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Insights into the mechanism of copper-tolerance in *Fibroporia radiculosa*: The biosynthesis of oxalate

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ABSTRACT

Copper is currently used as the key component in wood preservatives despite the known tolerance of many brown-rot Basidiomycetes. Copper-tolerant fungi, like *Fibroporia radiculosa*, produce and accumulate high levels of oxalate when exposed to copper. To gain insight into the mechanism of oxalate production, four *F. radiculosa* isolates decaying untreated and 1.2% ammoniacal copper citrate-treated wood were evaluated for the differential expression of citrate synthase, isocitrate lyase, glyoxylate dehydrogenase, a succinate/fumarate antiporter, and a copper resistance-associated ATPase pump. Samples were analyzed at 2, 4, 6, and 8 weeks for oxalate production and gene expression. ATPase pump expression increased in the presence of copper when initial oxalate concentrations were low, suggesting it functions in helping the fungus adapt to the copper-rich environment by pumping toxic copper ions out of the cell. A connection in expression levels between citrate synthase, the succinate/fumarate antiporter isocitrate lyase, and glyoxylate dehydrogenase for the four isolates was found suggesting the production of oxalate originates in the mitochondrial TCA cycle via citrate synthase, shunts to the glyoxysomal glyoxylate cycle via the succinate/fumarate antiporter, moves through a portion of the glyoxylate cycle (isocitrate lyase), and ultimately is made in the cytoplasm (gyloxylate dehydrogenase).
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1. Introduction

The mechanism of copper-tolerance in brown-rot fungi has often been associated with the production 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). High extracellular accumulation of oxalate initiates the precipitation of copper into the insoluble form of copper oxalate forming crystals and renders the copper ion inert (Pohleven et al., 1999; De Groot and Woodward, 1999; Humar et al., 2002). Both copper oxalate and calcium oxalate crystals are present in decayed wood treated with copper-based preservatives (Freeman and McIntyre, 2008). Another important factor related to copper tolerance is the presence of a low pH environment, which is also stimulated by oxalate production (Clausen et al., 2000; Humar et al., 2001; Clausen and Green, 2003; Green and Clausen, 2003, 2005). Choi et al. (2002) showed spores from copper-tolerant organisms did not germinate in the presence of copper because the spores lack the ability to actively produce oxalate (Freeman and McIntyre, 2008). Thus, oxalate accumulation and precipitation of copper oxalate crystals play an important role in the mechanism of copper tolerance.

The production of oxalate occurs by numerous classes of fungi and many accumulate it in high concentrations. The majority of brown-rot fungi produce oxalate in detectable amounts; however, its accumulation is limited in white-rot fungi most likely due to oxalate decarboxylase (Hastrup et al., 2006). In white-rot fungi, intracellular oxalate decarboxylase breaks down oxalate to carbon dioxide and formate (Espejo and Agosin, 1991; Hastrup et al., 2006). In brown-rot fungi, oxalate has the potential to accumulate as a function of fungal respiration (Schilling and Jellison, 2005). Some brown-rot fungi produce oxalate for initial decay but do not

Abbreviations: CS, citrate synthase; ICL, isocitrate lyase; GLOXDH, glyoxylate dehydrogenase; ANTI, succinate/fumarate antiporter; ATPase, copper resistance-associated ATPase pump; TCA, tricarboxylic acid cycle; GLOX, glyoxylate cycle; OXA, oxaloacetase; UN, untreated wood; CC, 1.2% ammoniacal copper citrate treated wood; RH, relative humidity; SP, Southern pine; CDS, coding sequence; RT-qPCR, Reverse Transcriptase Quantitative Polymerase Chain Reaction; HK, actin; C_T, threshold values.

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accumulate significant levels during the decay process, while other brown-rot fungi utilize oxalate throughout the decay process resulting in steady levels of accumulation (Goodell, 2003).

Several pathways have been proposed for the biosynthesis of oxalate in brown-rot fungi; however, in recent years, many researchers have linked it to the tricarboxylic acid (TCA) and glyoxylate (GLOX) cycles (Dutton and Evans, 1996; Munir et al., 2000; Munir et al., 2001; Yoon et al., 2002; Schilling and Jellison, 2005; Hastrup et al., 2006). The TCA cycle is localized in the mitochondria while the GLOX cycle takes place in the glyoxysome (Dutton and Evans, 1996). Munir et al., (2001) investigated oxalate biosynthesis in the copper-tolerant fungus *Tyromyces palustris*. This study hypothesized that 1 mol of glucose is converted to 2 mol of oxalic acid rather than producing the traditional CO_2 from the TCA cycle. Through their work, it was discovered that there are two major oxalate producing enzymes, oxaloacetase (OXA) and glyoxylate dehydrogenase (GLOXDH). Munir et al. (2001) discovered quantifiable OXA and GLOXDH enzymatic activity, with OXA synthesizing more oxalate than GLOXDH in *T. palustris*.

