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Utilizing pigment-producing fungi to add commercial value to American beech (*Fagus grandifolia*)

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Abstract American beech (*Fagus grandifolia*) is an abundant, underutilized tree in certain areas of North America, and methods to increase its market value are of considerable interest. This research utilized pigment-producing fungi to induce color in American beech to potentially establish its use as a decorative wood. Wood samples were inoculated with *Trametes versicolor*, *Xylaria polymorpha*, *Inonotus hispidus*, and *Arthrographis cuboidea* to induce fungal pigmentation. Black pigmentation (*T. versicolor*, *X. polymorpha*, *I. hispidus*) was sporadic, occurred primarily on the surfaces of the heartwood, but not internally. Pink pigmentation (*A. cuboidea*) occurred throughout all of the tested beech samples, but was difficult to see in the heartwood due to the darker color of the wood. To increase the visibility of the pink stain, beech blocks were pretreated with *T. versicolor* for 4 weeks before being inoculated with *A. cuboidea*. This method significantly increased the saturation of the pink stain on both beech heartwood and sapwood, creating coloration similar to that found on sugar maple. This value-adding process should be particularly effective for small-scale wood pigmentation, and should help establish a market for this currently underutilized wood species.

Keywords *Arthrographis cuboidea* · *Fagus grandifolia* · *Inonotus hispidus* · Spalting · *Trametes* · *versicolor* · *Xylaria polymorpha*

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Introduction

Pigments produced by fungi are routinely utilized as colorants for both foodstuffs and materials. Some of the more prominently utilized fungal pigments come from the water-soluble orange/red pigments produced by *Monascus* spp., frequently utilized in rice wines in eastern countries (De Carvalho et al. 2003). Pigments from *Monascus purpureus* Piedallu are used in wool dying (De Santis et al. 2005), and an anthraquinone pigment isolated from *Penicillium oxalicum* Currie & Thom is currently being developed for use as a 'natural' food additive that may have some anticancer effects (Dufossé et al. 2005; Mapari et al. 2005).

There is a long history of use of fungal-pigmented wood to add color to decorative wood products (Blanchette et al. 1992); however, the purposeful introduction of pigment-producing fungi to wood for the purpose of creating a value-added product is relatively new (Robinson et al. 2007a; Robinson and Laks 2010a; Robinson and Laks 2010b). Wood portraying this type of color (spalting) is becoming an increasingly popular specialty product. Several market surveys have noted a recent shift in consumer preferences towards nonuniform wood products (Donovan and Nicholls 2003; Kozak et al. 2004), and spalted wood continues to be sold at a high markup by mills and lumber stores, regardless of the wood species containing the spalting (Bell Forest Products 2011). Spalting is an ideal process for adding value to denser hardwoods as such species can withstand substantial fungal colonization without a significant loss in machinability (Robinson et al. 2007b).

Spalting occurs in two main forms. The first is formation of zone lines, which are usually lines of dark melanin, produced extracellularly by fungi in an effort to protect their resources from another fungus (Boddy 2000). A single

fungus may also produce zone lines in an attempt to delineate individuals within its own mass, or as a response to external factors such as changes in moisture content (Rayner and Todd 1978; Sharland et al. 1986; Lopez-Real and Swift 1975). Another type of dark melanin, produced by species within the *Ophiostoma* and *Ceratocystis* genera causes a penetrating dark blue/black stain instead of delineating lines (Davidson 1935; Dowding 1969; Gibbs 1993; Hedgcock 1906; Miller and Goodell 1981; Seifert 1993; Zink and Fengel 1988).

