

GENE EXPRESSION ANALYSIS OF WOOD DECAY FUNGUS *FIBROPORIA RADICULOSA* GROWN IN ACQ-TREATED WOOD

Ayfer Akgul*†

E-mail: aa1625@msstate.edu

Ali Akgul

Mississippi State University
Mississippi State, MS

E-mail: aa1116@msstate.edu

Juliet D. Tang

USDA Forest Service
Forest Products Laboratory
Starkville, MS

E-mail: julietdtang@fs.fed.us

Susan V. Diehl

Mississippi State University
Mississippi State, MS

E-mail: svd2@msstate.edu

(Received November 2017)

Abstract. Copper-tolerant brown-rot fungi are able to degrade wood treated with copper or copper-based wood preservatives. This research used quantitative reverse transcriptase polymerase chain reaction to explore what genes of the brown-rot fungus, *Fibroporia radiculosa*, were expressed when the fungus was overcoming the wood preservatives and decaying the wood. Aryl alcohol oxidase, catalase, oxalate decarboxylase 2, and copper resistance P-type ATPase pump had higher expression on alkaline copper quat type D treated wood compared with week I. In addition, two genes had high expression at week 5; glyceride hydrolase 5 and glyceride hydrolase 10 when wood strength loss was around 50%. Glyoxylate dehydrogenase had high expression until week 8. This gene might be involved in the production of oxalate. Laccase, oxalate decarboxylase I and isocitrate yase were not differentially expressed, suggesting that these genes were not involved in the decay process of alkaline copper quat-treated wood. These results are important to understand the genes that are involved in the mechanism of copper tolerance and wood decay in *F. radiculosa*.

Keywords: Copper tolerance, brown-rot decay, *Fibroporia radiculosa*, ACQ, gene expression.

INTRODUCTION

It is estimated that approximately 400 million cubic feet of preservative-treated wood is produced annually in the United States (Clausen et al 2014). Replacement of decayed wood products from homes and building costs more than \$5 billion annually with 10% of the annual production of forests going toward replacement of

decayed products (Schultz and Nicholas 2008). Copper-based preservatives are the primary biocide used to protect wood from ground contact exposure (Freeman and McIntyre 2008). Copper is an effective fungicide against many wood decay fungi, although certain brown-rot fungi have the ability to overcome or tolerate the copper component (Freeman and McIntyre 2008). Brown-rot fungi are the principal recyclers of dead wood in coniferous forests and select brown-rot fungi can decay wood treated with copper-based preservatives. When these decay fungi

* Corresponding author

† SWST member

attack, the wood loses physical and mechanical strength because of softening and weakening of the wood structure, leading to rapid failure. Premature failure of preservative treated wood due to copper tolerant fungi is a major concern of the wood preservation industry (Freeman and McIntyre 2008; Clausen et al 2014).

As brown-rot fungi are degrading the wood cell walls, the wood loses structural integrity because of the breakdown of hemicellulose and cellulose. Brown-rot decay is the major type of decay found in wood in residential or building applications (Eriksson et al 1990). Brown-rot decay involves

the generation of hydroxyl free radicals ($\cdot\text{OH}$) nonenzymatically via Fenton chemistry ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$) (Goodell et al 1997; Hummel et al 2002; Arantes et al 2012). Copper tolerance has been defined as 'the capacity of an organism to survive in the presence of high copper ions' (Hastrup et al 2005). Not all brown-rot fungal species are copper tolerant, and among those that are copper tolerant, there is variation in tolerance levels depending on the preservative formulation, fungal species as well as fungal isolate (Hastrup et al 2005; Freeman and McIntyre 2008). In addition, the mechanisms fungi use to overcome or tolerate high levels of copper are likely not the same, also depending on preservative, fungal species and isolate. Thus, there is unlikely a single mechanism used by fungi for copper tolerance.

Toxic levels of copper can denature proteins (including enzymes) and destroy the integrity of the cell membrane and cell walls (Gadd 1993). Changes that have been documented in some, but not all, fungi when adapting to high levels of copper include: increased production of oxalic acid, production of a thicker cell wall, an increase in *N*-acetyl glucosamine (chitin) in the cell wall, and an increase in the volume of the protective mucilaginous sheath (Green and Clausen 2003; Vesentini et al 2005; Hastrup et al 2006; Freeman and McIntyre 2008; Arango et al 2009).

A high level of oxalic acid is also believed to confer copper tolerance to some brown-rot fungi. Toxic levels of copper ions are chelated to the

oxalic acid forming copper: oxalate complexes. These complexes precipitate into copper oxalate crystals on the surface of the wood cell rendering the copper both insoluble and inert (Woodward and DeGroot 1999; Green and Clausen 2003; Schilling and Jellison 2006; Tang et al 2013). Biosynthesis of oxalate in brown-rot fungi potentially occurs through a coupling of the tricarboxylic acid (TCA) cycle located in the mitochondria and the glyoxylate (GLOX) cycle located in the peroxisome (Dutton and Evans 1996; Munir et al. 2001). Oxalate could be produced from excess malate originating from either the TCA or GLOX cycles (Fig 1) or from GLOX in the GLOX cycle (Munir et al 2001; Tang et al 2013).

Genomic sequence analysis of the brown-rot fungus *Fibroporia ridiculosa* (Peck) Parmasto resulted in putative annotations for many of the genes involved in wood decay (Tang et al 2012). Subsequently, expression of these genes by transcriptomics and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were studied during decay of wood treated with micronized copper quat (MCQ) (Tang et al 2013), another copper-based wood preservative. Increased expression of two pectin-degrading enzymes were detected during the early stages of decay (Tang et al 2013). As decay progressed, wood strength loss increased (Tang et al 2013) and degradation appeared to involve enzymatic hydrolysis. Several genes for different glyceride hydrolases were highly expressed during the late stages of decay by the brown-rot fungi, *F. ridiculosa* and *Postia placenta* (Fr.) M. J. Larsen & Lombard (Martinez et al 2009; Tang et al 2013; Wymelenberg et al 2010). One of the characteristics of brown-rot decay is the modification of the lignin and methanol generation. Methanol can be converted into formaldehyde and hydrogen peroxide by fungal alcohol oxidases. During this process, lignin is modified by demethylation of both the phenolic and nonphenolic portions and some loss of lignin in the cell walls and middle lamellae is observed (Eriksson et al 1990).

