The Effects of Copper in Large-Scale Single-Fungus and Dual-Fungi Wood Systems

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Abstract

Copper sulfate can be utilized to stimulate fungal pigment production for commercial spalting applications. This research explored two larger-scale uses for fungus stimulation by copper: ''drawing'' exclusion areas on sugar maple by surface application of copper sulfate and the use of copper sulfate in dual-fungi inoculation systems to increase the number of colors and types of spalting produced. Sugar maple boards treated with 2 mL of 0.13 percent copper sulfate in an S pattern and inoculated with *Xylaria polymorpha* showed a distinct area of clear wood, followed by thick dark zone lines and then by a general black pigmentation expanding outward from the S. Sugar maple blocks treated with 0.06 kg/m^3 copper sulfate and inoculated with *X. polymorpha*/*Arthrographis cuboidea* had fewer black zone lines than the control blocks but more pink zone lines and internal pink stain.

Unlike most types of character wood (bird's eye, ribbon figure, burls, knots, etc.), spalted wood has the potential to be produced on a large scale and mass marketed to the public. Although recent shifts in consumer preference for decorative wood (Nicholls 2002, Donovan and Nicholls 2003) have led to an increase in market availability for most types of character wood, spalted wood products are rarely available from specialty lumber retailers. Most of these retailers market spalted wood when they receive it, and there does not currently appear to be any sort of large-scale manufacturing of spalted wood (Loyalist Forest Products Inc. 2009, Bell Forest Products 2010).

Spalting is the process by which fungi color wood. The mechanisms vary but can be loosely broken down into the bleaching action caused by white rot fungi, pigmentation caused by growth of pigmented fungal hyphae colonization, and zone line formation from inter- and intrafungal antagonism. Spalting can occur on external surfaces or internally within wood, and different spalting fungi can be utilized based on the type and area of color desired. There are currently seven different species of fungus noted for their reliable spalting behavior: *Xylaria polymorpha* (Pers.) Grev, *Trametes versicolor* (L.) Lloyd, *Bjerkandera adusta* (Willd.) P. Karst, *Polyporus brumalis* (Pers.) Fr., *Arthrographis cuboidea* (Sacc. & Ellis) Sigler, *Chlorociboria aeruginascens* (Nyl.) Kanouse, and *Ceratocystis virescens* (R. W. Davidson) C. Moreau (Blanchette et al. 1992; Robinson et al. 2007, 2009b).

The recent increase in consumer interest in spalted wood has prompted research into its formation and possible commercial production methodology (Phillips 1987, Robinson et al. 2007, Robinson et al. 2010). Early research focused on the production of zone lines via interspecific antagonism between two different fungal isolates (Robinson et al. 2007). Most of the pairings in this experiment were between white rot fungi, which when inoculated on a sterile substrate, produce only bleaching. However, in the case of *T. versicolor* and *B. adusta* or *P. brumalis,* zone lines formed on intercolony contact. Blocks spalted under this method usually contained one well-defined zone line at the area of contact and bleaching throughout the rest of the block. Although incubation time was not long (8 to 10 wk), the number of zone lines produced was limited.

Research in the area of spalting stimulation found that certain fungi, especially *X. polymorpha,* increased production of black pigment when grown in the presence of sublethal copper sulfate concentrations (Robinson et al. 2010). The stimulation effect was remarkably strong when test blocks were incubated in soil, with zone line production occurring only on blocks pressure treated with copper sulfate pentahydrate retentions between 0.5 and 2.0 kg/m^3