The second type of pigmentation results from lower molecular weight pigments (not melanin) that penetrate the wood when released extracellularly. The release of pigments into the wood may be an attempt by a fungus to protect its territory (Schmidt and Dietz 1985; Sigler et al., 1990). In particular, various naphthoquinone pigments have been shown to be antagonistic towards some insects, bacteria, other fungi, and yeast (Durán et al. 2002). These types of pigments, such as those produced by *Chlorociboria* sp. (Saikawa et al. 2000; Sakaki et al. 2002) and *Arthrographis cuboidea* (Golinski et al. 1995) differ from pigments produced by surface mold fungi such as *Trichoderma* sp. and *Fusarium* sp. (Chidester 1940; Schmidt and Dietz 1985; Vesonder and Golinski 1989), which rarely penetrate the wood surface (Campbell 1959; Scheffer and Lindgren 1940).

Numerous hardwood species have been tested under controlled laboratory conditions against reliable pigment-producing fungi. Of the wood species tested, sugar maple (*Acer saccharum* Marsh) appears to appeal to a broad range of fungi, while most other wood species are preferentially colonized by either basidiomycete decay fungi or fungi that produce extracellular pigment (Robinson et al. 2011a). Several wood species, including basswood *Tilia americana* L. and yellow birch *Betula alleghaniensis* Britt., appear to be unsatisfactory hosts for common spalting fungi, displaying weight loss from fungal colonization without the associated pigmentation (Robinson and Laks 2010a; Robinson and Laks 2010b).

Due to the increase in value associated with spalted wood, efforts have recently been undertaken to utilize the process on low value hardwoods. Of particular interest in southeastern Canada is the potential for spalting American beech (*Fagus grandifolia* Ehrh.). This tree is native throughout the central and northeastern parts of North America, although it is also commonly found in urban areas as a planted tree. Lumber from American beech has few uses and has little commercial value due to a relatively high volumetric shrinkage (Stevens 1963). However, American beech is a dense hardwood (SG ~0.64) suitable for furniture, flooring, and other applications if properly processed (Vlosky and Chance 2000). American beech is readily available but currently underutilized in North America, making it an ideal wood for value-added processes.

This research attempted to identify fungi capable of pigmenting beech, utilizing established spalting fungi. Color visibility was a concern during this testing due to the high proportion of dark heartwood in beech. To address this potential issue, methods were also investigated in which white rot decay fungi were utilized to lighten the natural color of beech in order to heighten saturation and clarity of fungal pigments.

Materials and methods

Initial fungus trials

American beech from Ontario, Canada was cut into 14-mm cubes and separated into two groups based upon a visual assessment of heartwood and sapwood. The first set contained only lighter wood, presumed to be predominately sapwood (average oven dry SG=0.82), and the second set contained blocks with at least 70% darker wood, presumed to be predominately heartwood (average oven dry SG=0.89). Prior to inoculation, blocks were dried overnight in a forced air dryer (40 °C) and weighed. Blocks were then placed in vermiculite spalting jars and inoculated as described in Robinson et al. (2009b). Three blocks were placed in each jar and three jars were inoculated per fungus per wood type (total=72 blocks). An additional set of blocks was prepared with sugar maple sapwood, harvested from Ontario, Canada, to serve as controls in terms of standard pigment production with the utilized fungi.

The blocks were inoculated as monocultures within the jars. The following fungi were utilized in the experiment: *Trametes versicolor* (L.) Lloyd (UAMH11521; isolated from *A. saccharum* in Houghton, MI, USA; held in culture by the University of Alberta), *Xylaria polymorpha* (Pers.) Grev. (UAMH11520; isolated from *A. saccharum* in Alberta, MI, USA; held in culture by the University of Alberta), *A. cuboidea* (Sacc. & Ellis) Sigler (ELS-1; isolated from *Quercus* sp. in Memphis, TN, USA; held in culture by the US Forest Service Forest Products Lab in Madison, Wisconsin), and *Inonotus hispidus* (Bull.) P. Karst (F2037; isolation data unknown; held in culture by the University of Toronto). Prior to use, all fungi were grown and maintained at room temperature on 2% malt agar plates (95×15 mm Petri plates).