Using this discovery as a model, we hypothesized that *Fibroporia radiculosa*, another copper-tolerant fungus, may use a similar mechanism for oxalate production. Initial enzymatic analysis showed quantifiable GLOXDH, but not OXA, in four isolates of *F. radiculosa* (unpublished data). These results were related to the genome analysis of *F. radiculosa* isolate TFFH 294 showing up-regulation of GLOXDH, not OXA, in the early stages of decay (Tang, 2011). In addition to GLOXDH, Tang et al. (2012) found up-regulation of citrate synthase (CS), a succinate-fumarate antiporter (ANTI), isocitrate lyase (ICL), and a copper resistance-associated ATPase pump (ATPase) during the early stages of decay. Citrate synthase is found in the mitochondria as part of the TCA cycle and converts oxaloacetate to citrate (Fig. 1). The succinate/fumarate antiporter, found in the cytoplasm, functions as a shuttle from isocitrate in the TCA cycle to isocitrate in the GLOX cycle which is located in the glyoxysome. The ATPase pump is not associated with either the TCA or GLOX cycles; instead, it is found in the cytoplasm and functions to pump copper ions (Cu^{2+}) out of the cell (Fig. 1).

Co-biocides are needed to prevent these copper-tolerant organisms from circumventing the copper-based preventative measures in use today; therefore, it is critical that these mechanisms be investigated. Knowing the exact mechanisms and specific requirements of organisms involved in copper tolerance would give insight into the physiological intricacies of copper tolerance. A better understanding of this mechanism is crucial in developing targeted methods to control these particular organisms. The purpose of this study is to gain insight into the biosynthesis of oxalate by which the brown-rot decay fungus, *F. radiculosa*, regulates tolerance of copper-treated wood. Specific objectives of this study are to examine select *F. radiculosa* isolates during decay of untreated and copper-treated wood for the differential expression of genes associated with oxalate production, and to propose a potential pathway involved in the production of oxalate.

2. Materials and methods

2.1. Sample preparation

Four isolates of *F. radiculosa* (Peck) Gilb. & Ryvarden were used in this study: FP-90848-T, L-9414-SP, L-11659-SP, and TFFH 294 (USDA Forest Products Laboratory, Madison, WI). Cultures were maintained on 2% Malt Extract Agar (BD, Fisher Scientific). Isolates were subjected to an AWPA Standard E10-15 (2015) decay chamber at 26.7 °C and 70% relative humidity (RH) for 2 weeks to allow initial colonization (AWPA, 2015). Southern pine (SP) test wafers measuring 70 mm × 22 mm × 4 mm (t × r × l) were vacuum treated with 1.2% ammoniacal copper citrate (CC) for 40 min at −172 kPa. After initial colonization, two CC-treated SP test wafers were placed directly on top of the colonized feeder strips and incubated at 26.7 °C 70%RH for 8 weeks. Untreated SP test wafers were also exposed to the four isolates and incubated at 26.7 °C, 70% RH for 8 weeks. Both untreated and CC-treated SP test wafers