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(0.31 to 1.28 kg/m^3 copper sulfate). *X. polymorpha* produced its signature zone lines only on blocks treated to the aforementioned retentions; the other blocks, including the controls, did not contain zone lines. These findings were particularly interesting since vermiculite is the preferred medium for laboratory spalting incubation (Robinson et al. 2009b), but soil is commonly used both in laboratory testing (American Wood Protection Association 2009) and by do-it-yourself woodworkers. In addition, blocks inoculated with *X. polymorpha* did not produce zone lines when incubated in soil unless exposed to the aforementioned range of copper sulfate. This research also found that pigment production by *A. cuboidea* on sugar maple, which generally produces pink stain, can produce blue stain (an actual blue pigment, in contrast to the dark melanin produced by blue-stain fungi in the *Ceratocystis* and *Ophiostoma* genera) in the presence of copper sulfate (Robinson et al. 2010). Pigment production was stimulated on sugar maple blocks treated with 0.1 kg/m^3 copper sulfate pentahydrate (0.639 kg/m^3 copper sulfate) and inoculated with *A. cuboidea*.

Chemical stimulation of fungal pigment production is related to the detoxification mechanisms of the fungus. Some fungi produce more pigment in the presence of certain metals in an attempt to bind the metals, thereby causing their detoxification (Gadd and Griffiths 1980, Caesar-Tonthat et al. 1995). The functional groups on melanin (hydroxyl, amine, carboxyl, etc.) provide numerous binding sites for metal ions, although the binding capacity of these melanins varies depending on the metal (Fogarty and Tobin 1996). Copper has an especially high capacity to bind with fungal melanin because of its ability to bond with most of the functional groups present (Saiz-Jiminez and Shafizadeh 1984).

The research described in this article sought to develop methods using previous spalting research results that were anticipated to create products to be manufactured and marketed by the wood products industry. Several methods were evaluated. Topical treatments of copper sulfate were utilized to ''draw'' images on wood boards inoculated with *X. polymorpha*. As *X. polymorpha* will not produce zone lines on sugar maple when incubated in soil unless the wood had been treated with 0.31 to 1.28 kg/m^3 copper sulfate, this technique could produce zone lines that loosely follow the topical application areas, with zone lines decreasing in intensity as the copper diffused through the wood. Ideally, this would allow the designer to draw areas of exclusion on wood boards and create designated spalted areas based on the preference of the consumer.

In addition, pigment change and zone line stimulation were attempted by treating wood blocks with copper sulfate and subjecting them to dual-fungi inoculation. Maintaining multiple pure fungus cultures can be problematic for small wood-producing companies. Many may not have adequate space for sterile storage or have the equipment necessary for large culture maintenance. It is therefore important that one or two fungi can be utilized to produce a wide variety of colors and types of spalting. Based on previous research with dual-fungi interactions (Robinson et al. 2007) and zone line/pigment stimulation results from recent testing (Robinson et al. 2010), it was our hope that the interspecific antagonism response between the dual isolates would be stimulated in the presence of copper, creating an increased number of zone lines and the potential for multiple pigments from the interaction of only two fungi.

Experimental

Surface exclusions

Sugar maple (*Acer saccharum* Marsh.) logs were obtained from Houghton County, Michigan, in June 2007. Boards cut from the logs had an average 12 percent moisture content (MC) density of 728 kg/m^3. Eight boards (15.24 by 8.89 by 1.91 cm) were equilibrated to 12 percent MC in a humidity- and temperature-controlled room (21°C ± 1°C, 65% ± 10% relative humidity) and then sterilized for 35 minutes in an autoclave prior to treatment.

A plastic tub (32-liter capacity) was surface disinfected with a mixture of 80 percent ethanol and 10 percent bleach. It was then filled with 8.32 kg of oven-dried forest topsoil that had been autoclaved for 30 minutes and 26.61 liters of distilled water. This amount of water wetted the soil to 95 percent of its water-holding capacity, which is the ideal for spalting tests (Robinson et al. 2009b). The distilled water was boiled for 30 minutes prior to being poured onto the sterile soil.