Incubation time for jars was variable depending on fungus species (incubated in humidity and temperature controlled chamber: 27 °C±2 °C, 80%±5% relative humidity). Jars inoculated with *T. versicolor* were incubated for 8 weeks, jars incubated with *A. cuboidea* and *X.*

polymorpha were incubated for 10 weeks, and jars inoculated with *I. hispidus* were incubated for 12 weeks. After incubation, the blocks were removed from the jars, scrubbed with a soft-bristled brush to remove residual vermiculite and mycelium, weighed to determine moisture content, and then dried overnight in a forced air dryer (40 °C). Dry blocks were weighed to determine weight loss, and then scanned on an Epson V100 photo scanner at 1,200 dpi. The face of the block with the most spalting was scanned. After external scanning, blocks were cut in half to expose an internal radial face. One internal face was also scanned. Scanned images were evaluated using Scion Image Software as described by Robinson et al. (2009a).

Increasing visual clarity

Based upon the results from the initial fungus trials, a second set of testing was established to enhance the visibility of the pink stain produced by *A. cuboidea*. American beech cubes (37 mm) were cut from a board obtained in Ontario, Canada (average SG=0.74). Blocks were randomized before selection without consideration of heartwood/sapwood content. Blocks were placed, one block per jar, in vermiculite spalting jars and inoculated as described by Robinson et al. (2007a).

T. versicolor SR003 was utilized as a pretreatment in an attempt to lighten the color of the beech wood prior to colonization by *A. cuboidea* ELS-1. Three different methods were attempted, with ten replicates per method. In the first method, blocks were dual inoculated with *T. versicolor* on one transverse face, and *A. cuboidea* on the alternate transverse face at the same time. These jars were incubated for 10 weeks.

For the second and third methods, the beech blocks were preinoculated with *T. versicolor* on one transverse face. Jars were then sealed and placed in humidity and temperature-controlled room for 4 weeks. After 4 weeks, the second set was autoclaved for 35 min to ensure sterilization, allowed to cool overnight in a laminar flow hood, and then inoculated on the opposite transverse face with *A. cuboidea.* The third set of jars was not autoclaved before inoculation with *A. cuboidea.* After secondary inoculations, sets two and three were incubated for 10 weeks. After incubation, blocks were processed in an identical manner to the 14-mm cubes.

Saturation analysis

All blocks colonized by *A. cuboidea* were analyzed for red saturation (a*) using CIE Lab CM-2002 by Kinko Minolta following the procedure outlined in Pandey (2005). On 14-mm cubes, the entire external and internal surface areas were measured. On the 37-mm cubes, analysis was performed on the external and internal radial planes on the place with the most pink pigment.

Nitrogen analysis

Three randomly selected blocks of both beech heartwood and sapwood were dried at 40 °C overnight, ground in a Wiley Mill with a 30-mesh screen, and analyzed for carbon and nitrogen concentrations on a Leco TrueSpec model 630-100-400 C-N analyzer. The three replicate blocks of heartwood and sapwood were homogenized (within each group) before analysis to provide a mean amount of nitrogen concentration across the board, instead of three separate nitrogen readings from different board sections. As such, replicate analysis measurements were not made.

Statistical analysis

A one-way ANOVA was run to compare pigment production of each fungus on the 14-mm cubes of beech heartwood, sapwood, and sugar maple (serving as a pigment control), followed by Tukey's HSD at α=0.05. To determine if pretreatment of blocks with *T. versicolor* increased the saturation of pink stain, an additional one-way ANOVA was performed. This test compared the CIE Lab red output of the dual inoculation cubes (dual, sterilized, and unsterilized sets) against the red output from the 14-mm cubes of beech heartwood, sapwood, and sugar maple.

