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Wood species affects laboratory colonization rates of *Chlorociboria* sp.

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A B S T R A C T

This research attempted to determine conditions and wood species required to maximize xylindein production from a *Chlorociboria* sp. under laboratory conditions for use in induced spalting. A pure culture of a *Chlorociboria* species was isolated from fruiting bodies and grown on 2% malt extract agar. The colonies reached a maximum diameter of 18 mm in four weeks, although the blue-green xylindein pigment continued to diffuse through the culture media for at least eight months. In vermiculite jar tests, this *Chlorociboria* isolate colonized *Populus tremuloides* more heavily than *Acer saccharum* or *Betula alleghaniensis*, and did not colonize *Tilia americana* at all. Pre-inoculation of test blocks with white rot fungi did not significantly affect xylindein production, regardless of wood species.

1. Introduction

Chlorociboria spp. are green stain ascomycete fungi found throughout North America (Dixon, 1975). They produce small, blue-green disc-like apothecia on hardwood logs, although the fruiting bodies can be difficult to locate. Wood stained by *Chlorociboria* is abundant and can be found in many hardwood stands, commonly on wood that has already been colonized by white rot (Johnston and Park, 2005).

Only two species of *Chlorociboria* occur in North America: *Chlorociboria aeruginascens* (Nyl.) Kanouse and *Chlorociboria aeruginosa* (Oeder) Seaver. The species are primarily differentiated by spore size, with the ascospores of *C. aeruginascens* ranging from 5 to 7 μm and those of *C. aeruginosa* ranging from 8 to 14 μm (Dixon, 1975). In the literature, differences in growth rate and pigmentation between the two species are not noted, and it appears that the species are essentially identical in most aspects. As such, green stained wood is often identified as being colonized by *Chlorociboria* spp., and not by a specific species.

The unique blue-green pigment produced by *Chlorociboria* spp. is xylindein, which has been extensively researched (Blackburn et al., 1965; Edwards and Kale, 1965; Giles et al., 1979, 1990; Saikawa et al. 2000). Although produced within the hyphae, xylindein often diffuses into the surrounding substrate (Berthet, 1964). This diffusion leads to a greater amount of colored wood per colonization area as compared to fungi that do not produce diffusible pigments. Hence, the diffusion of xylindein can produce a relatively large volume of pigmented substrate without extensive colonization by the fungus. Despite the research by Berthet, however, this diffusion has not been confirmed by microscopy.

Little research has been conducted on laboratory growth of either of the North American *Chlorociboria* species. *C. aeruginascens* appears to be slightly more common than *C. aeruginosa*, grows slowly on culture plates, and does not consistently produce blue-green pigment (Fenwick, 1993; Dixon, 1975). Dixon notes that *C. aeruginascens* pigment typically diffuses through culture medium well beyond the colony diameter (1975). There is currently no published information on the growth rate or xylindein diffusion ability of *C. aeruginosa*. Although both species have been isolated from numerous hardwood and softwood species, the decay ability, wood species preference, and growth rate of North American *Chlorociboria* spp. on wood is currently unknown.

Wood colonized by *Chlorociboria* sp. has been utilized for artistic purposes since the 15th century, when it was used by intarsia artists for inlay pieces that required blue-green tones, such as plants and water (Blanchette et al., 1992). Sampling of stained pieces from intarsia works found that the green-stained wood pieces were all from the *Populus* species. The stained wood is still utilized today for artistic purposes; in fact the vernacular term 'green oak' has evolved due to the predominant growth of *Chlorociboria* sp. on oak (*Quercus* spp.) in some regions (Dennis, 1956).