Understanding the metabolic mechanisms that *F. ridiculosa* uses to detoxify the copper in treated

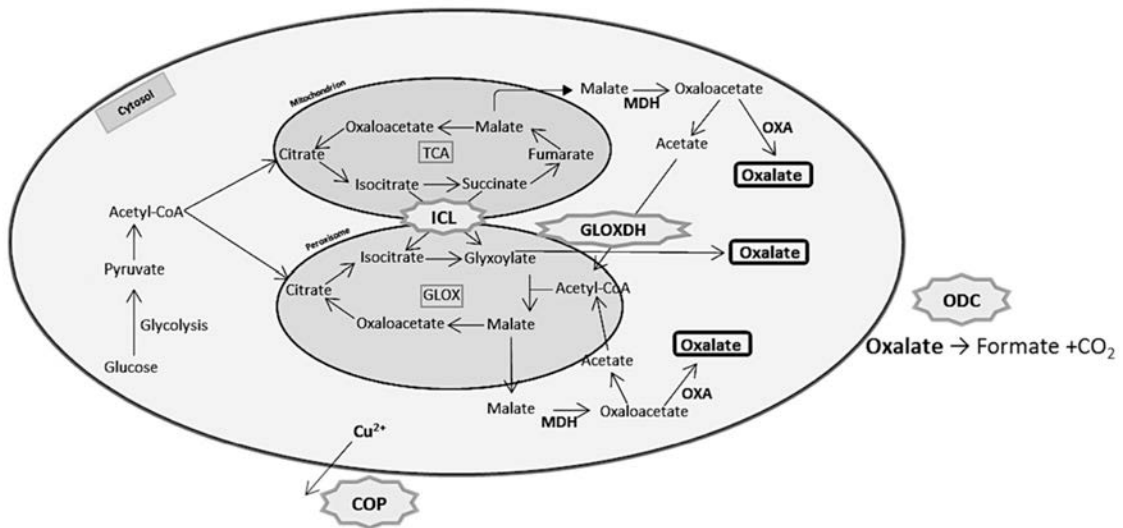


Figure 1. Proposed enzymatic pathway for growth on ACQ-treated wood. The mitochondrion and peroxisome are shown green and orange, respectively. The thick outer blue line denotes the cell membrane. The genes ICL, GLOXDH, and ODC2 are involved in oxalate metabolism, whereas COP is involved in copper homeostasis. All four genes were highly expressed on ACQ-treated wood (adapted from Munir et al 2001; Tang et al 2013). ACQ, alkaline copper quat; ICL, isocitrate lyase; GLOXDH, glyoxylate dehydrogenase; ODC2, oxalate decarboxylase 2; COP, copper resistance P-type ATPase pump.

wood, plus breakdown the wood cell walls to obtain food, is extremely complicated. Although some gene expression studies have been conducted in wood treated with MCQ (Tang et al 2013) and copper citrate (CC) (Ohno et al 2015), none have been reported yet from wood treated with alkaline copper quat (ACQ). This study examined gene expression analysis of ten genes in *F. ridiculous*, thought to play a role in copper tolerance and wood decay, when the fungus was growing on wood treated with a common wood preservative, alkaline copper quat type D (ACQD) to help further understand these complex mechanisms.

MATERIALS AND METHODS

Fungus Isolate

The strain of *F. ridiculous* TFFH 294 was provided by Carol Clausen, USDA Forest Service, Forest Products Laboratory, Madison, WI. The fungus was grown for 15 Da at 27°C on petridish plates containing malt extract agar (BD, Fisher Scientific, Pittsburg, PA).

Preservative Treatments and Wafer Preparation

Southern yellow pine (SYP) sapwood wafers measuring 19 by 19 by 5 mm ($l \times r \times t$) were vacuum treated at 0.25 pct for 30 min in ACQ-D. According to the American Wood Protection Standard U1 specification, 0.25 pct is an above ground retention for ACQ-D, where ACQ-D contains 66.7% copper oxide and 33.3% quat as dimethyldidecyl ammonium carbonate (AWPA 2014b). A matching set of wafers was treated the same way except with water to compare with exposed samples in the compression strength testing. ACQ-treated SYP test wafers were sterilized for 30 min by autoclaving before inoculation.

Fungal Growth on Copper Treated Wood Blocks

Soil block tests were set up according to American Wood Protection Association Standard E22-12 with minor modifications (AWPA 2012). Two test containers were prepared as biological replicates for each time point. Each test container

contained two (SYP) sapwood feeder strips measuring 73 by 22 by 2 mm ($l \times t \times r$) that were placed directly on the moist soil surface. The entire container was sterilized. After the 2 wk colonization period, six ACQ-treated SYP sapwood test wafers were placed directly on top of the SYP feeder strips and incubated at 27°C. Wafers were removed at days 7, 14, 22, 29, 36, 43, 50, and 57 for RNA extraction. One wafer from each container was reserved for the compression strength testing and the remaining five wafers were extracted individually and then merged into one RNA sample. Each wafer was reduced to small pieces using a razor blade, and 0.5 g of the cut wood was weighed into one 2-mL tube. Material was stored at -70°C for RNA isolation.

Wood Compression Testing

Wood compression strength testing was conducted according to American Wood Protection Association Standard E22-12 with minor modifications (AWPA 2012). Wafers were first stored in water until saturation. The testing apparatus compresses the wafers to 5% of their original dimension in the radial direction. The compression strength loss was calculated as a percentage of unexposed water-treated wafers.