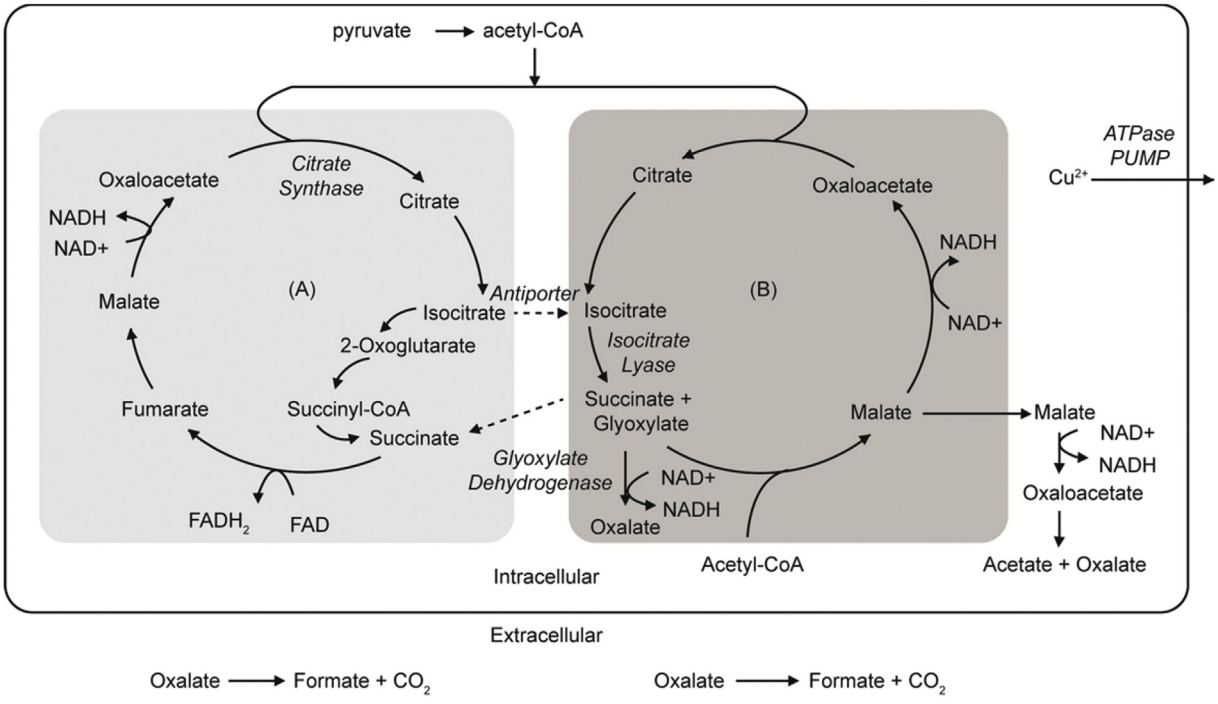

Fig. 1. Genes (*italics*) up-regulated in early decay. (A) TCA cycle; (B) GLOX cycle. Light gray box indicates TCA cycle in the mitochondria; dark gray box indicates GLOX cycle in the glyoxysome (Tang, 2011).

exposed to the four isolates were harvested at weeks 2, 4, 6, and 8. Test wafers were rasped into sawdust-like material and approximately 0.2 g of sawdust material was placed into 2.0 ml tubes containing two 5 mm glass beads. The 2.0 ml tubes were dipped into liquid nitrogen and stored at −80 °C until analysis.

2.2. Oxalate analysis

Cold 0.1 M phosphate buffer (1.5 mL) was added to each 2.0 ml tube and samples were homogenized for three minutes by a bead mill. After centrifugation to remove unwanted debris, the crude homogenate was placed directly on ice. Ten microliters of the crude homogenate from each sample was place in a 96-well microplate (Corning®, Corning, NY). In each well, 200 μl DMAB/oxalate oxidase reagent was added followed by 20 μl of MBTH reagent (Trinity Biotech, Wicklow, Ireland). Contents were mixed gently and incubated for 5 min at 37 °C (Clausen et al., 2008). Oxalate concentration was determined spectrophotometrically (Biotek Epoch, Winooski, VT) at 560 nm as specified (Trinity Biotech). Six replicates of each isolate at each time point for each treatment were analyzed for oxalate production ($n = 6$). Statistical analysis was performed using ANOVA ($p = 0.05$).

2.3. RNA extraction

RNA was isolated using the Ambion® RNAqueous™ Kit (Applied Biosystems, Carlsbad, CA) according to manufacturer's specifications with an added DNase I (Promega, Madison, WI) digestion step to remove unwanted genomic DNA contaminants for each isolate at each time point exposed to each treatments ($n = 1$). DNase I digestion was prepared by adding 40 μl DNase-free water, 5 μl DNase I 10× Buffer, and 5 μl DNase I enzyme. This step was included between the Wash Solution 1 step and Wash Solution 2 step of the RNAqueous™ Kit. Following elution, RNA was kept on ice at all times. RNA was quantified by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Quality was determined by chip electrophoresis using Experion™ RNA StdSens Analysis Kit following manufacture's specifications (Bio-Rad, Carlsbad, CA). All RNA was stored at −80 °C.

2.4. cDNA synthesis

RNA was synthesized to first strand cDNA using the SuperScript™ II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) according to manufacture's specifications. cDNA synthesis was carried out for each isolate at each time point exposed to each treatment ($n = 1$) using an Eppendorf® Mastercycler with the following settings: incubation at 42 °C for 90 min and incubation at 85 °C for 5 min. Both non-template controls and reverse transcriptase-free controls were also included.

2.5. Primer design

Expression levels of six specific genes were examined for the copper citrate treated and untreated test samples across all time points using real-time qPCR. Gene-specific primers were designed utilizing gene and coding (CDS) sequences of *F. radiculosa* TFFH 294 obtained from Tang (2012). CDS sequences for ICL, GLOXDH, CS, ANTI, and ATPase were matched against gene sequences for the respective genes. 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 uniquely specific for the desired region. Sequences used for real-time qPCR are listed in Table 1. Actin (HK) was used as the housekeeping gene.