The green stain of *Chlorociboria* is a type of spalting. Spalting is defined as any coloration of wood due to fungal colonization

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(Robinson et al., 2007). The three types of spalting include bleaching, caused by the removal of lignin and other colored wood constituents; zone lines, caused by intra- and inter-fungal antagonism; and pigmentation, caused by a large mass of pigmented fungal hyphae concentrated in one area and/or the diffusion of that pigment into the surrounding wood. The Ohio DNR notes that maple (*Acer* spp.), birch (*Betula* spp.), and beech (*Fagus* spp.) are the three most commonly spalted woods, with spalted oak (*Quercus* spp.) rarely occurring due to the high extractive content of the wood (Ohio DNR, 2009).

Research into controlled spalting for commercial and artistic purposes has expanded within the past five years. Most research has focused on zone line formation by white rot fungi, primarily because these fungi are capable of both forming zone lines and causing bleaching (Phillips, 1987; Robinson et al., 2007). However, pigment fungi have the potential to produce a higher quality of spalted wood, as most do not consume structural wood components. Recent studies have begun investigating the use of pigment fungi for spalting applications (Robinson et al., 2009a, 2009b), although results from these studies have not shown that fungal pigments readily penetrate into wood.

This research attempts to expand current knowledge of North American *Chlorociboria* species and investigate possible stimulatory mechanisms by which these fungi might be utilized in spalting applications.

2. Materials and methods

Fruiting bodies of *Chlorociboria* sp. were obtained in September 2008 from a *Populus* sp. log in a mixed hardwood forest in Keweenaw County, MI. The culture was grown on 2% malt extract agar and maintained at room temperature (22 °C). The culture was transferred to fresh agar plates every three months to maintain active growth.

Kiln-dried wood from four tree species was chosen for inoculation: sugar maple (*Acer saccharum* Marsh.) (12% SG = 0.66, 12% MC density = 710 kg/m^{-3}), basswood (*Tilia americana* L.) (12% SG = 0.41, 12% MC density = 430 kg/m^{-3}), paper birch (*Betula alleghaniensis* Britt.) (12% SG = 0.59, 12% MC density = 636 kg/m^{-3}), and trembling aspen (*Populus tremuloides* Michx.) (12% SG = 0.45, 12% MC density = 483 kg/m^{-3}). Sugar maple and yellow birch were chosen due to the prevalence of spalting occurring in these species (Robinson et al., 2007; Ohio DNR, 2009). Basswood was chosen due to its popularity among woodworkers as a low-density, easy-carving wood, and trembling aspen was chosen due to the test culture having been isolated from a *Populus* spp. wood. Although xylindein is commonly found in oak wood, this species was not utilized in this experiment due to its high extractive content and therefore its lack of potential to be utilized with any other spalting fungus.

Fifteen 14 mm cubes of each wood type were oven dried at 40 °C for 24 h and weighed. Blocks were steam sterilized for 30 min and then placed in vermiculite jars (4 × 4 × 12 cm flint jars), which were prepared as described in Robinson et al. (2009b). Jars were autoclaved for 30 min prior to block placement. The blocks were placed in the jars so that the vermiculite just covered the surface of the block. A strip of inoculum roughly 2 × 2 cm was placed on the top of the block and then covered with an aspen feeder strip measuring approximately 3 × 28 × 34 mm. A total of 45 jars were prepared, and each jar contained only one block.

Fifteen blocks were utilized per wood species. Five were pre-inoculated with *Trametes versicolor*, 5 with *Xylaria polymorpha*, and 5 were inoculated directly with *Chlorociboria* sp.

2.1. Pre-inoculation

Five blocks from each wood species were pre-inoculated with *T. versicolor* (L.) Lloyd SR003 and an additional five blocks from each were pre-inoculated with *X. polymorpha* (Pers.) Grev. SR001. *T. versicolor* was isolated from an *A. saccharum* log in Houghton Co., MI. *X. polymorpha* was isolated from an *A. saccharum* log in Baraga Co., MI. The pre-inoculated jars were incubated for eight weeks in a humidity and temperature-controlled room (27 °C ± 2 °C, 80% ± 5% relative humidity). After incubation, the jars were autoclaved for 35 min, allowed to cool, and then reinoculated with the *Chlorociboria* isolate. Jars that were not pre-inoculated were inoculated with *Chlorociboria* along with sterilized pre-inoculation jars. All 45 jars were then incubated for 24 weeks under the same conditions outlined above.

