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The effects of copper proximity on oxalate production in *Fibroporia radiculosa*

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ABSTRACT

Copper remains a key component used in wood preservatives available today. However, the observed tolerance of several critical wood rotting organisms continues to be problematic. Tolerance to copper has been linked to the production and accumulation of oxalate, which precipitates copper into insoluble copper-oxalate crystals, thus inactivating copper ions. The purpose of this study was to assess differences in oxalate production and decay capacity of four wood decay fungi (three copper-tolerant and one copper-sensitive) exposed to various applications of copper. Three *Fibroporia radiculosa* isolates and one *Gloeophyllum trabeum* isolate were subjected to one formulation of copper citrate presented to the test fungi by four different treatments in Southern pine wood blocks for an eight week period. Samples were evaluated for oxalate production and weight loss every two weeks. Two of the copper-tolerant isolates evaded the inhibitory effects of all four copper treatments by week eight. The copper-sensitive organism exhibited some limitations to actively decay blocks in two of the four copper treatments. These findings suggest that proximity to copper citrate, available in any form (i.e. impregnation, direct contact, free liquid or close proximity) generally, had no negative effect on fungal growth, oxalate production, and decay capacity of the copper-tolerant organisms. Results also suggested that the copper-sensitive fungus was restricted in its ability to effectively decay wood when copper was pressure treated or directly added to the surface of wood blocks. This study also suggested that close proximity to copper alone (i.e. not pressure treated) did not completely inhibit decay of the copper-tolerant or copper-sensitive test fungi.

Keywords: brown-rot decay, copper-tolerance, oxalate, *Fibroporia*, proximity to copper

1. INTRODUCTION

Copper is the primary component used to protect wood because it exhibits a wide spectrum of biocidal properties. A major concern associated with using copper as a primary active ingredient in wood preservatives is the potential for organisms, specifically brown-rot basidiomycetes, to tolerate or successfully resist the toxic effect (DeGroot and Woodward 1999, Clausen *et al.* 2000, Munir *et al.* 2001, Green and Clausen 2005, Freeman and McIntyre 2008, Arango *et al.* 2009, Clausen and Jenkins 2011; Schilling and Inda 2011). By evolving adaptation mechanisms, some brown-rot fungi have the ability to detoxify copper in copper-treated wood, which allows them to tolerate environments containing high concentrations of copper (Gadd 1993, DeGroot and Woodward 1999, Hall 2002, Hastrup *et al.* 2005). However, tolerance is particularly variable with respect to preservative formulations, fungal species, and even fungal isolates (Hastrup *et al.* 2005, Freeman and McIntyre 2008).

There are many theories attributed to the mechanism of copper tolerance. Clausen and Green (2003) stated that the “interaction of a number of diverse factors such as growth rate, pH, oxalic acid production, and decay capacity all contribute to copper tolerance”. High extracellular accumulation of oxalate initiates the precipitation of copper into the insoluble form of oxalate (copper oxalate crystals), and renders the copper ion inert (Green and Clausen 2003). Both copper oxalate and calcium oxalate crystals are present in decayed wood treated with copper-

based preservatives (Freeman and McIntyre 2008). The formation of calcium oxalate crystals plays a crucial role in regulating the pH of the environment and facilitating the occurrence of the lower pH environments typically observed in brown-rot decay (Goodell 2003). Oxalate also functions to depolymerize cellulose over a range of pH levels from 1.5 to 2.5 (Green *et al.* 1991). Oxalate production and the rapid decrease of pH are critical for the initial stages of decay by brown-rot fungi (Bech-Andersen 1987, Green *et al.* 1991, Shimada *et al.* 1994, Green and Clausen 2003). However, oxalate production is not always directly correlated to the capability of an organism to actively decay wood (Micales and Highley 1988, Green and Clausen 2003).

The production of oxalate tends to vary with the type of copper treatment and fungal species (Arango *et al.* 2009). Most brown-rot fungi produce oxalate routinely; however, copper-tolerant fungi produce it in much greater quantities as an induced response to copper. Green and Clausen (2001) evaluated the varying degrees of copper tolerance in brown-rot organisms from various genera including *Fibroporia*, *Postia*, *Serpula*, *Meruliporia*, *Tyromyces*, *Coniophora*, *Wolfiporia*, and *Gloeophyllum*. It was determined that out of those eight genera *Gloeophyllum* was recognized as the only copper-sensitive organism. A consistent demonstration of higher levels of oxalate production and accumulation when exposed to copper has been shown in the other seven genera (DeGroot and Woodward 1999, Clausen and Green 2003, Green and Clausen 2005). Additionally, it was found that copper-tolerant fungi produce 2-17 times more oxalate in copper citrate-treated blocks compared to untreated controls (Green and Clausen 2005).