2.6. Gene expression

To determine gene expression, Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) was monitored in real time for the six genes in question. RT-qPCR reactions were carried out in an iCycler iQ™ 96 well PCR plates with Microseal® 'B' Film using an iQ™ 5 Real-Time PCR Detection System (Bio-Rad, Carlsbad, CA). Two genes of interest were analyzed on one plate and each sample ($n = 1$) was measured in triplicate. The 20 μl reactions contained 10 μl 2× iQ™ SYBR® Green Supermix (Bio-Rad, Carlsbad, CA), 8 μl sterile molecular grade water, 0.5 μl of both forward and reverse primers (10 μM), 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 s 95 °C denaturation, 30 s annealing at 65 °C, and a 30 s 72 °C extension. 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 s with temperatures increase 0.5 °C each cycle. Using the actin (HK) mean C_T, average replicate C_T values were normalized for all plates. 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).

3. Results

3.1. Oxalate analysis

The overall goal of this research was to determine the potential genes involved in the production of oxalate by the four *F. radiculosa* isolates during decay of 1.2% copper citrate (CC) treated and untreated (UN) wood. Oxalate production was evaluated every 2 weeks over an 8 week period for L-11659-SP (Fig. 2a), FP-90848-T (Fig. 2b), TFFH 294 (Fig. 2c), and L-9414-SP (Fig. 2d). Oxalate produced by the four isolates exposed to both treatments was significantly higher at week 6 ($p = 0.05$). Oxalate production was significantly higher when each isolate was exposed to the CC treatment ($p = 0.05$). FP-90848-T produced significantly higher amounts of oxalate compared to L-11659-SP and TFFH 294. There was no significant difference in oxalate production between FP-90848-T and L-9414-SP ($p = 0.05$). TFFH 294 produced significantly lower amounts of oxalate compared to the other three isolates over the course of this study ($p = 0.05$); however, TFFH 294 at week 6 exposed to CC had the overall highest oxalate concentration value (1.375 mg/mL) for any isolate.

3.2. Gene expression analysis

Real-time qPCR was used to analyze C_T values of the four *F. radiculosa* isolates for CS (Fig. 3a), ANTI (Fig. 3b), ICL (Fig. 3c), GLOXDH (Fig. 3d), and ATPase genes (Fig. 4) undergoing decay of 1.2% copper citrate (CC) treated and untreated (UN) SP wafers every 2 weeks over an 8 week period. For each isolate, the UN controls at each respective week were used as the endogenous controls for all calculations. Therefore, the values expressed in the following graphs reflect the mean fold change (difference) between the UN and CC treatments. For example, L-9414-SP expressed 7.6 times higher CS when exposed to CC at week 2 (i.e. there was 7.6 times more CS expression when this isolate was exposed to the CC treatment) (Fig. 3a). If the mean fold change is above a value of 2, the expression of that particular gene is considered significant. Expression levels of the four genes were seen in all isolates throughout the 8 week period; however, expression was somewhat

Table 1
Target gene, nucleotide sequence, and reference gene accession number for all oligonucleotide primers used for gene expression.

Target gene	Primer sequence	Accession number
HK	Forward 5′-GTGATGGTTGGTATGGGTCAGAAGG Reverse 5′-GAAGCTCGTTGTAGAAAGTGTGATGC	XP_003026150.1
ICL	Forward 5′-CAGTACTGCATCCAGCACGAACGAGC Reverse 5′-CGTGACTCTGCTTGCTTGCGGTCGTG	BAD93181.1
GLOXDH	Forward 5′-CGATGCGATCACGACAAACCTGCGCC Reverse 5′-CGAGGAACTTCTGCCGCCGCTTCTC	BAH29964.1
CS	Forward 5′-CATCCTATGAGCCAATTCAGTTTGGC Reverse 5′-CCATGCAGTCTTCGAATACCGGCTTC	EGN99006.1
ANTI	Forward 5′-CAAGGGATGGCTAGCAGACAAGGAG Reverse 5′-GCCGAATCTTCACAACCTCCATGGGC	EGO00768.1
ATPase	Forward 5′-GCGCTGCGTCGTGGCTATGGTTGGCG Reverse 5′-CCGATACAAGGATGAACGCGGCGCTG	XP_002473512.1

