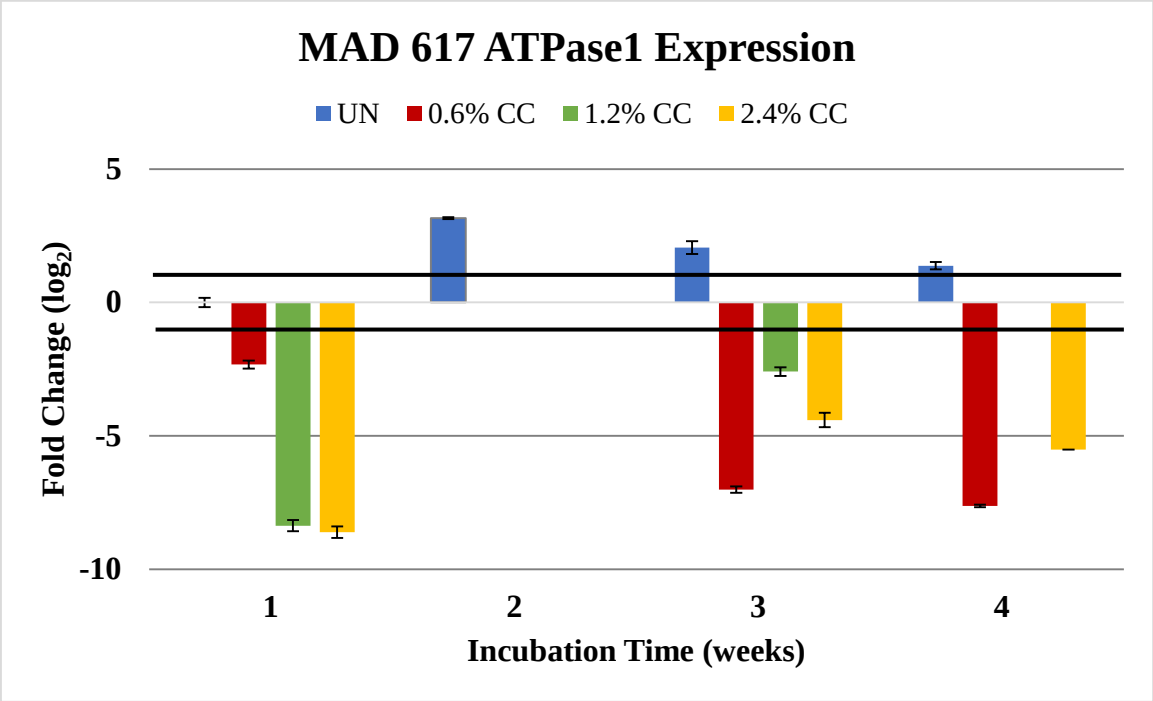

Figure 4: Gene expression values (fold change) of ATPase1 for copper-sensitive *G. trabeum* MAD 617 exposed to untreated (UN) (), 0.6% pressure-treated copper citrate (CC) (), 1.2% pressure-treated CC () , and 2.4% pressure-treated CC () over time. Error bars represent the mean of three biological replicates.

ATPase1 expression in copper-tolerant *F. radiculosa* is depicted in Figure 5. Generally, ATPase1 was up-regulated in *F. radiculosa* when exposed to the three copper treatments. At week 4, ATPase1 was down-regulated when *F. radiculosa* was exposed to 1.2% CC. There was an upward trend in ATPase1 up-regulation when *F. radiculosa* was exposed to copper for the first three time points.