2.2. Analysis

After incubation, the blocks were removed from the jars, washed to remove any adhering mycelium and vermiculite, dried overnight, and then weighed to determine weight loss. After drying, the transverse face with the most green stain was scanned at 2400 dpi using an Epson Perfection V100 photo scanner. Blocks were then cut in half to expose a radial face, then scanned again for internal spalting. Spalting amounts (amount of visible pigmentation per block) were analyzed using the procedure outlined in Robinson et al. (2009a). All data were analyzed using a two-way analysis of variance (ANOVA) with wood species and pre-treatment as independent variables. If differences were found, a Tukey HSD was run to determine the location of the significant difference. Percentage data were transformed using arcsine square root to meet ANOVA assumptions of error term variance and normality. All statistical procedures were performed using SAS, version 9.2 of the SAS (2009) system for Windows.

3. Results

Growth of the *Chlorociboria* isolate on agar plates was slow, with colonies never exceeding 18 mm in diameter regardless of incubation time or plate size (Fig. 1). Xylindein diffusion into the culture media began after approximately four weeks of incubation, and continued up to four months, with colony size not increasing after four weeks of incubation. The cultures never completely covered the surface of the plate, however xylindein diffused throughout an entire 60 × 15 mm plate by eight months.

Of the wood species tested, the *Chlorociboria* isolate colonized all but basswood (Table 1), with significantly more colonization on aspen than on any other wood species ($\alpha = 0.05$). No weight loss occurred with any wood species. Birch was lightly colonized with only three of the fifteen blocks showing any amount of external green stain – one from the *X. polymorpha* pre-treatment group (3.5% green surface area) and two from the control group (3.5% and 7%). Of those three blocks, only the one from the pre-treatment group had internal green stain (5%).

The sugar maple blocks as a group were colonized moderately well by the *Chlorociboria* isolate. The control group and *T. versicolor* pre-treatment group had almost the same amount of external green stain (around 2.5% ± 4.75% SD). External green stain occurred much more frequently in the *X. polymorpha* pretreated blocks, which had an average of 6.79 ± 4.45% surface area covered by green stain. However, the green stain did not penetrate internally into the blocks.

The *Chlorociboria* isolate was most successful at colonizing the aspen blocks (Fig. 2, Fig. 3). Blocks pretreated with *T. versicolor* were most heavily stained (17.98 ± 15.01% SD external green and 8.34 ± 10.66% SD internal green). There was more external green stain on the control blocks than the *X. polymorpha* pre-treatment blocks, and of the two, only the control blocks exhibited any internal green stain. None of the pre-treatment results were statistically significant.