The use of copper as a primary component for the development of wood preservatives continues to prevail despite the ability of tolerant organisms to circumvent copper toxicity. It is critical to gain a better understanding of the mechanism of copper tolerance to develop techniques that are effective in stopping all forms of biological attack by copper-tolerant organisms. The purpose of this study was to evaluate decay capability and oxalate production of three copper-tolerant *Fibroporia radiculosa* isolates compared with the copper-sensitive *Gloeophyllum trabeum* isolate exposed to Southern pine blocks treated with different applications of copper citrate. Specific objectives of this project were to determine response characteristics of these four organisms to the various applications (i.e. impregnation, direct contact, free liquid, or close proximity) of copper and to gain additional information about the biological intricacies of decay by *F. radiculosa*.

2. MATERIALS AND METHODS

2.1 Fungal isolates

Three *Fibroporia radiculosa* (Peck) Gilb. & Ryvardeen strains were used in this study: L-11659-SP, FP-90848-T, and L-9414-SP (USDA Forest Products Laboratory, Madison, WI). The copper-sensitive fungus, *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. (MAD 617), (USDA Forest Products Laboratory, Madison, WI) was included for comparison. All test fungi were maintained on malt extract agar at 27°C.

2.2 Preservative treatment and test block preparation

Southern pine (SP) test blocks (10 mm³) were vacuumed treated with 1.2% ammoniacal copper citrate according to the AWWA E10-12 Standard (AWWA 2013). Untreated SP test blocks (10mm³) were also used for untreated controls. Both treated and untreated blocks were conditioned at 27°C and 70% relative humidity (RH) for 2 weeks. Following conditioning, all blocks were steam-sterilized for 20 minutes at 122°C prior to fungal exposure.

2.3 Soil block decay test

The decay tests were set up according to the AWPA E10-12 Standard (AWPA 2013) and incubated at 27°C and 70% RH up to 8 weeks. Copper citrate-treated SP test blocks were placed in various locations adjacent to untreated SP test blocks to determine differences in oxalate production and decay rate when exposed to the four fungi. Treatment 1 included untreated SP blocks to serve as the negative control (UN). Treatment 2 included copper citrate-treated SP blocks to serve as the positive control (CC). Treatment 3 included copper citrate-treated SP blocks placed directly on top of untreated SP blocks, which were in contact with the feeder strip (ST). Treatment 4 included 100 µl of liquid copper citrate (not pressure treated) added directly to the surface of the untreated SP blocks (LQ). Treatment 5 included untreated SP blocks that were adjacent to and in direct contact with copper citrate-treated SP blocks; the copper-treated blocks were introduced every two weeks (RP). Figure 1 shows the design set up for each of the experiments.