Before being buried in the soil, the boards were placed in a sterile laminar flow hood and treated with a 0.13 percent solution of copper sulfate (a 0.2% solution of copper[II] sulfate pentahydrate [98+%] dissolved in distilled water) on only one face. The solution was mixed to provide a target retention of 1.0 kg/m^3 copper sulfate pentahydrate if applied using a pressure-treating cylinder. Two milliliters of solution was placed on the boards with a pipette in the shape of the letter S, with the size of the letter maximized for the size of the board. The boards sat for 2 hours in the sterile hood until the wood had absorbed all of the solution. They were then stacked in three columns, with three boards in two columns and two boards in the final column. Boards were stacked with the broad face toward the bottom of the tub and with the opposite face in contact with the next board. Treated faces were always oriented toward the lid so that no two treated faces were against one another. The contents of an entire 95 by 15-mm Petri plate containing actively growing *X. polymorpha* SR001 (Table 1) on 2 percent malt agar, with colonies completely covering the surface of the plate, were sandwiched between each board, with an additional plate placed on the top and bottom of each column. Colonies were applied intact on their slab of agar. All broad faces of the boards were in direct contact with the mycelium.

The boards were buried in the soil so that the top board was just covered by the substrate. A surface-disinfected lid was placed on the top of the tub, which was then incubated in a humidity- and temperature-controlled room (27°C ± 2°C, 80% ± 5% relative humidity) for 10 weeks. After incubation, the boards were removed from the tub, scrubbed free of soil and mycelium, and dried in a forced-air dryer.

Dual inoculations

The dual-inoculation tests followed the procedure described in Robinson et al. (2007). Sugar maple was cut into 37-mm cubes and placed in pint Mason jars containing 20 g of vermiculite and 67 mL of water. The sugar maple cubes were inoculated on one transverse face with one fungus and on the opposite end with a different fungus and oriented within the jars so that the vermiculite just covered the surface of each block, with the two transverse faces toward the sides of the jar. Blocks were incubated for 8 to 20 weeks

Table 1.—Fungi utilized in surface exclusion and dual-inoculation testing (type of spalting produced by each fungus is noted in parentheses).

Fungus	Location	Collector	Substrate/host
Xylaria polymorpha SR001 (zone lines, white rot)	Alberta, MI	S. C. Robinson; held in culture at Michigan Technological University	*Acer saccharum*
X. polymorpha SR010 (zone lines, white rot)	Alberta, MI	S. C. Robinson; held in culture at Michigan Technological University	*A. saccharum*
Trametes versicolor SR003 (white rot, zone lines)	Houghton, MI	S. C. Robinson; held in culture at Michigan Technological University	*A. saccharum*
Ceratocystis virescens C252 (blue stain)	Mantle, NY	D. Houston; held in culture by Dr. T. Harrington	*A. saccharum*
Arthrographis cuboidea ELS-1 (pink stain, blue stain)	Memphis, TN	E. L. Schmidt; held in culture by the Northern Research Station, USDA Forest Service, and Michigan Technological University	*Quercus* sp.
Bjerkandera adusta FP-101236-Sp (white rot)	Oneida, WI	F. F. Lombard; held in culture by the Northern Research Station, USDA Forest Service, and Michigan Technological University	*Populus* sp.
Polyporus brumalis FP-102443-Sp (white rot)	Baraga, MI	T. J. Volk; held in culture by the Northern Research Station, USDA Forest Service, and Michigan Technological University	*Betula papyrifera*

and then removed, scrubbed free of vermiculite and mycelium, and analyzed for external and internal spalting.

A total of 100 blocks were tested. Before inoculation, 20 blocks were pressure treated with 6.39×10^{-6}, 6.39×10^{-4}, 6.39×10^{-3}, or 6.39×10^{-2} copper sulfate (0.00001, 0.001, 0.01, or 0.1 kg/m^3 copper sulfate pentahydrate, respectively; a solution of copper[II] sulfate pentahydrate [98+%] dissolved in distilled water) with 20 blocks left as untreated controls. Each retention for each pairing contained five replicates.