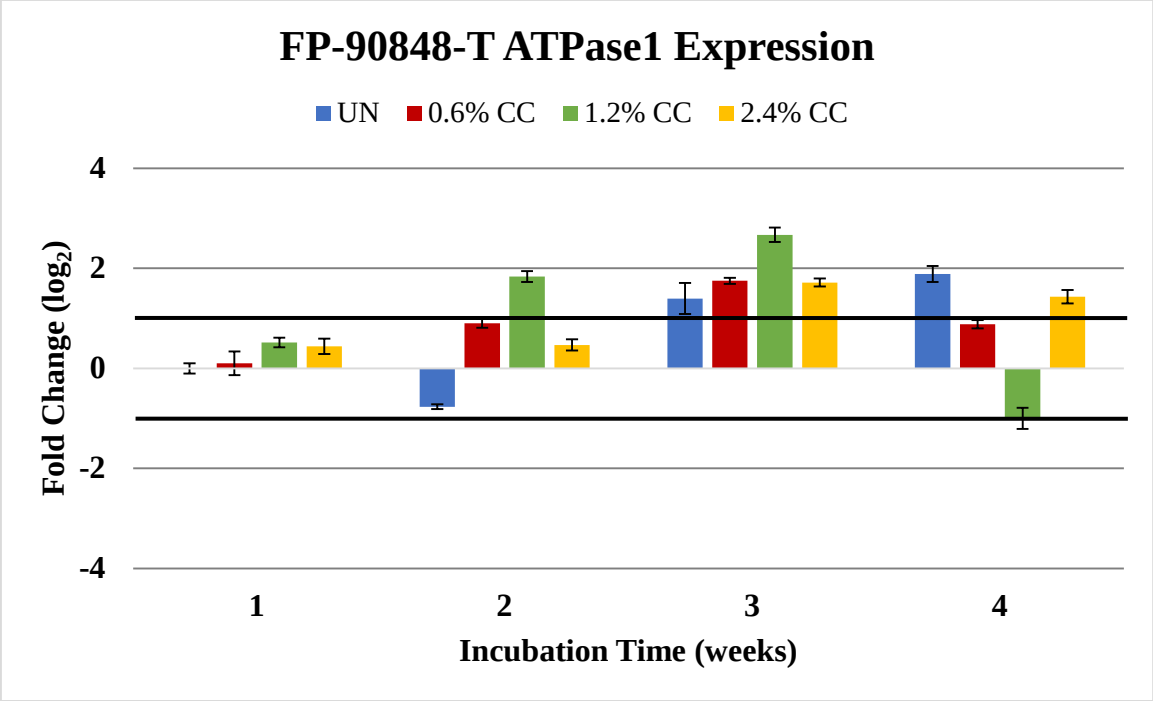

Figure 5: Gene expression values (fold change) of ATPase1 for copper-sensitive *F. radiculosa* FP-90848-T exposed to UN ( ), 0.6% pressure-treated CC ( ), 1.2% pressure-treated CC ( ), and 2.4% pressure-treated CC ( ) over time. Error bars represent the mean of three biological replicates.

3.3.2 ATPase2 Expression

ATPase2 expression in *G. trabeum* is shown in Figure 6. Slight up-regulation of ATPase2 when *G. trabeum* is exposed to UN is shown at week 2. When exposed to the three copper treatments, ATPase2 is down-regulated at weeks 1, 2, and 3. In addition, there was no detectable expression of ATPase2 at week 4 when *G. trabeum* was exposed to any treatment.