RNA Isolation

RNA isolation was accomplished using the Ambion RNAqueous Kit (Ambion, Austin, TX). RNA was extracted from the five test wafers and merged into one RNA sample per test container. To each sampling tube; 1 ml lysis buffer was added to the 0.5 g rasped wood, and each tube was placed on ice. Test samples were homogenized twice (2-3 min beating separated by a 3 min incubation on ice) using a Mini beadbeater 16 (Bio Spec Products, Bartlesville, OK). Centrifugation and washing steps were performed according to the RNAqueous Kit instructions. DNA digestion was performed on the spin column with the TURBO DNA-free™ kit (Ambion). The DNA digestion mixture was prepared by

mixing together 42 μ L RNase-free water, 5 μ L 10x DNase I Buffer, and 3 μ L DNase I enzyme. After eluting the RNA from the spin column, its concentration was determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA quality was evaluated by Experion chip electrophoresis (RNA StdSens Analysis Kit; Bio-Rad, Hercules, CA). All RNA samples were stored at -70°C.

qRT-PCR

RNA (1 μ g RNA for 20 μ L of reaction mix) was converted to first strand cDNA using the Invitrogen Superscript II Reverse Transcriptase Kit (Carlsbad, CA) with random hexanucleotide primers. The reaction was performed in a Mastercycler (Eppendorf, Hauppauge, NY) programmed to hold 25°C for 10min, 42°C for 90 min, and then 85°C for 5 min.

Primers for each gene-specific transcript and 18S rRNA (the internal reference gene) were designed by Tang et al (2013) (Table 1). One primer of each pair spanned an exon-exon boundary, and each primer pair spanned at least one intron. Polymerase chain reaction (PCR) amplification was examined in real time for the ten genes to determine gene expression levels. An iQ5 real-time PCR detection system (Bio-Rad) was used to perform the amplification. The master mix consisted of forward (0.5 μ L) and reverse (0.5 μ L) primers (final concentration; 0.25 μ M), 10 μ L 2x iQ SYBR Green Supermix, 8 μ L sterile molecular grade water, and 1 ml of first strand cDNA. The first strand cDNA was diluted 30x for amplification of 18S rRNA (the internal reference gene), diluted 4x for genes isocitrate lyase (ICL), oxalate decarboxylase 1 (ODC1), glyoxylate dehydrogenase (GLOXDH) and undiluted for genes laccase (LCC), oxalate decarboxylase 2 (ODC2), glyceride hydrolase 5 (GH5), glyceride hydrolase 10 (GH10), aryl alcohol oxidase (AAOX), catalase (CAT), and copper resistance P-type ATPase pump (COP). Amplification conditions were: 95°C for 3 min, 45 cycles of three steps (95°C for 15 s, 62°C for 30 s, and 72°C for 30s), and 72°C for 3 min for final extension. After

Table I. Primers used for qRT-PCR to amplify target genes from first strand cDNA (Tang et al 2013).

Gene	Primers	n-mer	Sequence (5'-3')	PCR length (bp)
18S rRNA	F	21	TCTTAGTTGGTGGAGTGATTGTCTGG	152
	R	21	ACAGACCTGTTATTGCCTCAAACCTCC	
ICL	F	20	TTCTGCTGGCAGTTCATTACGTTGGCTGG	192
	R	18	CATGAGGTTGTCCATGTAGTCCGCACC	
GLOXDH	F	21	GTCTCCCCGTCTACATTTCCGGCGACTGC	194
	R	21	CTTGTTACGTACAACCTGCAGGTAGAGC	
LCC	F	21	AGTCCCCATTCTGCAAAATCCGGACTCT	199
	R	21	GTCAGCTTCTATTATTGTGAAATTGTCGTGG	
ODC1	F	20	AAAAGAGCTCTACATCTTCCCGCTGCAC	190
	R	20	TGCGACGGCAATTGTCGTTGACGCCTT	
ODC2	F	22	TCGCCAAACCAATATAAATCAACTGTCC	187
	R	22	GCCAAATTGTTCCCAGGAAATTTCTACC	
GH10	F	21	GGCATGATGCTCAGAGGGCACAACCTGG	183
	R	21	ATTATCATTAGGGGTTTCGTTGATGACG	
GH5	F	22	CTGTTGTTTCGACGTTCAAAATATCTGG	188
	R	22	TCTTGAGCAACGTCAGTGTAGCAACTGG	
AAOX	F	21	GGAATGGCTCTTCGCGAATGGTTTTGTG	182
	R	21	GGTCGAAGGGGCTTGAGAGTAGACCAGG	
CAT	F	20	TGTTCTGGATCTTACCAAGGCTTGCGCG	207
	R	19	AGTCGGTATCGTTGCGTGTCTGGGTAGG	
COP	F	21	ATTGCAATTGGCTCAGGGAGCGATGTGG	188
	R	21	TATATCACACCAGCTGCGATCGGTACAGC	

ICL, isocitrate lyase; GLOXDH, glyoxylatedehydrogenase; LCC, lac case ODC1, oxalate decarboxylase 1; ODC2, oxalatedecarboxylase 2; GH10, glyceride hydrolase 10; GH5, glyceride hydrolase 5; AAOX, eryl alcohol oxidase; CAT, catalyzes COP, copper resistance P-type ATPase pump; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

amplification, a melt curve program was set to increase 0.5°C every 15 s from 60 to 95°C. Each plate contained two biological replicates and three technical replicates of all time points tested against one target gene and the internal reference gene and at least three no template control. Formation of primer dimers were detected by melt curve analysis for each reaction. The $\Delta\Delta C_T$ method, as described in Livak and Schmittgen (2001), was used to calculate relative gene expression levels, where $\Delta\Delta C_T = C_{T,target} - C_{T,18S}$ week $X - (C_{T,target} - C_{T,18S})_{week 1}$. In the formula, C_T was the cycle number when the SYBR green fluorescence signal increased more than background levels and week X was week 2-8. The fold change in expression or relative expression value was $\Delta\Delta C_T$. This method was used because it normalized expression of the target gene against an internal reference gene and expressed the time course results relative to our reference condition, which was week 1. The ten target genes examined were: ODC1, GLOXDH, LCC, COP, ICL, CAT, GH10, GH5, AAOX, and ODC2. If relative expression levels changed by

more than a factor of ± 2 , gene expression was considered to be significant.