The pairings consisted of the following: *T. versicolor* × *P. brumalis* (10 wk of incubation), *T. versicolor* × *B. adusta* (8 wk), *X. polymorpha* (SR010) × *A. cuboidea* (20 wk), and *X. polymorpha* (SR010) × *C. virescens* (20 wk). Additional information on the test fungi can be found in Table 1.

For external spalting analysis, the longitudinal face with the greatest amount of zone lines and/or pigmentation was scanned at 2,400 dpi. After external analysis, the blocks were cut in half, and the internal radial face with the most spalting was scanned. Color analysis was performed using Scion Image software, as outlined in Robinson et al. (2009a).

Analysis

Surface exclusion boards were visually evaluated based on presence or absence of all or part of an S outline on the board and the presence of zone lines. Boards that contained zone lines on an untreated face or that did not display an area of fungal exclusion generally conforming to the area treated with copper sulfate were considered failures.

After a visual evaluation, the boards were put through a surface planer to determine penetration depth of the zone lines. The boards were glued end to end on a piece of scrap lumber and fed through a Delta 15-inch 62-175 planer. The planer removed surface wood in 1-mm increments. Boards were continually run through the planer until no zone lines remained on any of the surfaces.

Dual-inoculation data were analyzed using a one-way analysis of variance (ANOVA) performed using SAS software, version 9.2 (SAS Institute Inc. 2009), for Windows, with copper retention as the treatment. If differences were found, a Tukey honestly significant difference test was run. All percentage data were transformed before analysis using arcsine square root to meet ANOVA assumptions of normality and variance of the error term.

Results and Discussion

Surface exclusions

X. polymorpha produced zone lines on all the wood surfaces treated with copper sulfate. Opposite faces did not contain zone lines, while zone lines occurred on the board edges only if the copper sulfate dripped over the broad face during treatment. Although the solution tended to stray from the intended S-shape during application, parts of the original treatment area were still visible on the boards as areas of uncolonized wood, surrounded by thick, well-defined zone lines (Fig. 1). Areas of diffused black pigmentation often surrounded the zone lines, with color intensity decreasing with increasing distance from the line itself.

The diffused areas of black pigmentation most likely followed the diffusion or flow of the copper sulfate into the wood. As the concentration of copper sulfate decreased from the point of treatment, so too did the zone lines. This response to copper sulfate is not surprising, as *X. polymorpha* is known to produce zone lines on blocks incubated in soil only when in the presence of copper sulfate (Robinson et al. 2010). The higher-intensity black pigment found at the treatment point was probably caused by *X. polymorpha* producing melanin to sequester the copper from the wood. Hence, as the amount of copper decreased, so too did the intensity of the zone line.

The zone lines persisted up to 8 mm into the wood on six of the eight boards, although the intended design was no longer distinguishable after 3 mm of wood had been removed. The zone lines produced by *X. polymorpha* typically do not penetrate deeply into the wood surface, so this amount of penetration was expected (Robinson et al. 2010). Spalting from these types of decorative surface methods needs to progress only deep enough into the wood that it will not be removed when the wood is planed or sanded for finishing.

Dual inoculations

White rot combinations.—Neither *T. versicolor*/*B. adusta* nor *T. versicolor*/*P. brumalis* produced zone lines on treated blocks; zone lines were produced only on the controls. Although both these combinations are known to produce