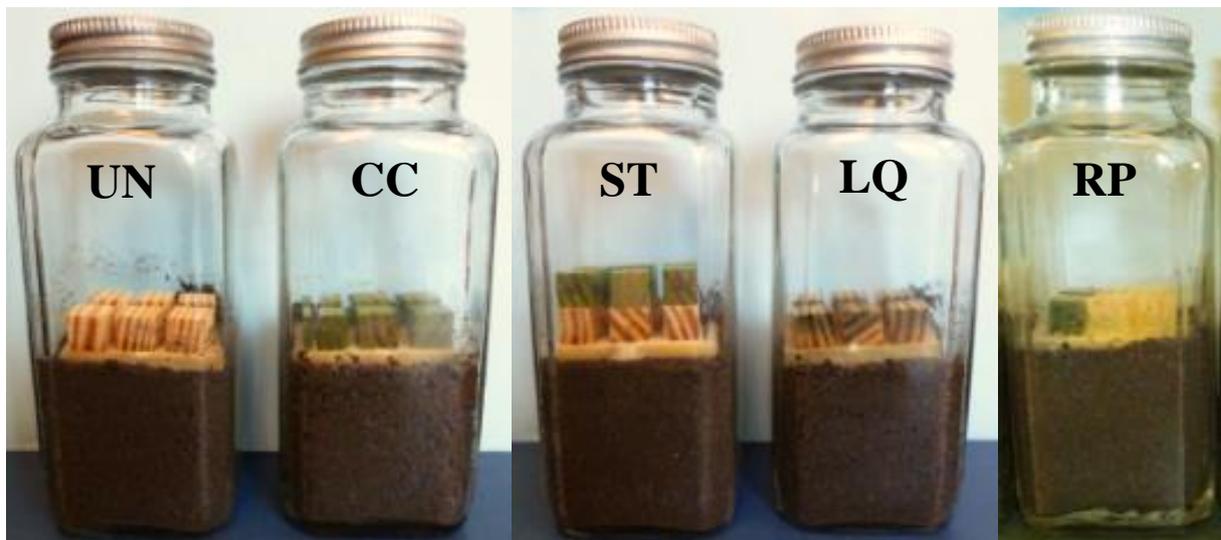


Figure 1: Variations on copper citrate-treated test blocks. Bottle 1: untreated SP (UN); Bottle 2: copper citrate-treated SP (CC); Bottle 3: copper citrate-treated SP on top of untreated SP (ST); Bottle 4: liquid copper citrate-treated SP on top of untreated SP (LQ); Bottle 5: copper citrate-treated SP adjacent to and in direct contact with untreated SP (RP).

2.4 Oxalate analysis and mass loss

Six SP blocks were removed from each experimental set-up after 2, 4, 6, and 8 weeks of incubation and were gently brushed free of surface mycelia. Blocks that were tested for oxalate production were: untreated blocks (treatment 1); copper citrate-treated blocks (treatment 2); untreated blocks (treatment 3); untreated blocks with 100 µl liquid copper citrate (treatment 4); and untreated blocks (treatment 5). Each block was placed separately in a 50 mL disposable sterile centrifuge tube containing 3 mL of 0.1M phosphate buffer (pH 7.0) and gently shaken for 2 hr. After agitation, oxalate concentration was analyzed in triplicate spectrophotometrically at 560 nm (Biotek Epoch) as specified (Trinity Biotech). After oxalate concentration was determined, test blocks were oven-dried overnight at 60°C then reconditioned at 27°C and 70% RH for two weeks before calculating weight loss.

3. RESULTS AND DISCUSSION

The overall goal of this study was to determine if various exposures to copper citrate led to differences in decay via weight loss and oxalate production of the organisms over time. In the

subsequent graphs, each organism is represented individually with respect to decay (A) and oxalate production (B) over time. Results for the copper-tolerant *F. radiculosa* isolates, L-11659-SP, FP-90848-T, and L-9414-SP, are shown in Figures 2, 3, and 4, respectively. Results for the copper-sensitive *G. trabeum* isolate, MAD 617, are shown in Figure 5. Both decay rate and oxalate concentration values shown in the graphs are the average of six biological replicates. Generally, decay increased with respect to time for all test organisms for all treatments except MAD 617 exposed to the CC treatment. There was no apparent increasing or decreasing trend of oxalate production with respect to time for all organisms for all treatments. Similar trends seen in these graphs were also found when the ratio of oxalate (mg) to mass loss (mg) was determined for these organisms (graphs not shown).

L-11659-SP exhibited a much lower decay rate than the other two *F. radiculosa* isolates. When exposed to the three variations of the copper citrate treatment (ST, LQ, and RP), L-11659-SP showed greater weight loss than the UN and CC controls (Figure 2A). Oxalate production increased over time when this isolate was exposed to the ST and RP treatments (Figure 2B). However, when it was exposed to the LQ treatment, oxalate production remained steady after week 4. When exposed to the UN control, oxalate production remained constant for the four sampling time points, but increased through week 4 and was followed by reduced oxalate production at weeks 6 and 8 when the fungus was exposed to the CC control.