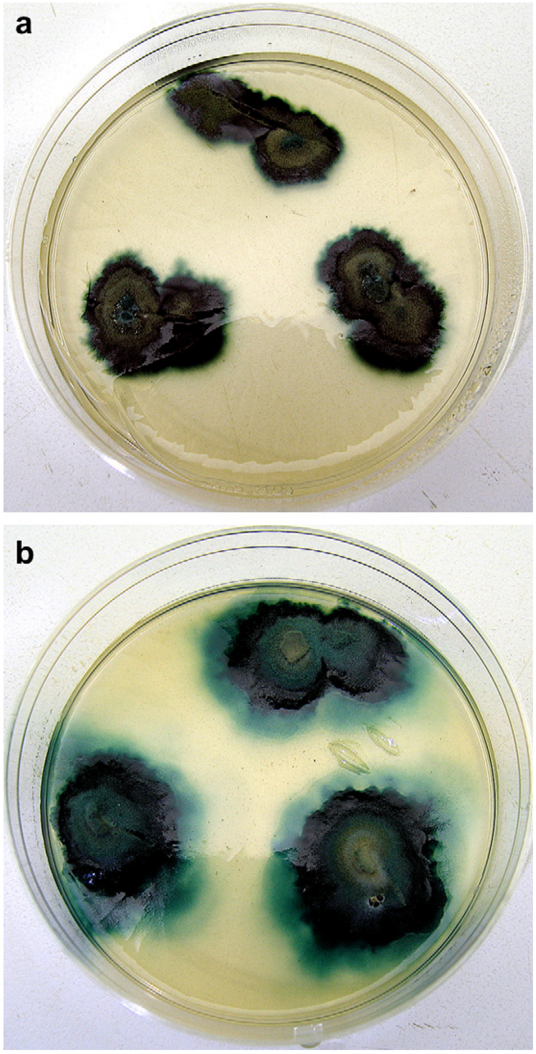

Fig. 1. *Chlorociboria* isolate growth on 2% malt agar plates at (A) four weeks and (B) four months (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

4. Discussion

Although the diameter growth of the *Chlorociboria* isolate was small compared to other pigment fungi, the growth rate of this isolate was far quicker than Fenwick's (1993) isolates, which grew to their maximum diameter of 5 mm in four weeks and failed to produce xylindein.

The *Chlorociboria* isolate produced significantly more external green stain on aspen than on any other wood species. This result is not surprising, as the *Chlorociboria* species isolated from 15th century intarsia works were all from *Populus* sp. (Blanchette et al., 1992) and the culture used in these experiments was also isolated from aspen. Noting the slow growth of our isolate, it is possible that this fungus preferentially colonizes low-density wood species with low extractive levels. *Populus* sp. have very low decay resistance (USDA Forest Service, 1967), making them perhaps more attractive as a colonizable substrate. Sugar maple also has a low decay resistance, however its higher specific gravity (0.63 compared to 0.38 in aspen) may have played a role in the failure of the *Chlorociboria* isolate to colonize internally.

Table 1
Number of green stained blocks per treatment per wood species. Total number of blocks tested per wood species is 15; total number of blocks tested per treatment is 5. * indicates statistically significant differences between species at ($\alpha = 0.05$).

Wood Species	Pre-treatment	Total # Spalted	# Externally Spalted	# Internally Spalted
Aspen*	*T. versicolor*	4	4	3
	X. polymorpha	2	1	0
	None	3	3	1
Sugar Maple	*T. versicolor*	1	1	0
	X. polymorpha	4	4	0
	None	2	2	0
Yellow Birch	*T. versicolor*	0	0	0
	X. polymorpha	1	1	1
	None	2	2	0
Basswood	*T. versicolor*	0	0	0
	X. polymorpha	0	0	0
	None	0	0	0

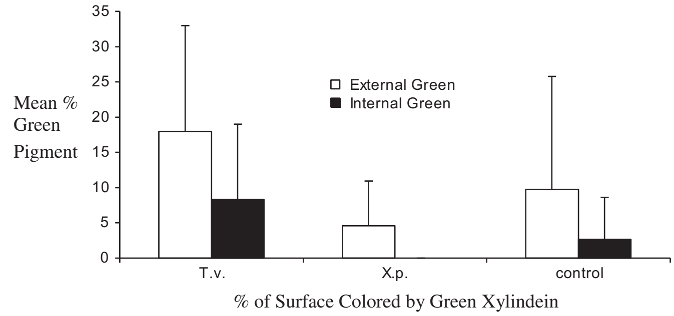

Fig. 2. External and internal colonization of the *Chlorociboria* isolate on aspen blocks (T.v = *Trametes versicolor*, X.p. = *Xylaria polymorpha*). Error bars represent one standard deviation.