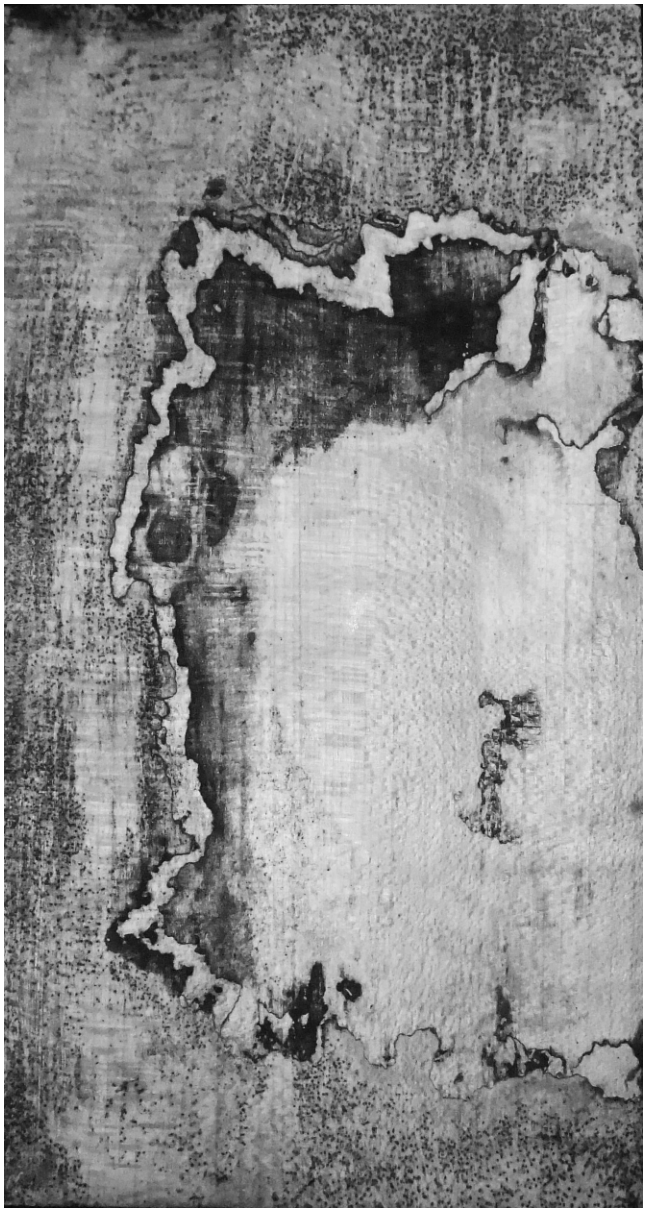

Figure 1.—Sugar maple board treated with a 13 percent solution of copper sulfate and inoculated with Xylaria polymorpha *SR001 showing treatment pattern.*

zone lines when paired together, these results suggest that copper sulfate interferes with the zone line–producing antagonism between the paired fungi.

Zone line production.—Previous research on *X. polymorpha* found that external zone line production was stimulated on sugar maple treated to 6.39×10^{-4} and 6.39×10^{-3} kg/m^3 copper sulfate (0.001 and 0.01 kg/m^3 copper sulfate pentahydrate, respectively; Robinson et al. 2010). This stimulation effect was observed with two separate *X. polymorpha* isolates when incubated in vermiculite. However, in both dual-inoculation tests, zone line stimulation by *X. polymorpha* did not occur at any retention (although zone lines still did occur), and zone line production was statistically significantly reduced at 6.39×10^{-2} kg/m^3 copper sulfate ($\alpha = 0.05$).

The reduction and lack of stimulation of zone lines on *A. cuboidea*–inoculated blocks is most likely due to the quicker growth rate of *A. cuboidea*. Malt agar plates inoculated with this fungus were fully colonized within 3 days, whereas plates inoculated with *X. polymorpha* required 10 days to completely colonize. It is probable that *A. cuboidea* quickly colonized the wood surface once inoculated, gaining first access to the resources. *X. polymorpha*, which typically does not penetrate deeply into the wood, then had limited resources to utilize. It is also possible that *A. cuboidea* detoxified the wood as it colonized, as none of the copper sulfate retentions affected the amount of pigment produced by this fungus. If the copper were bound within the pigment as the colonization progressed, there would not have been much (if any) left to stimulate zone line production in *X. polymorpha* once it reached the same area.