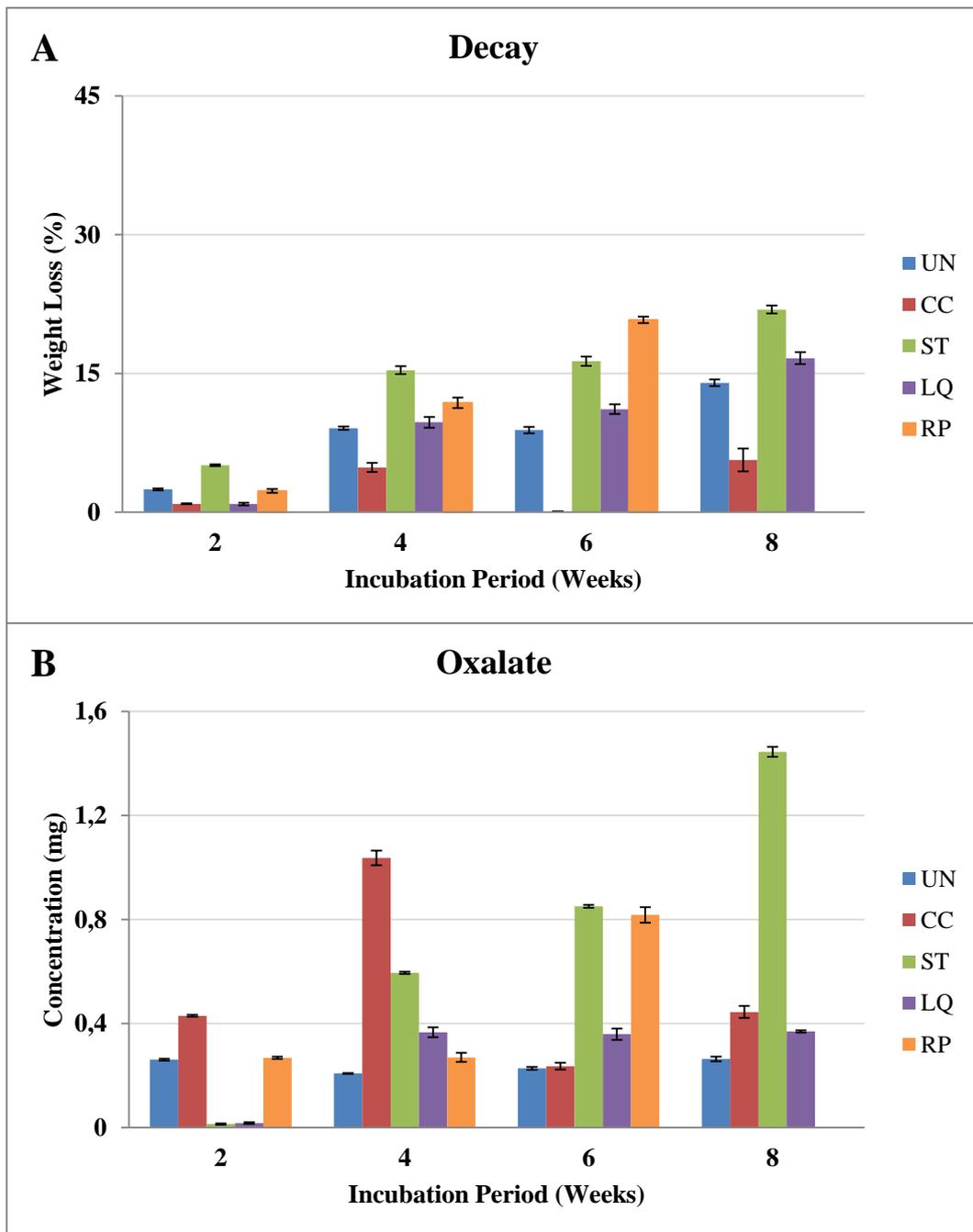


Figure 2: Decay in % weight loss (A) and oxalate concentration in milligrams (B) of copper-tolerant *F. radiculosa* isolate L-11659-SP exposed to untreated blocks (UN) in blue, vacuum treated copper citrate blocks (CC) in red, vacuum treated copper citrate blocks stacked directly on top of untreated blocks (ST) in green, 100 μ l of liquid copper citrate added directly to the surface of untreated blocks (LQ) in purple, and untreated blocks adjacent to and in direct contact with vacuum treated copper citrate blocks that were introduced every two weeks (RP) in orange. Bars represent the average of six biological replicates.

FP-90848-T exhibited slightly lower decay for all treatments by week 8 compared to L-9414-SP. When exposed to the three copper variations (ST, LQ, and RP), FP-90848-T demonstrated higher decay than when the fungus was exposed to the CC control, and generally correlated to the decay seen when exposed to the UN control (Figure 3A). It is important to note that this isolate accumulated much higher oxalate (greater than 4 mg) than the other two *F. radiculosa* isolates (less than 1.6 mg). Oxalate production increased through week 6 when the fungus was exposed to the ST, LQ, and RP treatments (Figure 3B). Highest oxalate production was seen at

week 6 when the fungus was exposed to all treatments, which was followed by a reduced production of oxalate at week 8 when the fungus was exposed to all treatments except the CC controls, which remained constant.