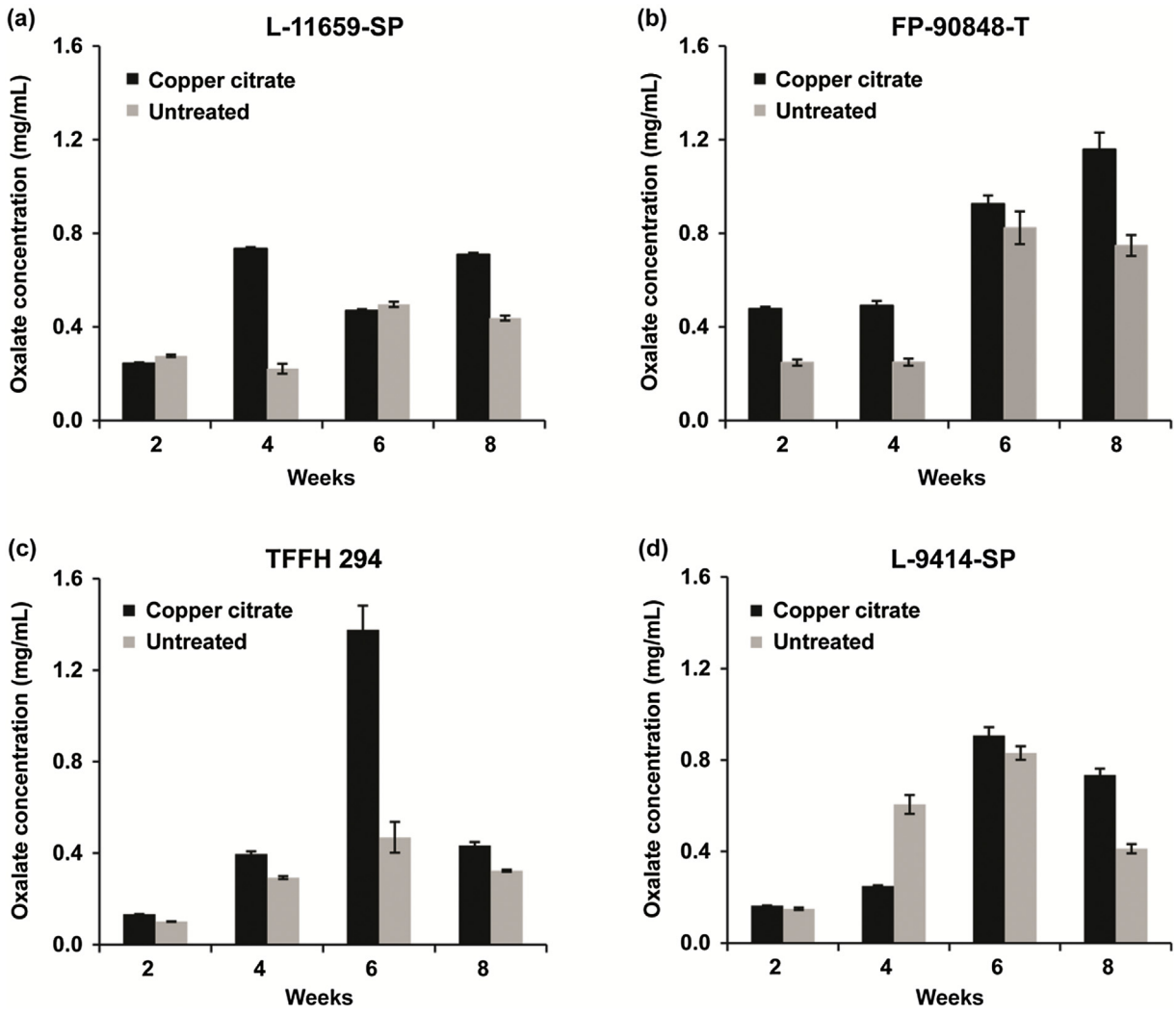

Fig. 2. Oxalate concentration values (mg/mL) of L-11659-SP (a), FP-90848-T (b), TFFH 294 (c), and L-9414-SP (d) exposed to copper citrate (black) and untreated (gray) test wafers for 2, 4, 6, and 8 weeks. Standard deviation is shown above each bar (n = 6).

variable with respect to isolate and time. Most notably, L-9414-SP showed significant fold change differences in CS (7.6), ANTI (9.7), ICL (21.8), and GLOXDH (42.8) expression levels at week 2. L-11659-SP showed significant fold change differences in ICL (5.2, 2.3) and GLOXDH (6.6, 2.1) expression levels at weeks 2 and 6, respectively. TFFH 294 also showed a significant fold change difference in GLOXDH (3.9) expression level at week 2.

Expression levels of the ATPase pump were also evaluated every 2 weeks for an 8 week period in all four isolates. Fold change difference in expression of ATPase for all isolates can be seen in Fig. 4. L-11659-SP showed significant fold change differences in ATPase expression at week 2 (2.5) and week 4 (10.1). FP-90848-T showed significant fold change differences in ATPase expression at week 2 (2.3), week 4 (11.8), week 6 (13.5), and week 8 (2.5). TFFH 294 showed significant fold change differences in ATPase expression at week 2 (7.5), week 4 (3.0), and week 8 (6.0). L-9414-SP showed significant fold change differences in ATPase expression at week 2 (2.2), week 4 (2.5), week 6 (2.8), and week 8 (3.7).