Surprisingly, external pink zone lines occurred on *X. polymorpha*/*A. cuboidea* at 6.39×10^{-3} kg/m^3 copper sulfate (Fig. 2). The zone lines did not occur at any other retention, and the difference between retentions was statistically significant. Previous tests with *A. cuboidea* and copper did not produce any zone lines (Robinson et al. 2010), although since a different *A. cuboidea* isolate was utilized, it is unknown whether the pink zone line production is an artifact of a dual inoculation in the presence of copper or whether this isolate would form pink zone lines in a single-inoculation test.

Pigment production.—*A. cuboidea* produced a pink stain that penetrated into the entire block regardless of copper level. Previous testing of *A. cuboidea* in the presence of copper noted that primarily blue stain was produced by this fungus instead of the usual pink stain (Robinson et al. 2010). Again, as different isolates were utilized between these two tests, it is not possible to determine whether the pigmentation difference was due to the dual inoculations or to isolate differences.

Internal pink stain by *A. cuboidea* was highest at 6.39×10^{-2} kg/m^3 copper sulfate (Fig. 3), although the differences were not statistically significant. However, this is the same retention level that produced pink zone lines and signifi-

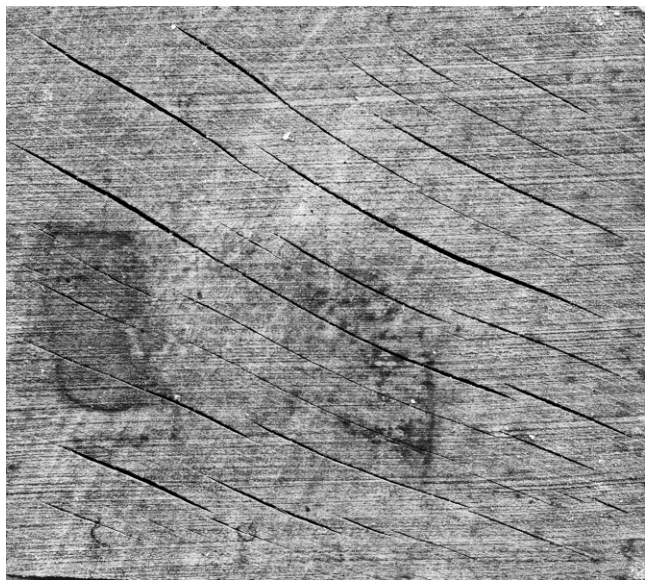

Figure 2.—External pink zone lines on Xylaria polymorpha/Arthrographis cuboidea–*inoculated blocks treated with 6.39 $\times$ 10^{-2} kg/m^3 copper sulfate.*

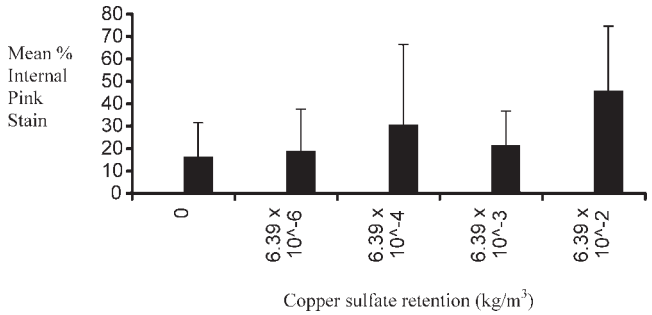

Figure 3.—Mean percent internal pink stain in sugar maple blocks inoculated with Xylaria polymorpha/Arthrographis cuboidea. *Error bars indicate one standard deviation.*

cantly inhibited black zone line formation in *X. polymorpha*. This same retention stimulated pigment production of *A. cuboidea* ELS-1 from the single-inoculation study with copper sulfate (Robinson 2011). This retention level appears to stimulate pigment production by both isolates, while the concurrent inoculation of *X. polymorpha* may play a role in the production of pink pigment instead of blue and the production of pink zone lines.