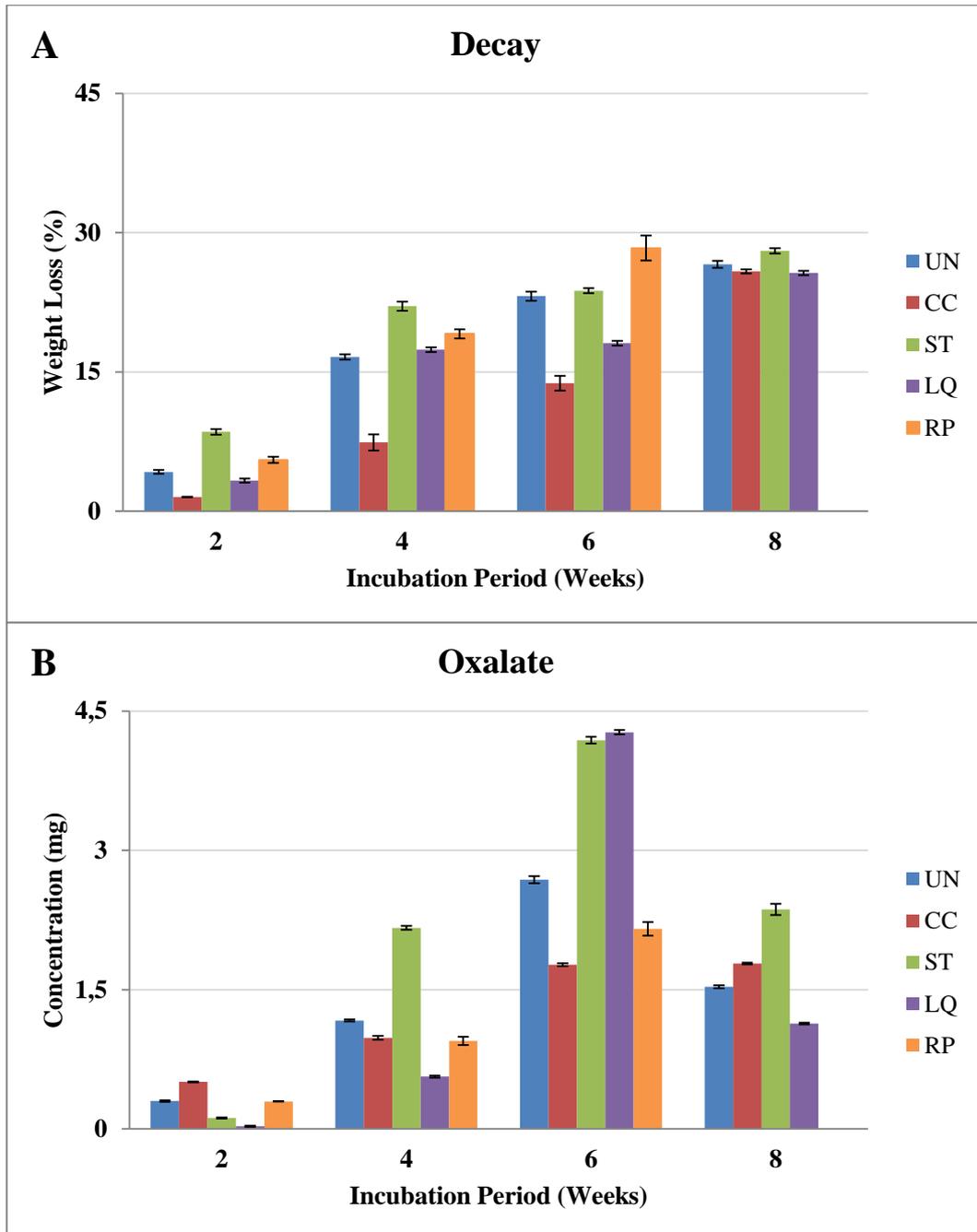


Figure 3: Decay in % weight loss (A) and oxalate concentration in milligrams (B) of copper-tolerant *F. radiculosa* isolate FP-90848-T exposed to untreated blocks (UN) in blue, vacuum treated copper citrate blocks (CC) in red, vacuum treated copper citrate blocks stacked directly on top of untreated blocks (ST) in green, 100 μ l of liquid copper citrate added directly to the surface of untreated blocks (LQ) in purple, and untreated blocks adjacent to and in direct contact with vacuum treated copper citrate blocks that were introduced every two weeks (RP) in orange. Bars represent the average of six biological replicates.