RESULTS AND DISCUSSION

Wood Compression Strength Loss

Comparison of the matching set of wafers treated with water and ACQ-treated unexposed wafers, and there was no loss on ACQ unexposed samples. The effect of exposure to *F. ridiculous* on percent compression strength loss of the ACQ-treated wafers is plotted in Fig 2. At weeks 1 and 2, the ACQ-treated wafers showed 0% and 5% compression strength loss, respectively. Between 3 and 5 wk of fungal exposure, strength loss showed a roughly linear increase from 10% to 48%. At 6 wk, strength loss showed a small dip back down to 32%. At weeks 7 and 8, strength loss exhibited a plateau around 83-85%. The overall trend in compression strength loss was an initial lag period until 3 wk followed by a more rapid rate of increase until a plateau of 85% strength loss was reached at 7 wk. When the compression strength loss hit around 48% at

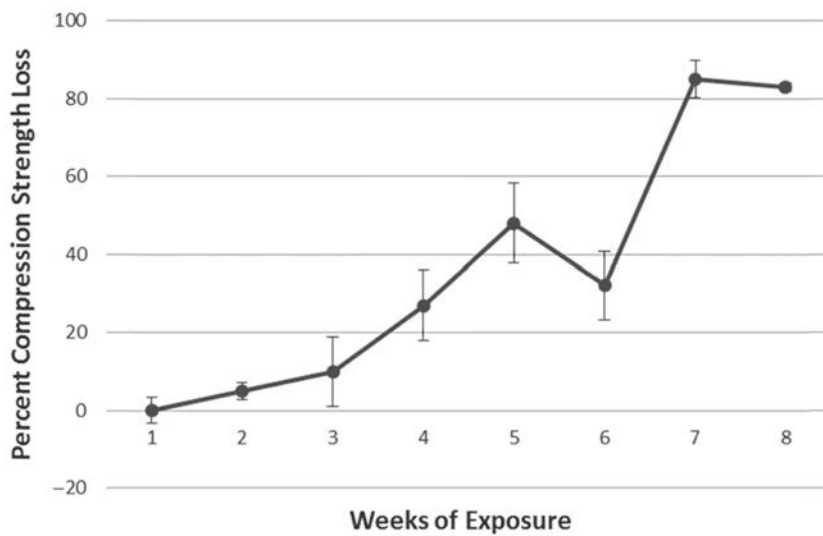


Figure 2. Percent compression strengthloss of ACQ-treated wafers after exposure to *Fibroporiaridiculosa*. Mean SD bar is plotted for each time point. ACQ, alkaline copper quat.

week 5, GH5 and GH10 reached the highest level of gene expression. This means that there is a strong correlation between strength loss and gene expression level in fungal body.

Gene Expression Analysis (qRT-PCR)

qRT-PCR was used to evaluate gene expression of ten genes for their responses to the treated wood (Figs 3-6). Four genes had higher expression on ACQ-D treated wood compared with week 1 (Fig 3). These were AAOX, CAT, ODC2, and COP. The pattern of increased expression was similar, however, the levels differed. Expression of these four genes increased from week 1 to 2, then remained relatively stable until week 7, when levels of all four genes dropped. By week 8, expression levels returned to those exhibited during week 2-6. The relative increase was around 9-10-fold for AAOX. AAOX catalyzes the reaction that uses methanol as a substrate to produce an aromatic aldehyde and hydrogen peroxide (Farmer et al 1960). The expression level increase for CAT was around 17-fold. CAT is responsible for the degradation of hydrogen peroxide to water and oxygen (Chelikani et al 2004). ODC2 had the highest change in relative

expression, increasing by 22-fold. ODC functions in the breakdown of oxalic acid by converting it to formic acid and carbon dioxide (Hayaishi et al 1956). COP increased approximately 16-17-fold but showed the greatest decrease in expression at week 7, which was down to 5-fold. COP moves copper into and of cell, thus providing copper to copper-reliant enzymes and eliminating excess copper, respectively. This suggests that COP was actively involving in to tolerate excess amount of copper to protect fungal cells by moving copper ions in or out.

Fold change values relative to week 1 for GLOXDH are graphed in Fig 3. GLOXDH exhibited the highest significant down-regulation around 7-fold at week 8, and, similar to ODC2, was among the highest significantly up-regulated at week 2 with around 22 fold change. GLOXDH has a function in GLOX and dicarboxylate metabolism, which includes biosynthesis of oxalate.

Fold change values in gene expression of LCC, ICL, and ODC1 did not significantly fluctuate during the 8th week of exposure to ACQ-treated wood (Fig 5). The observed trends, however, were expression peaks at weeks 4 and 8 for LCC, and a peak at week 2 ODC1 and ICL. ICL