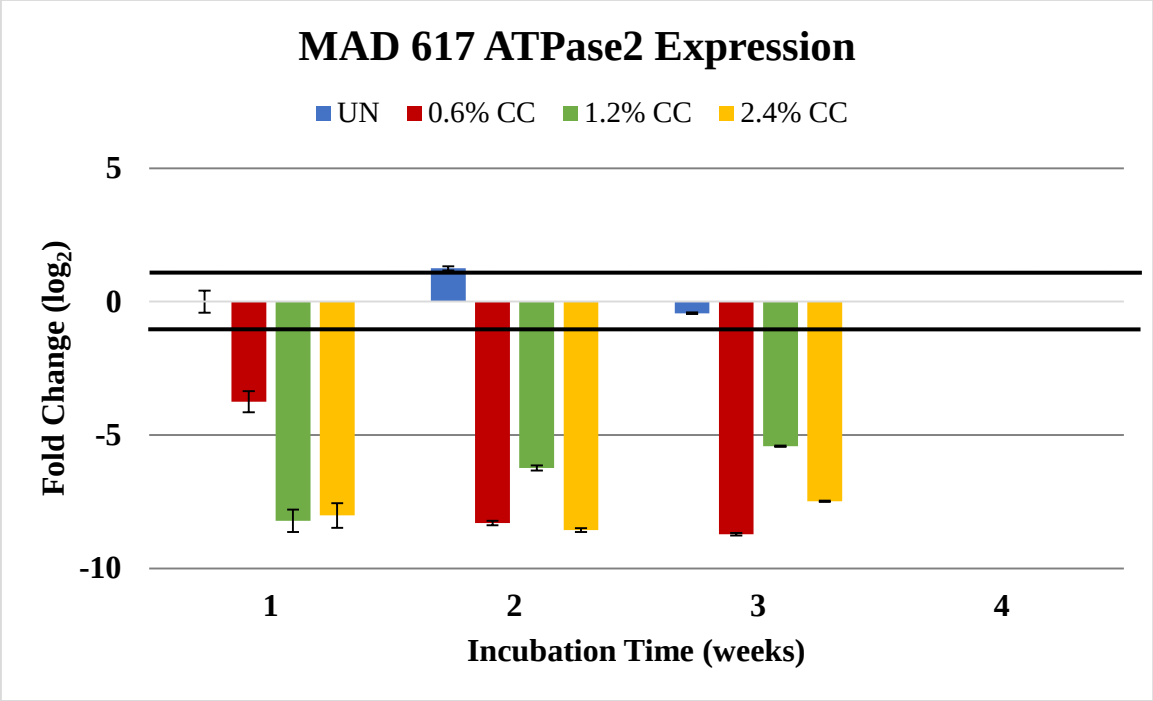

Figure 6: Gene expression values (fold change) of ATPase2 for copper-sensitive *G. trabeum* MAD 617 exposed to UN ( ), 0.6% pressure-treated CC ( ), 1.2% pressure-treated CC ( ), and 2.4% pressure-treated CC ( ) over time. Error bars represent the mean of three biological replicates.

ATPase2 expression in *F. radiculosa* is shown in Figure 7. ATPase2 was up-regulated when *F. radiculosa* was exposed to the three copper treatments at weeks 1, 2, and 3. However by week 4, ATPase2 was down-regulated in the copper treatments. Overall, there was a downward trend in ATPase2 up-regulation with increasing incubation time.