L-9414-SP was the *F. radiculosa* isolate that exhibited the highest decay. Like FP-90848-T, when L-9414-SP was exposed to the three copper variations (ST, LQ, and RP), higher decay was demonstrated compared to the CC control. The decay seen on the ST, LQ, and RP also correlated

to the decay seen when the fungus was exposed to the UN control (Figure 4A). Overall, oxalate production increased through week 6 then either dropped or remained constant by week 8 when the fungus was exposed to all treatments (Figure 4B). Generally, highest oxalate production was seen at week 6 when the fungus was exposed to all treatments with the exception of exposure to the CC controls, which remained constant after week 4.

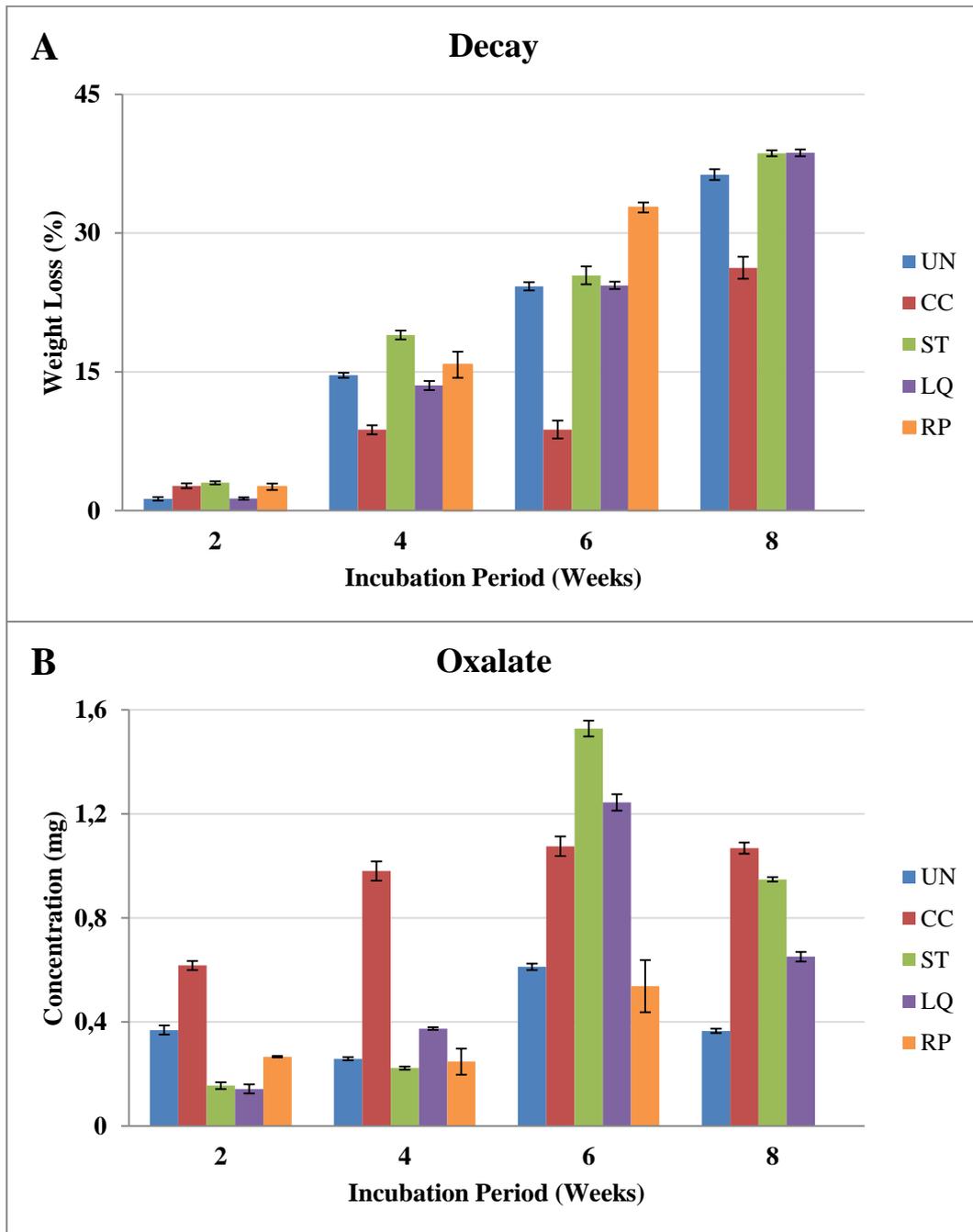


Figure 4: Decay in % weight loss (A) and oxalate concentration in milligrams (B) of copper-tolerant *F. radiculosa* isolate L-9414-SP exposed to untreated blocks (UN) in blue, vacuum treated copper citrate blocks (CC) in red, vacuum treated copper citrate blocks stacked directly on top of untreated blocks (ST) in green, 100 μ l of liquid copper citrate added directly to the surface of untreated blocks (LQ) in purple, and untreated blocks adjacent to and in direct contact with vacuum treated copper citrate blocks that were introduced every two weeks (RP) in orange. Bars represent the average of six biological replicates.