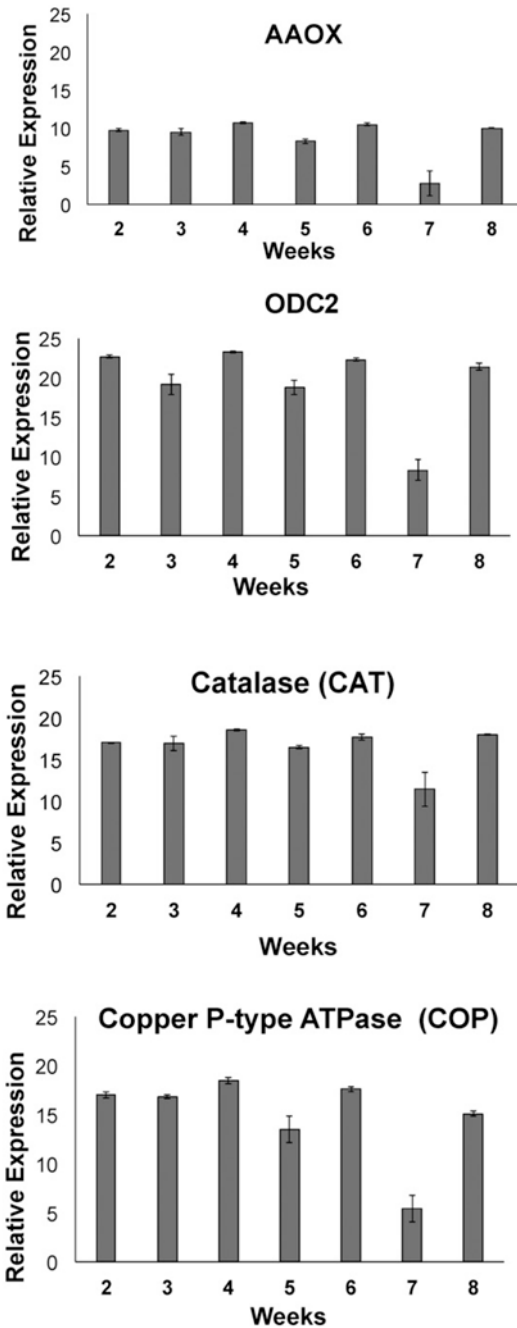


Figure 3. Relative gene expression values of AAOX, ODC2, CAT, and COP in *F. ridiculous* exposed to ACQ-D treated test wafers at 1, 2, 3, 4, 5, 6, 7, and 8 wk. Expression values were normalized against 18S rRNA and expressed relative to week 1. AAOX, aryl alcohol oxidase; ODC2, oxalate decarboxylase 2; CAT, catalyzes COP, copper resistance P-type ATPase pump; ACQ-D, alkaline copper quat type D.

catalyzes the conversion of isocitrate to succinate and GLOX. Succinate is an intermediate of the TCA cycle, whereas GLOX is an intermediate in the GLOX cycle and is the direct precursor to oxalate. ICL is a GLOX-producing enzyme involved in both TCA and GLOX cycles (Munir et al 2001). ODC functions in the breakdown of oxalate, producing formic acid and carbon dioxide. LCC plays an important role in oxidation and partial cleavage of the lignin to generate methanol during brown-rot fungal attack (Wei et al 2010). Relative gene expression of GH10 and GH5 are shown in Fig 6. These two genes displayed similar patterns of change with a significant spike in the relative expression level at week 5 around 7-fold. Other weeks were not significantly different from week 1. GH5 and GH10 are common enzymes involved in degradation of cellulose and hemicellulose (Pérez et al 2002).

Understanding the metabolic mechanisms that *F. ridiculous* uses to detoxify the copper in ACQ-treated wood plus breakdown the wood cell walls to obtain food, is extremely complicated. There are some complications caused by fungus grown on wood because it is very important to obtain all at the stages in the copper tolerance/wood decay process, and when extracting mycelia for RNA, a mixture of activities might be taken. In the study, we presented gene expression analysis of *F. ridiculous* when growing on ACQ-treated wood over an 8 wk period. This study compared week 2-8 to the expression levels determined in the week 1 ACQ-treated samples.

As *F. ridiculous* adapts to copper and initiates the wood decay process, increases in the concentration of oxalate (Ohno et al 2015) appear to be the result of increased transcription of key genes in oxalate metabolism (Tang et al 2013). Although oxalate could be produced from excess malate originating from either the TCA or GLOX cycles or from GLOX in the GLOX cycle (Munir et al 2001), gene expression data suggest that oxalate production in response to copper preservative occurred primarily through the GLOX cycle (Tang et al 2013). This study measured the expression levels of two genes likely involved in the production of oxalate, ICL and GLOXDH,

and two genes involved in the breakdown of oxalate, ODC1 and ODC2. As Fig 5 shows, the levels of ICL gene expression did not significantly

was a distinct trend. Expression of ICL was increased about 2-fold at week 2, then there was a steady reduction of expression until week 5, when there was a repression of expression during weeks 6 and 7. Ohno et al (2015) found increased expression of ICL at week 2 from three of four *F. ridiculous* isolates tested, then a drop of expression by week 4. In comparison, Tang et al (2013) found increased expression of ICL on MCQ-treated wood at week 3, followed by a steady decline in expression levels through week 22. Low levels of ICL expression were found on untreated wood throughout the MCQ study. Both studies measured an increase in ICL early in the decay process in the presence of copper, followed by a decline in expression level over time. ICL is a key enzyme in the TCA cycle which generates needed energy for fungal metabolism, and because both energy and oxalate are needed for wood decay to occur, it can be difficult to directly link this enzyme to copper tolerance unless gene regulation studies are performed.

GLOXDH converts GLOX from the GLOX cycle into oxalate. In this study, expression of GLOXDH increased 20-fold in week 2, relative to the comparison of week 1 (Fig 4). Expression levels remained up through week 8. However, slowly declined thereafter until lowest at week 8. GLOXDH was highly expressed at week 3 when the fungus was growing on MCQ-treated wood, and expression levels decreased over time (Tang et al 2013). On CC-treated wood, three isolates showed increased expression of GLOXDH at week 2, and expression levels drastically dropped at week 4 (Ohno et al 2015). Thus, GLOXDH shows similar expression patterns to ICL, in that the highest levels are detected very early in decay and adaptation. Tang et al (2013) correlated high ICL and GLOXDH expression levels with the early period of copper adaptation period when the treated wood was still exhibiting zero percent strength loss. In this study, the highest expression level of GLOXDH occurred at zero percent

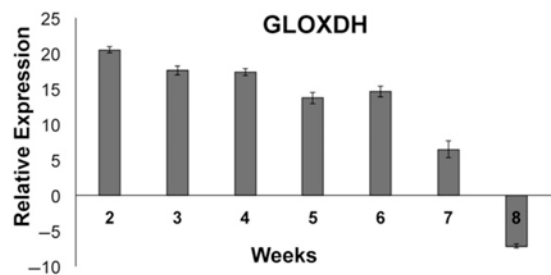


Figure 4. Relative gene expression value of the gene GLOXDH. Fungus was grown on ACQ-treated wafers for 8 wk. Expression normalized against 18S rRNA and expressed relative to week 1. GLOXDH, glyoxylate dehydrogenase; ACQ, alkaline copper quat.

strength loss, whereas the highest levels of ICL expression occurred at five percent strength loss.