Results

Initial fungus trials

Weight loss was minimal on all blocks, with the exception of those inoculated with *T. versicolor* (Table 1). Amount of zone lines produced by the tested fungi on beech (14-mm

Table 1 Mean percent weight loss in initial block testing by *Trametes versicolor*, *Inonotus hispidus*, *Arthrographis cuboidea*, and *Xylaria polymorpha* on beech sapwood and heartwood

	Trametes versicolor	*Inonotus hispidus*	*Arthrographis cuboidea*	*Xylaria polymorpha*
Beech sapwood	30±3	6±2	1±1	7±1
Beech heartwood	10±12	2±3	0±1	3±3

cubes) varied with fungus species and, with *T. versicolor*, between heartwood and sapwood sections. *T. versicolor* produced significantly more external zone lines on beech heartwood than beech sapwood or the sugar maple control (4.5±2%; $P<0.0001$). External zone lines produced by *X. polymorpha* did not differ between beech heartwood and sapwood, and both had significantly fewer zone lines than sugar maple (3.9±6.6% for heartwood, 0.5±1.7% for sapwood, 12.1±13.8% for sugar maple; $P=0.0008$). External zone lines produced by *I. hispidus* did not differ significantly between beech heartwood, sapwood, or sugar maple, with beech heartwood displaying the most zone lines at 8.3± 10.5%. Internal zone lines did not occur on beech with *T. versicolor*, *X. polymorpha*, or *I. hispidus*.

Amount of pink stain produced by *A. cuboidea* did not differ between beech heartwood and sapwood either externally or internally. However, the amount of pink pigment on beech sapwood (79.9±22.4% external, 43.5± 25.4% internal) and heartwood (58.6±19.5% external, 21.5±33.3% internal) was significantly less than the amount of pink pigment on the sugar maple controls (87.6±33.3% external, 88.9±29.7% internal; $P=0.01$ for external, $P<0.0001$ for internal).

Beech heartwood contained a higher percentage of nitrogen (0.33%) than beech sapwood (0.27%). Carbon amounts were not different, with beech heartwood containing 50.36% carbon and beech sapwood containing 50.33% carbon.

Increasing visual clarity

External and internal red saturation (a*) differed significantly by treatment ($P<0.0001$ for both, Fig. 1). Beech blocks pretreated with *T. versicolor*, sterilized, then inoculated with *A. cuboidea* had the highest amount of red saturation, and did not differ significantly from the red saturation of the sugar maple controls. Both sugar maple and the sterilized blocks' red saturations were significantly higher than those measured on the 14-mm heartwood and sapwood samples. The dual inoculation blocks showed a moderately high red saturation; however, the levels were only significantly different from the unsterilized 37-mm cubes (external only).

Fig. 1 External and internal red (a*) saturation amounts on beech and sugar maple cubes. *Different letters* indicate significant differences at $\alpha=0.05$ for each surface. $N=10$

There was a significant difference in total amounts of pink pigment on the 37-mm cubes by treatment and when compared to the control sugar maple blocks ($P<0.0001$). The sterilized 37-mm cubes (79.3±18.2%) and the sugar maple controls (87.6±33.3%) had significantly more surface area externally covered by pink pigment than did the dual-inoculated (26.5±6.7%) and unsterilized blocks (no pink stain). Internally, treatment did not appear to affect pink stain production, with the sugar maple controls having significantly more pink stain (88.9±33%) than any of the treatments (sterilized: 13.2±8.6%; dual: no pink stain; unsterilized: no pink stain).

Although not expected on the 37-mm cubes, zone lines did occur externally and differed significantly between treatment ($P<0.0001$). Zone lines only occurred on the unsterilized beech blocks with an average of 4.0±2.4%.

Discussion

Zone line development in beech proved to be difficult, and varied depending on fungus species and heartwood/sapwood placement. The basidiomycetes *T. versicolor* and *I. hispidus* produced more zone lines on beech heartwood than on sapwood or the sugar maple controls; however, this difference was only significant with *T. versicolor.* It is possible that the increase in zone line production on beech heartwood is a response of the fungus to various extractives not found in sugar maple or beech sapwood. Many basidiomycetes form interaction zones due to external stimuli (Rayner and Boddy 1978), such as the presence of other fungi or changes in moisture conditions. In addition, *T. versicolor* is well known for its ability to produce zone lines under a range of conditions (Rayner and Todd 1978; Rayner 1977). In contrast, *X. polymorpha*, an ascomycete that causes soft rot in hardwoods (Worrall et al. 1997), produced more zone lines on the sugar maple controls, and did not appear to prefer one type of beech wood to another. This fungus routinely produces zone lines without being subjected to an antagonist or adverse conditions (Campbell 1933). It has been suggested that it may utilize different pathways for pigment production (Durán et al. 2002) than basidiomycetes. The results of this study suggest that *X. polymorpha* produces zone lines under more ideal conditions (the sugar maple controls) versus the potentially more inhospitable conditions of beech sapwood, whereas *T. versicolor* and *I.*