The response of *C. virescens* in the dual-inoculation tests was similar to its response in the former single-inoculation tests. In both sets of tests, *C. virescens* was not stimulated by copper sulfate (vermiculite incubation) regardless of retention, and blue pigment production was inhibited at 6.39×10^{-2} kg/m^3. Despite the lack of stimulation however, an interesting colonization pattern developed on *X. polymorpha*/*C. virescens* blocks: blue stain occurred only in small areas bound by *X. polymorpha* zone lines (Fig. 4). This effect was not due to copper presence, as it occurred on treated and untreated blocks.

The reason for the unusual blue stain colonization is unknown. It is possible that the white rot produced by *X. polymorpha* was more heavily concentrated in those areas, creating an altered wood structure that was favorable to *C. virescens* colonization. This type of pretreatment effect has been shown to help pigment-producing fungi colonize wood (Robinson and Laks 2010). No blue coloration was produced by copper oxidation.

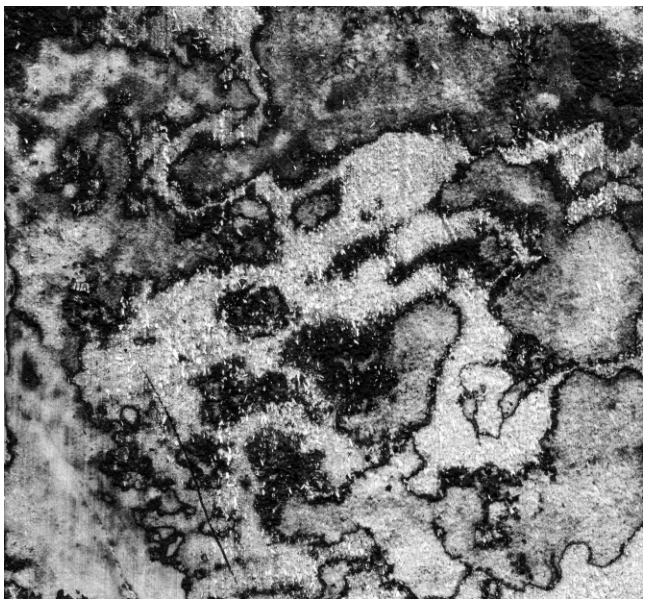

Figure 4.—Sugar maple block inoculated with Xylaria polymorpha/Ceratocystis virescens. *Blue stain from* C. virescens *is contained with areas enclosed by* X. polymorpha *zone lines.*

Conclusion

Results from these tests indicate that copper sulfate can be successfully utilized to alter pigment production by spalting fungi. Surface treatment of sugar maple with a 0.13 percent solution of copper sulfate produced a clear zone of inhibition, followed by thick, dark zone lines that diffused outward away from the treatment center. Zone lines occurred only in areas where copper had spread and penetrated up to 8 mm into the wood. This type of topical treatment, combined with a relatively short incubation time, should provide a quick, reproducible method for directing zone line production in *X. polymorpha*. This directed zone line production should enable drawing on sugar maple lumber, a procedure that would produce specific designs within the spalted area.

In addition to drawing, copper sulfate may also be utilized in dual-fungi systems to change types and colors of pigmentation. For the fungal pairing of *X. polymorpha*/*A. cuboidea,* external pink streaking, similar to zone line formation, occurred only at 6.39×10^{-2} kg/m^3 copper sulfate (0.1 kg/m^3 copper sulfate pentahydrate), and internal amounts of pink stain were also stimulated at this retention. Black zone lines produced by *X. polymorpha* were also present at this retention. It is possible that the pink pigment and pink streak stimulation are due to the presence of *X. polymorpha* on the blocks, as previous research with *A. cuboidea* and copper sulfate found that *A. cuboidea* produces a blue pigment in the presence of copper and does not produce zone lines (Robinson et al. 2010). Utilizing copper-treated wood in a dual-inoculation system with *X. polymorpha*/*A. cuboidea* produces two different colors of zone lines and a penetrating pink stain. The multiple colors of zone lines and pink pigment provide three different spalting effects with the use of only two fungi.

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