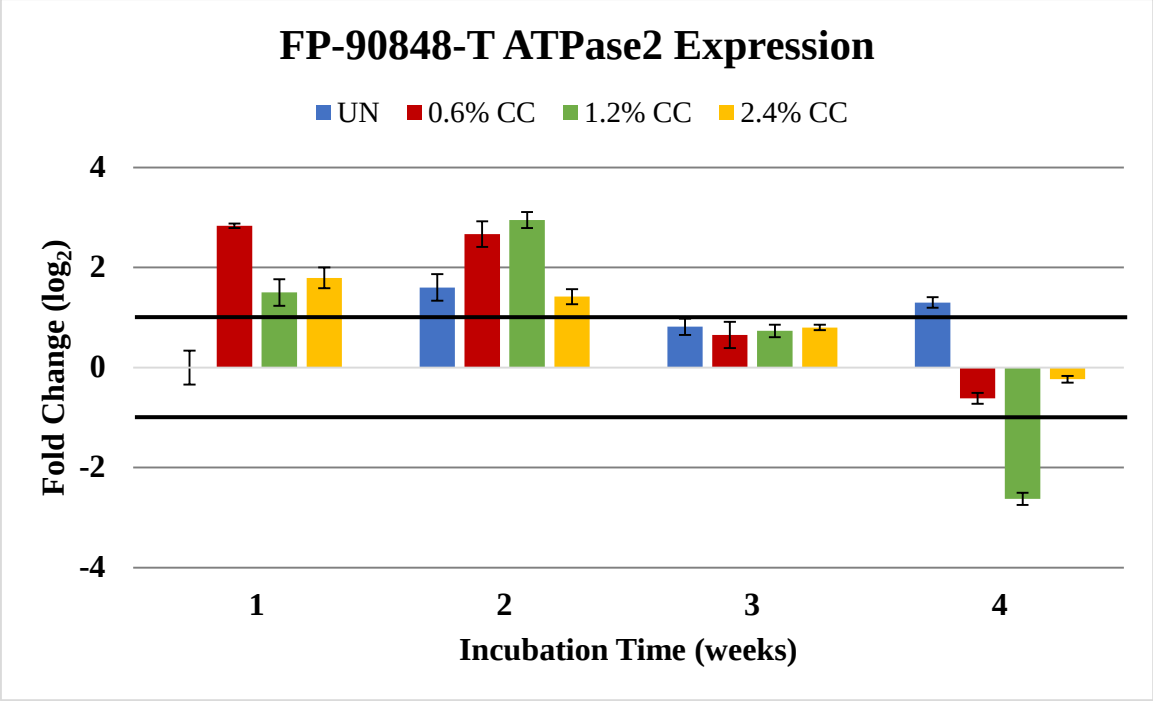

Figure 7: Gene expression values (fold change) of ATPase2 for copper-sensitive *F. radiculosa* FP-90848-T exposed to UN (), 0.6% pressure-treated CC (), 1.2% pressure-treated CC (), and 2.4% pressure-treated CC () over time. Error bars represent the mean of three biological replicates.

3.3.3 ATPase3 Expression

There was no detectable ATPase3 expression in *G. trabeum* for any treatment. The initial primer specificity check pointed to the presence of this protein (Fig. 1, lane 5); however, the lack of expression indicates ATPase3 is non-functional in this fungus.

ATPase3 expression in *F. radiculosa* is depicted in Figure 8. There was minimal up-regulation of ATPase3 (0.6% CC at week 1 and 2 and 1.2% CC at week 2) in this fungus. At weeks 3 and 4, ATPase3 was down-regulated when *F. radiculosa* was exposed to the three copper treatments.

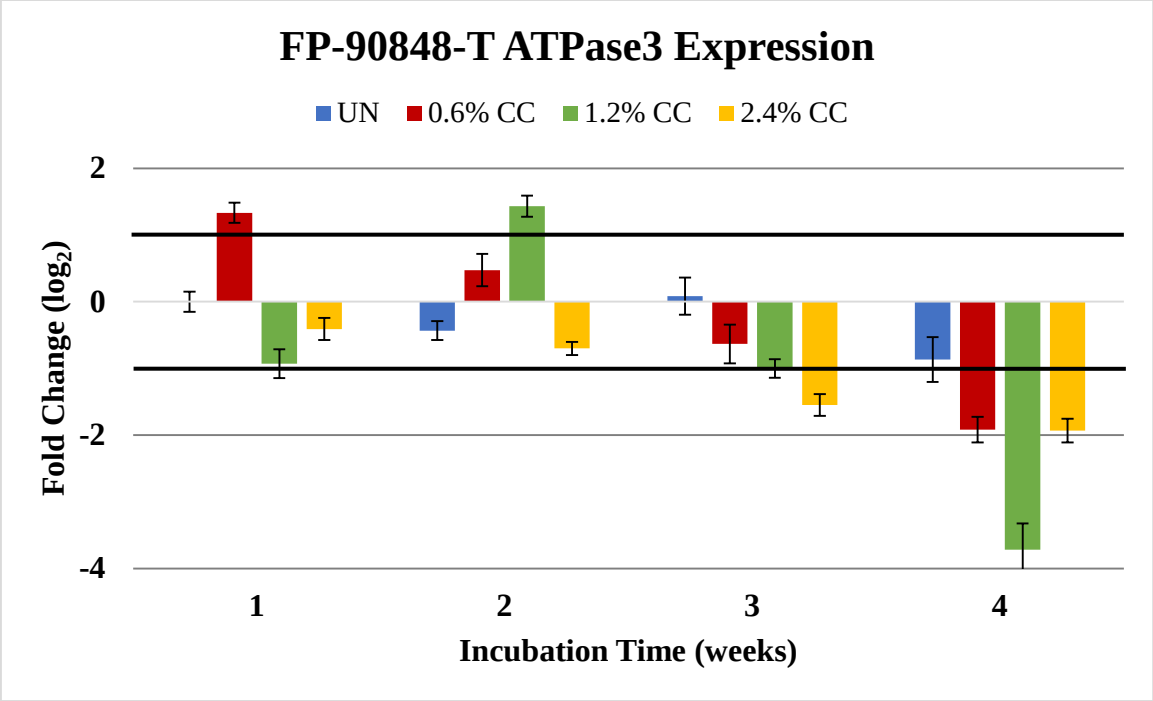

Figure 8: Gene expression values (fold change) of ATPase3 for copper-sensitive *F. radiculosa* FP-90848-T exposed to UN ( ), 0.6% pressure-treated CC ( ), 1.2% pressure-treated CC ( ), and 2.4% pressure-treated CC ( ) over time. Error bars represent the mean of three biological replicates.