4. Discussion

4.1. Oxalate analysis

Oxalate is a critical component of decay by brown-rot fungi

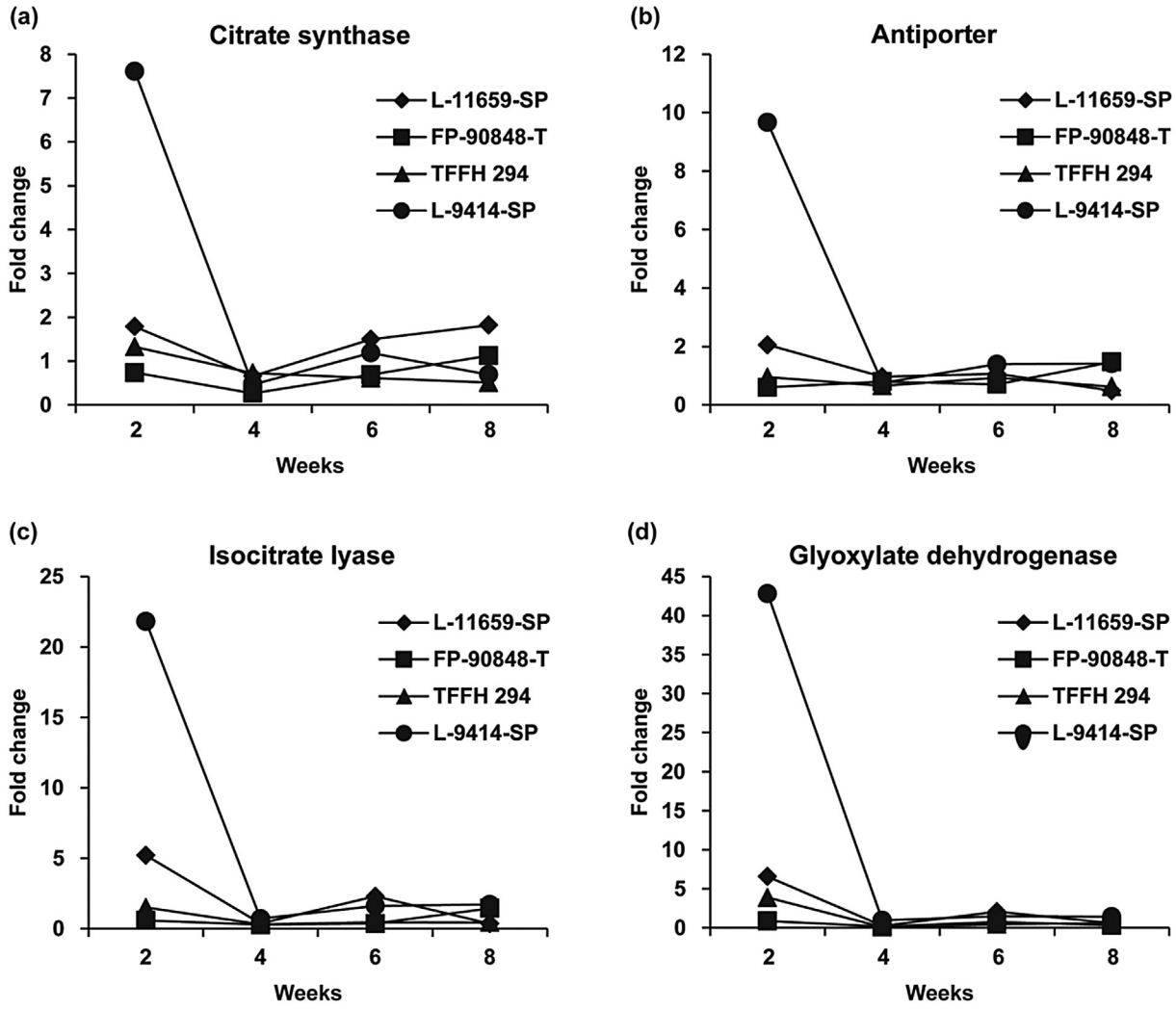

Fig. 3. Gene expression values (fold change) of citrate synthase (a), succinate-fumarate antiporter (b), isocitrate lyase (c), and glyoxylate dehydrogenase (d) between isolates exposed to copper-treated and untreated test wafers for 2, 4, 6, and 8 weeks (n = 1). L-11659-SP (◆); FP-90848-T (∎); TFFH 294 (▲); L-9414-SP (●).