As expected, MAD 617 was unable to actively decay the CC treatment over the 8-wk period (Figure 5A).

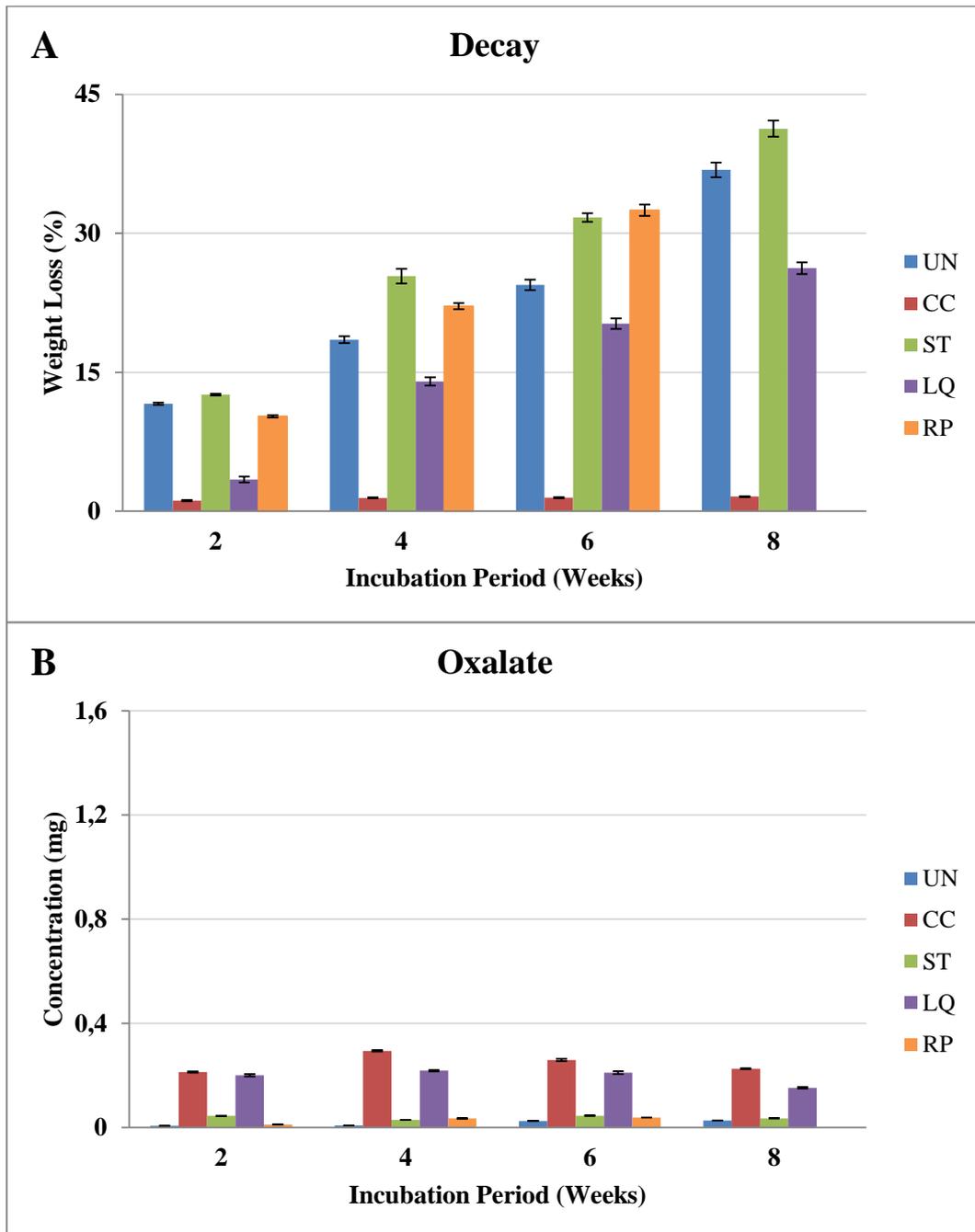


Figure 5: Decay in % weight loss (A) and oxalate concentration in milligrams (B) of copper-sensitive *G. trabeum* isolate MAD 617 exposed to untreated blocks (UN) in blue, vacuum treated copper citrate blocks (CC) in red, vacuum treated copper citrate blocks stacked directly on top of untreated blocks (ST) in green, 100 μ l of liquid copper citrate added directly to the surface of untreated blocks (LQ) in purple, and untreated blocks adjacent to and in direct contact with vacuum treated copper citrate blocks that were introduced every two weeks (RP) in orange. Bars represent the average of six biological replicates.

Interestingly, when *G. trabeum* was exposed to the LQ treatment, MAD 617 showed some capability of active breakdown, but was somewhat inhibited by the liquid copper addition to the test blocks. Exposure of MAD 617 to the ST and RP treatments generally matched the decay seen in the UN controls over the course of the study. When compared to the three *F. radiculosa*

isolates, MAD 617 produced much lower quantities of oxalate when the fungus was exposed to all treatments (Figure 5B). However, when the fungus exposed to the CC and LQ treatments, oxalate production in MAD 617 was higher than when the fungus was exposed to the UN control for all the sampling time points in spite of the lower weight loss observed.

Differences in the three *F. radiculosa* isolates to efficiently decay wood treated with the various copper treatments could be indicative of an individual isolate's metabolic response to a particular environment. For example, L-1159-SP did not surpass 22% weight loss when exposed to any of the five treatments, while FP-90848-T and L-9414-SP caused approximately 30% weight loss by week 8. The same holds true for oxalate production in these three organisms. FP-90848-T accumulated much greater quantities of oxalate than L-11659-SP and L-9414-SP. Additionally; there were no apparent similarities in oxalate production between the five treatments over time for these three copper-tolerant isolates. These trends (or lack thereof) suggest that each isolate's metabolism is unique to a particular environment, time frame, and ability to adapt to a certain situation.

4. CONCLUSIONS