ODC breaks down oxalate to formic acid and carbon dioxide, thus allowing the fungus to control the levels of oxalate in its surrounding environment. Tang et al (2012) detected four different ODC genes in the genome of *F. radiculosa*. Of these, two were differentially expressed on MCQ-treated wood, but in opposing patterns (Tang et al 2013). ODC1 showed much higher levels of expression on treated than untreated wood exhibiting peak expression during the period of copper adaptation when the treated wood was still exhibiting 0% strength loss. ODC2, on the other hand, was barely detected on MCQ-treated wood but exhibited extremely high levels of expression on untreated wood, with peak expression occurring early during the decay process (Tang et al 2013). These results contrast with those presented for ACQ-D treated wood in this study, where ODC2 expression levels were higher at week 2 and remained high through week 8 (Fig 3), whereas ODC1 expression levels were slightly increased at week 2 and steadily decreased through week 8 (Fig 5). In both studies, however, the expression pattern of ICL and ODC1 were roughly similar. Ohno et al (2015) did not measure ODC expression. At this time, there is not enough information to speculate why these two studies found different patterns of expression, especially for ODC2. It should be pointed out that the earlier study contained in-soluble copper carbonate in the micronized form

treated at a ground contact retention (Tang et al 2013) whereas this study used a soluble copper ethanolamine treated at an above ground retention. The two forms of copper differ both chemically and physically. The treating solution of micronized copper is an opaque green color and is an emulsion of insoluble copper carbonate particles. Copper ethanolamine, on the other hand, is soluble in water and appears as a clear dark blue solution. Because of the different retentions, the rate of decay as measured by strength loss was also very different. Fifty-four percent strength loss occurred at week 22 on MCQ-treated wood (Tang et al 2013), whereas 50% strength loss occurred at week 5 in this study on ACQ-treated wood. Further studies are needed that directly compare these preservative treatments to understand the regulation patterns of these two genes.

For wood decay to proceed, the fungus needs to generate the necessary components for the Fenton reaction to occur and for hydroxyl radicals to be produced. Thus, the fungus needs to produce hydrogen peroxide. Two of the genes measured in the present study may have a role in the production of hydrogen peroxide, AAOX and LCC, whereas a third gene, CAT, breaks down hydrogen peroxide. In this study, both AAOX and CAT expression patterns were similar (Fig 3). Both genes significantly increased from week 1 to 2, remained high until week 7, when they dropped, but increased again at week 8. By contrast, Tang et al (2013) found that expression of AAOX on MCQ-treated wood was not detectable or low. Only the week 22 time point measured AAOX expression. Expression of CAT was also low on MCQ-treated wood, with detectable levels at weeks 3, 10, and 22. However, both AAOX and CAT expression was significantly greater on untreated wood. These authors postulated that perhaps AAOX was not generating hydrogen peroxide for the Fenton reaction in *F. ridiculous*, but in fact, it may be laccase driving the Fenton system. In the Tang et al (2013) study, the expression of LCC increased from week 3 until week 10, and then dropped at week 22. The present study found that LCC expression was low and not significant at all time

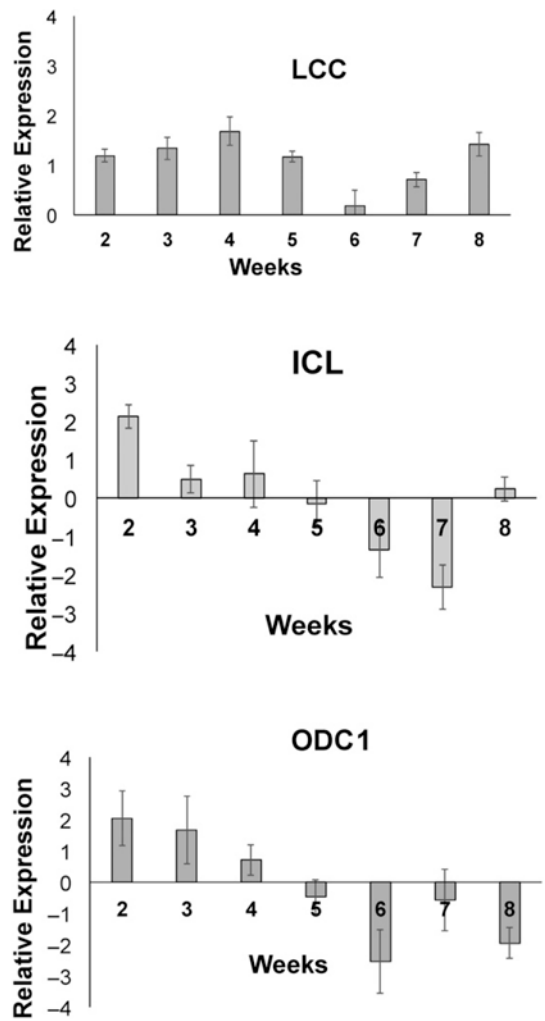


Figure 5. Relative gene expression values of LCC, ODCI, and ICL when the fungus was grown on ACQ-treated wafers for 8 wk. Expressions were normalized against 18S rRNA and expressed relative to week 1. LCC, lac case ODCI, oxalate decarboxylase I; ICL, isocitrate lyase; ACQ, alkaline copper quat.

points (Fig 5). There was a 1-fold increase from week 1 to 2, and then expression remained about the same until week 6 when expression levels dropped. They rose again slightly at weeks 7 and 8. Thus, the present study points to AAOX as the possible source to drive Fenton chemistry during decay.

The last gene measured in this study that may be involved in the copper tolerance mechanism is the COP, which potentially moves copper both in and

of cell. The four isolates of *F. ridiculous* in Ohno et al (2015) study each showed different expression patterns for COP. The isolate TFFH-294, which is the same as in the current study, showed the highest expression level at week 2, then a steady decrease in expression levels until week 8. Tang et al (2013) measured a highest expression of COP at first time point with a decline over time. Expression levels on untreated wood were higher than on MCQ-treated wood, but were still relatively low. In the present study, COP showed a very similar pattern of expression as AAOX, CAT, and ODC2. The level of expression significantly increased at week 2, remained high until week 7, and increased again at week 8 (Fig 3). This suggests that during week 2-6, hydrogen peroxide is being produced (AAOX) and concentrations regulated (CAT), oxalate levels are being regulated (ODC2) and copper levels are being regulated (COP).