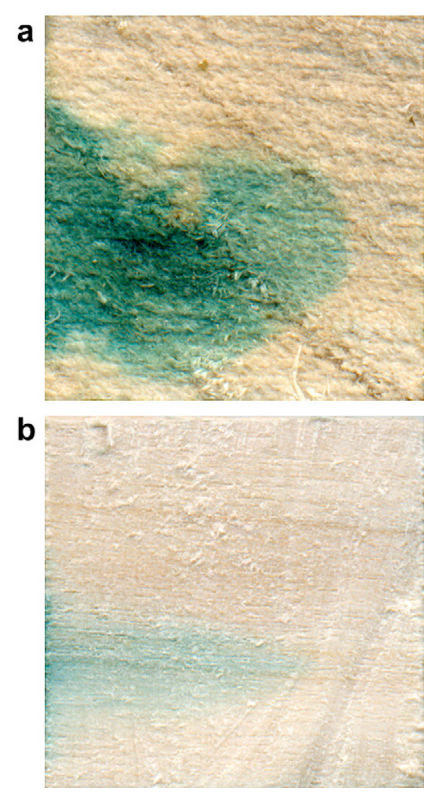

Fig. 3. External (A) and internal (B) colonization of the *Chlorociboria* isolate on non-pretreated aspen blocks (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

It is unknown why the *Chlorociboria* isolate failed to colonize basswood, and barely colonized birch. Neither of these species is particularly decay resistant, and both have specific gravities comparable to maple and aspen, respectively (basswood SG: 0.37, yellow birch SG: 0.62). All four of the wood species used are diffuse porous hardwoods with similar pore sizes. In addition, aspen and basswood have very similar chemical compositions (Petterson, 1984).

This research suggests that pre-treatment of wood by a white-rot fungus probably does not increase the colonization rates of *Chlorociboria* sp. However, although not statistically significant, pre-treatment of sugar maple by *X. polymorpha* resulted in a four percent increase in external green stain over the control amount, and pre-treatment of aspen by *T. versicolor* caused an eight percent increase. This pre-treatment effect may be due to the decay of lignin and cellulose by white rot fungi. The removal of these two wood components 'opens up' the matrix of the wood, which may facilitate faster or additional avenues for xylindein diffusion or mycelial colonization. The increased colonization may also be due to changes in block moisture content after autoclaving.

No weight loss was caused by the *Chlorociboria* isolate regardless of wood species. Although Johnston and Park (2005) proposed that *Chlorociboria* spp. may cause white rot due to its prevalence on decayed wood, it is apparent from this research that our isolate did not cause decay.

Based upon the results outlined above, it appears that *Chlorociboria* is an ideal fungus for laboratory spalting under certain conditions. Unlike some other pigment fungi whose hyphae must penetrate the substrate to affect a color change (*Ceratocystis* spp. in particular), *Chlorociboria* sp. needs only to colonize the surface, owing to the diffusion of xylindein throughout the wood substrate. Although our *Chlorociboria* isolate preferentially colonized *P. tremuloides*, pre-treatment of test blocks with a white rot fungus and/or autoclaving may allow standard laboratory spalting tests on sugar maple to be conducted once the wood has been conditioned. There is currently no other tested spalting fungus that can produce the strong blue-green color of xylindein, and the use of this pigment in spalting applications should provide an excellent addition to the known spalting fungi.

5. Conclusions

The *Chlorociboria* isolate is a slow-growing fungus that reaches its maximum colony diameter at four weeks. However, xylindein appears to continuously diffuse from the hyphae after colony growth has subsided, making deep wood colonization of this fungus unnecessary to achieve a penetrating stain. This research suggests *Chlorociboria* sp. may preferentially colonize *Populus* species over other hardwoods, including others of low extractive content and low specific gravity. Although pre-treatment of the wood species with white rot fungi did not significantly impact *Chlorociboria* colonization, a small stimulatory effect did occur with some wood species. Future research on *Chlorociboria* species should continue to investigate the possible effects of white rot pre-treatment in the colonization rates of *Chlorociboria* under laboratory conditions.

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