4. DISCUSSION

It is evident that these copper-transporting ATPase pumps play some role in the intricate mechanism of copper-tolerance. Ohno *et al*. (2015) suggests the ATPase pumps work to expel copper first, copper ions are chelated via oxalate, followed by active utilization of the wood. Down-regulation of ATPase1 (Fig. 4) and ATPase2 (Fig. 5) when *G. trabeum* is exposed to copper-treated samples is correlated with the low decay of the copper-treated samples (Fig. 2). Because *G. trabeum* down-regulates ATPase1 and ATPase2, has no detectable ATPase3 expression, and is unable to effectively decay copper-treated wood, this could be indicative of the importance of these proteins as a key component in the mechanism of copper-tolerance.

Up-regulation of ATPase1 (Fig. 5) and ATPase2 (Fig. 7) when *F. radiculosa* is exposed to copper is comparable to decay when exposed to the three copper treatments (Fig. 3), and indicates the significance of these proteins in copper-tolerance. The presence of an upward

trend in ATPase1 up-regulation with increasing incubation time contrasts the downward trend in ATPase2 up-regulation seen in *F. radiculosa*. It seems as though ATPase2 is functioning to expel copper at earlier stages than ATPase1. This suggests that ATPase1 and ATPase2 are working in tandem to expel copper so *F. radiculosa* can efficiently decay copper-treated wood. There was an overall downward trend in ATPase3 expression with increasing incubation time in *F. radiculosa* (Fig. 8). It is possible ATPase3 is highly functional at a time point unrepresented in this study (even earlier than weeks 1 and 2). This data still suggests the importance of ATPase3 to the mechanism of copper-tolerance, especially since ATPase3 was undetectable in copper-sensitive *G. trabeum*.

5. CONCLUSIONS

Copper-tolerant organisms are cause for concern because of their inherent ability to destroy copper-treated wood products in service. It is critical that this mechanism be investigated in order to attempt to control these organisms. In this study, it was suggested that ATPase1, ATPase2, and ATPase3 function as key components in the mechanism of copper-tolerance. Copper-sensitive *G. trabeum* down-regulated ATPase1 and ATPase2 and ATPase3 was not functional, which is related to the inability to decay copper-treated wood. Copper-tolerant *F. radiculosa* highly up-regulated ATPase1 and ATPase2 at specific time points in this study, which is correlated to the ability to decay copper-treated wood. It was suggested that ATPase3 is a key component in copper-tolerance; however, ATPase3 up-regulation in *F. radiculosa* was not clearly represented in this study.

This research begins to untangle the complex mechanism of copper-tolerance by suggesting these copper-transporting ATPase proteins play a key role in the success of copper-tolerant fungi, like *F. radiculosa*. In order to fully understand copper-tolerance, additional research in this area is needed. Determining the necessary components and their functionality in the mechanism of copper-tolerance will prove extremely valuable in developing a more targeted approach in lessening and possibly halting the destruction of wood-based materials caused by copper-tolerant fungi. This study marks one of the first efforts in tying copper-tolerance to the activities of these copper-transporting ATPase pumps in a copper-tolerant wood decay fungus. Future studies plan to analyze residual copper levels in the test blocks used in this study. In addition, future studies plan to explore structural differences between these three ATPase pumps and to locate these pumps to a particular site in the cell.

6. REFERENCES

American Wood Protection Association (2015): Standard methods of testing wood preservatives by laboratory soil-block cultures. Standard E10-15. Book of Standards. American Wood-Preservers Association, Birmingham, AL, pp. 418-428.

Arango, R A, Lebow, P, Green, F (2009): Correlation between oxalic acid production and tolerance of *Tyromyces palustris* strain TYP-6137 to N', N-naphthaloylhydroxamine. *International Biodeterioration and Biodegradation*, **63**: 46-51.

Daniel, G (1994): Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiology Reviews* **13:** 199-233.

DeGroot, R and Woodward, B (1999): Using copper-tolerant fungi to bidegrade wood treated with copper based preservatives. *International Biodeterioration and Biodegradation*, **44**: 17-27.

Freeman, M and McIntyre, C (2008): A comprehensive review of copper-based wood preservatives with a focus on new micronized or dispersed copper systems. *Forest Products Journal*, **58**: 6-27.