The polysaccharide genes measured in this study are two glyceride hydro lases (GH5 and GH10), which play a role in the degradation of cellulose and hemicellulose. In the present study, the only significant increase in level of expression of both GH5 and GH10 was at week 5 when there was fifty percent loss of wood strength (Fig 6). This same result was found in Tang et al (2013) where the highest expression of these two genes were at week 22, when there was fifty-four percent loss of wood strength. This suggests that at fifty percent strength loss, the wood cells have degraded to the extent that the enzymes responsible for cellulose and hemicellulose degradation can penetrate into the walls and access their substrates. What was surprising is that in this present study, the expression of both genes significantly dropped after week 5. One might have expected a continuation of gene expression in the subsequent week. Further transcriptions studies will unravel our curiosity on how these genes being regulated by copper or organic based wood treatments (Akgul 2016).

CONCLUSIONS

This study compared the expression level of ten genes thought to be involved in the mechanism of copper tolerance and wood decay in the

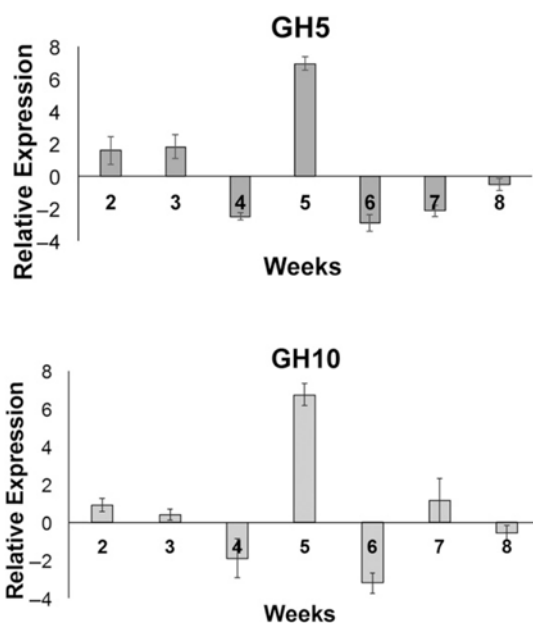


Figure 6. Relative gene expression values of GH5 and GH10 in *F. ridiculous* exposed to ACQ-D treated test wafers at 1, 2, 3, 4, 5, 6, 7, and 8 wk. Expression values were normalized against 18S rRNA and expressed relative to week 1. GH5, glyceride hydro lase 5; GH10, glyceride hydro lase 10; ACQ-D, alkaline copper quat type D.

brown-rot fungus, *F. ridiculous* (Table 2). Some similarities and differences were seen among this study and a previous study. Tang et al (2013) postulated that *F. ridiculous* is using a laccase-driven Fenton chemistry to both overcome the copper and initiate decay the wood. Laccase is believed to be involved in the oxidation and partial cleavage of lignin, generating methanol, during brown-rot fungal attack (Irbe et al 2001; Yelle et al 2008). A purified laccase from *P. placenta* was able to oxidize 2,5-dimethoxyhydroquinone to a per hydroxyl radical at a ratio of one radical produced for each hydroquinone supplied (Wei et al 2010). The brown-rot fungi *F. ridiculous* and *P. placenta* have been found to encode two putative laccases (Martinez et al 2009; Tang et al 2012). Tang et al (2013) found simultaneous upregulation of laccase and oxalate by *F. ridiculous* on copper treated wood providing further evidence for a laccase-driven Fenton system in a high oxalate environment.

Table 2. Summary of gene expression levels for 10 genes.

Gene ID ↓	Weeks →						
	2	3	4	5	6	7	8
AAOX	9.75	9.50	10.72	8.28	10.50	2.75	10
ODC2	22.7	19.1	23.2	18.7	22.3	8.3	21.3
CAT	17	17	18.6	16.4	17.7	11.43	18
COP	17.03	16.8	18.48	13.5	17.62	5.4	15.09
GLOXDH	20.5	17.58	17.37	13.71	14.6	6.44	-7.16
LCC	1.19	1.34	1.68	1.17	0.175	0.71	1.42
ICL	2.1	0.5	0.6	-0.2	-1.4	-2.3	0.2
ODC1	2.03	1.67	0.71	-0.47	-2.54	-0.58	-1.95
GH5	1.57	1.81	-2.48	6.94	-2.89	-2.14	-0.49
GH10	0.9	0.4	-1.9	6.8	-3.2	1.2	-0.6

ICL, isocitrate lyase; GLOXDH, glyoxylate dehydrogenase; LCC, laccase; ODC1, oxalate decarboxylase 1; ODC2, oxalate decarboxylase 2; GH10, glycoside hydrolase 10; GH5, glycoside hydrolase 5; AAOX, aryl alcohol oxidase; CAT, catalase; COP, copper resistance P-type ATPase pump.

Based on the results from this study, *F. radiculosa* appears to regulate Fenton chemistry through the production and breakdown of hydrogen peroxide by AAOX and CAT. The production of oxalate correlated to GLOXDH activity and the alternative enzyme, oxaloacetic hydrolase, was not measured. Break down of oxalate through ODC2 did correlate to both AAOX and CAT activity, as did the activity of the copper pump, COP. By contrast to Tang et al (2013), LCC did not appear to be driving the mechanism for *F. radiculosa* when grown on ACQ-treated wood. Each of the three studies compared here provided different answers to the same questions. Whether these differences stem from the different treatments tested or the extreme complexities involved in trying to control the metabolic state of the fungus and stage of decay is not known. In this study, gene expression levels have been analyzed in an actively decaying fungus. However, this may not be related to direct production of proteins. Future studies investigating protein production in these same conditions would serve to bridge the knowledge gap in our understanding of biosynthetic pathways that confer copper tolerance.

ACKNOWLEDGMENTS

We are grateful to Linda Sites and Army Rowlen for excellent assistance in preparation of soil block test, and special thanks to Dr. Dragica Jeremic Nikolic for her guidance.