Three isolates of *Fibroporia radiculosa* and one isolate of *Gloeophyllum trabeum* varied in decay and oxalate production when compared to one another over the course of this study. This could be credited to a number of factors including adaptability, metabolism, growth, and exposure time. The results of this experiment suggest that each individual organism is capable of its own unique metabolic breakdown process (i.e. dissimilar metabolism), which is reflected in the different decay rates and quantity of oxalate produced between the four organisms. The different trends found between the test fungi could be attributed to the metabolism of the specific fungal strain and its ability to initiate specific mechanisms (i.e. adaptation) to overcome an unfavourable environment. The different responses of the four test fungi suggest variation in active growth of the individual organism in response to the copper-rich environment. It is probable that the ability of the test fungi to grow and adapt to a particular environment plays an important role in how effectively they could initiate decay during exposure to the different copper treatments used in this study.

This study was designed to evaluate differences in the response of *F. radiculosa* isolates to different applications of copper citrate. Results suggest that FP-90848-T and L-9414-SP circumvented the inhibitory effects of all copper citrate treatments by week 8, which was demonstrated by the approximately 30% decay rate. However L-11659-SP, oxalate production fluctuated between weeks and decay was below 25% by week 8, which suggested this organism had yet to adapt to the copper-rich environment under the conditions of this study. Copper sensitivity was reflected in *G. trabeum* MAD 617 when it was cultured in direct contact with vacuum treated copper citrate (CC) SP blocks. Interestingly, MAD 617 demonstrated limited production of oxalate when it was exposed to the CC (less than 0.30 mg) and LQ (less than 0.22 mg) treatments over the course of the study. However, the decay capability was non-existent on CC (less than 2%) and reduced on LQ (less than 26%) treatments. These results confirm that copper-sensitive *G. trabeum* is limited in its ability to successfully decay SP blocks that are vacuum treated with copper citrate (CC) or when it is directly added to the surface (LQ) of wood samples. These results also suggest that *G. trabeum* uses a completely separate mechanism to initiate decay (i.e. different from the typical brown-rot mechanism). Proximity to copper in the other treatments used in this study (ST, LQ, and RP) generally had no negative effect on fungal growth, oxalate production, or decay capacity for all four test fungi. This could indicate that close proximity of copper does not affect the decay capability of copper-tolerant and copper-sensitive organisms.

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