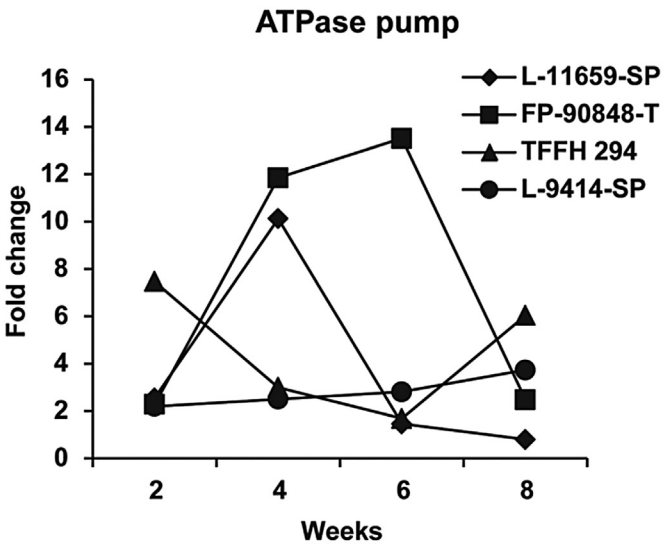

Fig. 4. Gene expression values (fold change) of the ATPase pump between isolates exposed to copper-treated and untreated test wafers for 2, 4, 6, and 8 weeks (n = 1). L-11659-SP (◆); FP-90848-T (∎); TFFH 294 (▲); L-9414-SP (●).

because of its ability to establish the organism within a particular woody environment (Dutton and Evans, 1996; Gadd, 1993; Jarosz-Wilkolazka and Gadd, 2003). Because oxalate is crucial in allowing the organism to survive a certain environment, production should be higher in the initial stages of decay. It is possible that these isolates are accumulating high oxalate concentrations at week 6 because that is the time point in which oxalate is most needed. The slight drop in oxalate production at week 8 could be perceived as the time point to which the organism is beginning to establish itself in that environment and no longer needs oxalate in high quantities. It should be noted that oxalate in too high a concentration could be toxic to certain fungi (Dutton and Evans, 1996).

An organism will react to a particular environment as needed. In untreated wood, the production of oxalate is still necessary to reduce the pH of the surrounding environment. High levels of oxalate should not be required in untreated wood because the organism does not need to overcome any extraneous chemicals. Brown-rot fungi have evolved a mechanism to bypass the toxic effect of copper by increasing oxalate production in the presence of a copper-rich environment. In copper-treated wood, the organism stimulates higher amounts of oxalate in order to tie-up the copper ions (De Groot and Woodward, 1999; Green and Clausen, 2003). Because oxalate production in CC treated wood is higher than the

UN controls, it can be deduced that copper is in some form stimulating the production of oxalate in these four isolates. But, the response of an individual organisms' ability to produce higher amounts of oxalate on CC treated wood varied with respect to time and isolate.

Interestingly, only FP-90848-T (Fig. 2b) and TFFH 294 (Fig. 2c) showed higher oxalate production in CC treated wood at all four time points when compared to the UN controls. At week 2, FP-90848-T showed higher oxalate accumulation in CC treated wood when compared to the UN controls. By week 4, FP-11659-SP (Fig. 2a) showed higher oxalate production in CC treated wood than the UN controls. In TFFH 294, week 6 showed much higher oxalate production in CC treated wood than the UN controls. However, L-9414-SP (Fig. 2d) did not show higher oxalate production on CC treated wood until week 8. This could be attributed to the metabolism of the specific isolate and its ability to initiate specific mechanisms to overcome copper in that environment. The different responses of the four isolates could also suggest a variation in the active growth of an individual organism in response to the copper-rich environment. In addition, oxalate decarboxylase could be present and could be limiting extracellular oxalate in this isolate.

4.2. Gene expression analysis

Expression patterns did not directly correlate to the patterns seen in oxalate production (Fig. 2). For CS, ANTI, ICL, and GLOXDH, expression levels were highest in L-9414-SP at week 2 when compared to the other isolates (Fig. 3). Highest oxalate production for L-9414-SP was not seen until week 6 (Fig. 2d). It is possible that the genes are being expressed much sooner than the appearance of end product. For example, elevated ICL expression could occur after a few days, which may not be reflected in oxalate production until week 4 or 6. The same could hold true for GLOXDH expression. It would be expected that elevated ICL expression would occur before elevated GLOXDH expression since glyoxylate needs to be produced before oxalate can be produced (Fig. 1). However, both of these genes could have been expressed much higher prior to the first time point evaluated in this study. Also, it would be expected that CS, ANTI, ICL, and GLOXDH expression levels would be connected based on their location in the TCA cycle (Fig. 1), which can be seen in the expression levels of all four genes for L-9414-SP (Fig. 3).