Gadd, G (1993): Tansley review no. 47: interactions of fungi with toxic metals. *New Phytologists* **124**: 25-60.

Green, F and Clausen, C A (2003): Copper tolerance of brown-rot fungi: time course of oxalic acid production. *International Biodeterioration and Biodegradation*, **51**: 145-149.

Green, F and Clausen, C A (2005): Copper tolerance of brown-rot fungi: oxalic acid production in southern pine treated with arsenic-free preservatives. *International Biodeterioration and Biodegradation*, **56**: 75-79.

Goodell, B (2003): Brown-rot fungal degradation of wood: our evolving view. In: *Wood Deterioration and Preservation, Advances in Our Changing World*, eds. B. Goodell, D. Nicholas, and T. Schultz. ACS Symposium Series 845, pp. 34-72. American Chemical Society, Washington, D.C.

Hall, J (2002): Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, **53**: 1-11.

Hastrup, A, Green, F, Clausen, C A, Jensen, B (2005): Tolerance of *Serpula lacrymans* to copper-based wood preservatives. *International Biodeterioration and Biodegradation*, **56**: 173-177.

Hastrup, A, Jensen, B, Clausen, C A, Green, F (2006): The effect of CaCl2 on growth rate, wood decay and oxalic acid accumulation in *Serpula lacrymans* and related brown-rot fungi. *Holzforschung*, **60**: 339-345.

Humar, M, Petrič, M, Pohleven, F (2001): Changes of pH of impregnated wood during exposure to wood-rotting fungi. *Holz als Roh-und Werkstoff*, **59**: 288-293.

Humar, M, Petrič, M, Pohleven, F, Šentjurc, M, Kalan, P (2002): Changes of EPR spectra of wood, impregnated with copper based preservatives, during exposure to several wood rotting fungi. *Holzforschung*, **56**: 229-238.

Leithoff, H, Stephan, I, Lenz, M T, Peek, R D (1995): Growth of the copper tolerant brown rot fungus *Antrodia vaillantii* on different substrates. *Proceedings IRG Annual Meeting*, IRG/WP 95-10121.

Livak, K. and Schmittgen, T. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods*, **25**:402-408.

Murphy, R J and Levy, J F (1983): Production of copper oxalate by some copper tolerant fungi. *Transactions of the British Mycological Society* **81**: 165-168.

Ohno, K M, Clausen, C A, Green, F, Diehl, S V (2015): Insights into the mechanism of copper-tolerance in *Fibroporia radiculosa*: The biosynthesis of oxalate. *International Biodeterioration and Biodegradation*, **105**: 90-96.

Pohleven, F, Breznikar, Š Kalan, P, Petrič, M (1999): Determination of absorption, accumulation and transport of copper in mycelium of some wood decay fungi. *Proceedings IRG Annual Meeting*, IRG/WP 99-10323.

Pohleven, F, Humar, M, Amartey, S, Benedik, J (2002): Tolerance of wood decay fungi to commercial copper based wood preservatives. *Proceedings IRG Annual Meeting*, IRG/WP 02-30291.

Schilling, J and Inda, J (2011): Assessing the relative bioavailability of copper to fungi degrading treated wood. *International Biodeterioration and Biodegradation*, **56**: 18-22.

Sutter, H P, Jones, E B G, Wälchli, O (1983): The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.) Fr. *Material Und Organismen* **18**: 241-262.

Tang, Juliet Dao-May. (2011): A genomic and transcriptomics analysis of wood decay and copper tolerance in the brown rot fungus *Fibroporia radiculosa*. Ph.D. Dissertation, Mississippi State University 122 Pages.

Tang, J D, Perkins, A D, Sonstegard, T S, Schroeder, S G., Burgess, S C, and Diehl, S V (2012): Short-read sequencing for genomic analysis of the brown-rot fungus *Fibroporia radiculosa*. *Applied and Environmental Microbiology*, **78**(7): 2272-2281.

Weissman, Z, Berdicevsky, I, Cavari, B Z, Kornitzer, D (2000): The high copper tolerance of Candida albicans is mediated by a P-type ATPase. *Proceeding of the National Academy of Sciences of the United States of America*, **97**: 3520-3525.