REFERENCES

- Akgul A (2016) Gene expression profiling of wood decay fungus *Fibropora ridiculous* grown on different organic and copper based preservatives. Ph.D. Dissertation, Mississippi State University, p. 224.
- Arango R, Lebow P, Green F III (2009) Correlation between oxalic acid production and tolerance of *Tyromyces palustris* strain TYP-6137 to N,N-naphthaloylhydroxamine. *Int Biodeterior Biodegradation* 63:46-51.
- Arantes V, Jellison J, Goodell B (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. *Appl Microbiol Biotechnol* 94:323-338.
- American Wood Protection Association (AWPA) (2012) E22-12 Standard accelerated laboratory method for testing the efficacy of preservatives against wood decay fungi using compression strength. *Book of Standards*. American Wood Protection Association, Birmingham, AL. 8 pp.
- American Wood Protection Association (AWPA) (2014b) P29-14 Standard for alkaline copper quat type D (ACQ-D). *Book of Standards*. American Wood-Preservers Association, Birmingham, AL.
- Clausen C, Green F III, Kirker GT, Lebow S (2014) Wood protection research council: Research priorities 2013. General technical report FPL-GTR-232. U.S. Department of Agriculture, Forest Service, Forest Products Laboratory, Madison, WI. 11 pp.
- Chelikani P, Fita I, Loewen PC (2004) Diversity of structures and properties among catalases. *Cell Mol Life Sci* 61(2): 192-208.
- Button MV, Evans CS (1996) Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. *Can J Microbiol* 42:881-895.
- Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer Verlag Publishers, Berlin. 407 pp.

- Farmer VC, Henderson MEK, Russell JD (1960) Aromatic-alcohol-oxidase activity in the growth medium of *Polystictus versicolor*. *Biochem J* 74(2):257-262.
- Freeman M, McIntyre C (2008) A comprehensive review of copper-based wood preservatives with a focus on new micronized or dispersed copper systems. *Forest Products Society* 58:6-27.
- Gadd GM (1993) Interactions of fungi with toxic metals. *New Phytol* 124:25-60.
- Goodell B, Liu J, Daniel G, Paszczynski A, Fekete F, Krishnamurthy JL, Xu G (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J Biotechnol* 53:133-162.
- Green F III, Clausen CA (2003) Copper tolerance of brown-rot fungi: Time course of oxalic acid production. *Int Biodeterior Biodegradation* 51:145-149.
- Hummel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb Technol* 30:445-453.
- Hastmp ACS, Green F III, Clausen C, Jensen B (2005) Tolerance of *Serpula commons* to copper-based wood preservatives. *Int Biodeterior Biodegradation* 56:173-177.
- Hastmp ACS, Jensen B, Clausen C, Green F I" (2006) The effect of CaCl₂ on growth rate, wood decay and oxalic acid accumulation in *Serpula commons* and related brown-rot fungi. *Holzforschung* 60:339-345.
- Hayaishi O, Jakoby WB, Ohmura E (1956) Enzymatic decarboxylation of oxalic acid. *J Biol Chem* 222(1):435-446.
- Irbe I, Andersone I, Andersons B, Chirkova J (2001) Use of ¹³C NMR, sorption and chemical analyses for characteristics of brown-rotted Scots pine. *Int Biodeterior Biodegradation* 47:37-45.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25:402-408.
- Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P (2009) Genome, transcriptome, and secretions analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc Natl Acad Sci USA* 106:1954-1959.
- Munir E, Yoon JJ, Tokimatsu T, Hattori T, Shimada M (2001) A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustris*. *Proc Natl Acad Sci USA* 98:11126-11130.
- Ohno KM, Clausen CA, Green F, Diehl SV (2015) Insights into the mechanism of copper-tolerance in *Fibroporia ridiculosa* The biosynthesis of oxalate. *Int Biodeterior Biodegradation* 105:90-96.
- Pérez J, Munoz-Dorado J, Rubia De La T (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An overview. *Int Microbiol* 5:53.
- Schilling JS, Jellison J (2006) Metal accumulation without enhanced oxalate secretion in wooddegraded by brown rot fungi. *App1 Environ Microbiol* 72(8):5662-5665.
- Schultz TP, Nicholas DD (2008) Introduction to developing wood preservative systems and molds in homes. ACS Symposium series, American Chemical Society, Washington, DC, 2008.
- Tang JD, Parker LA, Perkins AD, Sonstegard TS, Schroeder SG, Nicholas DD, Diehl SV (2013) Gene expression analysis of copper tolerance and wood decay in the brown rot fungus *Fibroporia ridiculosa*. *Appl Environ Microbiol* 79:1523-1533.
- Tang JD, Perkins AD, Sonstegard TS, Schroeder SG, Burgess SC, Diehl SV (2012) Shotgun-read sequencing for genomic analysis of the brown rot fungus *Fibroporia ridiculosa*. *App1 Environ Microbiol* 78:2272-2281.
- Vesentini D, Dickinson DJ, Murphy RJ (2005) The production of extracellular mucilaginous material (ECMM) in two wood-rotting basidiomycetes is affected by growth conditions. *Mycologia* 97(6):1163-1170.
- Wei D, Houtman CJ, Kapich AN, Hunt CG, Cullen D, Hummel KE (2010) Laccase and its role in production of extracellular reactive oxygen species during wood decay by the brown rot basidiomycete *Postia placenta*. *Appl Environ Microbiol* 76:2091-2097.
- Woodward BM, DeGroot RC (1999) Tolerance of *Wolfiporia cocos* isolates to copper in agar media *Forest Prod J* 49: 87-94.
- Wymelenberg AV, Gaskell J, Mozuch M, Sabat G, Ralph J, Skyba O, Mansfield SD, Blanchette RA, Martinez D, Grigoriev I, Kersten PJ (2010) Comparative transcriptome and secretions analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. *App1 Environ Microbiol* 76:3599-3610.
- Yelle DJ, Ralph J, Lu F, Hammel KE (2008) Evidence for cleavage of lignin by a brown rot basidiomycete. *Environ Microbiol* 10(7):1844-1849.