L-11659-SP exposed to CC at week 4 shows higher oxalate production (Fig. 2a), but by week 6 oxalate production was nearly equal between the two treatments. This could suggest that by week 6, L-11659-SP has bound up the excess copper and no longer needs to produce higher oxalate amounts. FP-90848-T exposed to CC at all four time points produces higher oxalate (Fig. 2b) indicating it requires oxalate through week 8. Oxalate production in TFFH 294 exposed to CC is higher at weeks 6 and 8 (Fig. 2c) suggesting it has not fully adapted to the environment by week 8 and still requires oxalate to some degree. Also, L-9414-SP exposed to CC at weeks 6 and 8 produce higher oxalate (Fig. 2d) suggesting it has not fully adapted to the environment.

It is likely that the presence of copper in the CC treatment causes the increase in expression of ATPase through week 8 for all isolates. Since the ATPase pumps copper out of the cell, it would be expected that in a copper-rich environment expression would be higher. The organism would need to efficiently pump the copper ions out to keep the copper from building to a toxic level in the cell. It can also be deduced that the organism would need to pump out the copper ions at the earliest stage possible, which results in much higher ATPase expression levels. If copper functions to initiate a response in elevated oxalate production, it should be reflected in the expression levels of all five genes. However, it is possible for levels of expression to fluctuate based on isolate and time, which could be dependent on the metabolism of the particular isolate.

One possible explanation for the role of these ATPase pumps suggests that they work in tandem with oxalate. First, the pump must expel the toxic levels of copper out of the cell (early stages of decay). These expelled copper ions are then chelated by oxalate (late stages of decay), rendering them insoluble and inactivated. With the copper ions chelated by oxalate, the organism has overcome this preventative measure and is capable of active breakdown of the treated wood. If it is assumed that ATPase expression indicates the adaptation phase in a copper rich environment certain inferences can be made for each isolate. L-11659-SP seems to have adapted by week 6 based on the decrease in ATPase expression by week 6 (Fig. 4). FP-90848-T seems to have adapted by week 8 as a result of the decrease in ATPase expression by week 8 (Fig. 4). TFFH 294 has not adapted by week 8 indicated by the spike in ATPase expression at week 8 (Fig. 4). Also, L-9414-SP has yet to adapt due to the continuous increase in ATPase expression over the 8 week period (Fig. 4).

Interestingly, the data represented here could indicate the possibility of two distinct mechanisms for decay of copper treated wood in these four isolates. L-11659-SP, FP-90848-T, and TFFH 294 seem to rely heavily on ATPase (Fig. 4) to pump toxic copper ions out of the cell during the early stages of decay before oxalate (Fig. 2) can be produced in large enough quantities to tie-up copper ions. However, L-9414-SP seems to wait for the oxalate production via the TCA and GLOX cycles indicated by the high expression levels seen for CS, ANTI, ICL, and GLOXDH at week 2 (Fig. 3). This could indicate that L-11659-SP, FP-90848-T, and TFFH 294 utilize the ATPase pump initially while L-9414-SP relies more on the metabolically derived oxalate to aid in the breakdown of copper-treated wood.

5. Conclusions

This research tracked the production of oxalate from expression of four key genes (CS, ANTI, ICL, GLOXDH) to detection of oxalate outside the fungal cell walls in four *F. radiculosa* isolates. The isolates showed variation in oxalate production and gene expression when compared to each other throughout the course of the study, which could be credited to numerous factors: adaptability, environment, growth, metabolism, and exposure time.

It has been theorized that in copper-rich environments, oxalate is necessary in order to render the toxic copper ions inert. Overall, isolates exposed to the CC treatment produced significantly higher amounts of oxalate throughout the course of the study. If oxalate is needed to detoxify copper, it would be expected that individual isolates need to continue producing oxalate until the majority of the copper ions are bound.

Metabolically, CS, ANTI, ICL, and GLOXDH feed into each other. Expression levels of these four genes are connected in individual isolates. For example, in L-9414-SP, expression levels of the four genes at week 2: CS (7.6), ANTI (9.7), ICL (21.8), and GLOXDH (42.8), increase proportionally indicating a possible connection of these genes. Based on this connection, it is likely that these four genes play a key role in the synthesis of oxalate as an adaptive mechanism when exposed to copper. From these findings, it was hypothesized oxalate biosynthesis in *F. radiculosa* is dependent upon the mitochondrial TCA cycle (CS), shunts to the glyoxysomal GLOX cycle (ANTI), moves through the GLOX cycle (ICL), and ultimately is made in the cytoplasm (GLOXDH). Thus, GLOXDH appears to be a crucial element in the direct production of oxalate.

It can also be hypothesized that the ATPase pump functions in the organisms' ability to adapt to a copper-rich environment based on the fold change differences expressed in all isolates. It is possible

to suggest the function of the ATPase pump is an important aspect of the mechanism of copper-tolerance.

In order to ultimately define the mechanism of copper-tolerance, further studies are needed to explore the differences and similarities of oxalate biosynthesis in other copper-tolerant organisms.

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