hispidus, in monoculture systems, may require external stimuli to produce zone lines.

The difference in nitrogen amounts between the beech heartwood and sapwood may play a role in the black pigmentation primarily occurring in the heartwood on blocks inoculated with *T. versicolor*. Since nitrogen is required in the formation of melanin, the difference in zone line production between heartwood and sapwood (with no significant difference in zone line amounts with the other two zone lines producing fungi *X. polymorpha* and *I. hispidus*) may be due to different pathways utilized for melanin biosynthesis, and their different nitrogen requirements. Although relatively little is known about specific fungi and their utilized pathway for melanin synthesis, Wheeler (1983) found that, in general, ascomycetes appear to utilize the pentaketide pathway for melanin biosynthesis, and that basidiomycetes appear to produce an entirely different type of melanin than ascomycetes. This difference was recently confirmed by Babitskaya and Shcherba (2002), who also found that the basidiomycetes *Inonotus obliquus* (Ach. Ex Pers.) and *Phellinus robustus* (P. Karst.) produced melanin via the shikimate pathway. No specific information is available on the pathways utilized by *T. versicolor*, *I. hispidus*, and *X. polymorpha*. It is possible that *I. hispidus*, a basidiomycete, and *X. polymorpha*, an ascomycete, both capable of producing soft rot, utilize different melanin biosynthesis pathways than *T. versicolor*, a basidiomycete capable of very aggressive white rot. The differences in percent nitrogen may explain the increase in zone lines in beech heartwood inoculated with *T. versicolor* if this fungus does utilize a different pathway than the other two, and if such a pathway requires a higher amount of nitrogen.

No zone lines were produced internally. Although some applications exist for pigmenting the surface of wood (Robinson 2011; Robinson and Laks 2011), pigments that penetrate into the wood allow for a broader use of spalted wood products. This research suggests that American beech wood may not be suitable for controlled spalting experiments that aim to produce zone lines. However, the staining of beech with fungal pigments, specifically *A. cuboidea*, appears to be possible. When directly inoculated onto beech, the pink stain produced by *A. cuboidea* occurred both externally and internally on both heartwood and sapwood.

The saturation of the pink stain was an issue in the direct inoculation technique. The darker color of beech heartwood compared to sugar maple made the pink pigment difficult to see, and potentially not as appealing to consumers. Pretreatment of beech with *T. versicolor* alleviated this issue by bleaching the wood; however, the pretreatment conditions played a large role (Figs. 2 and 3). *A. cuboidea* and *T. versicolor* are both capable of quickly colonizing wood (Anagnost et al. 1994; Rayner 1977), although *A. cuboidea* is less effective under unsterile conditions (Robinson et al 2011b). In the pretreatment tests, blocks that were inoculated concurrently with *A. cuboidea* and *T. versicolor* showed no visual effects of bleaching, and had no differences in red saturation from the 14-mm heartwood/sapwood blocks. It appears that, when inoculated concurrently, *A. cuboidea* more effectively captures primary resources than *T. versicolor*, and is able to successfully maintain control of its resources. This can also be seen in the weight loss data, as weight loss was less than 4% for all treatments. Overall, dual inoculations of *T. versicolor* and *A. cuboidea* did not significantly alter the visual clarity of the pink stain on beech.

The treatment that proved most effective for increasing pink saturation was the sterilization method. Blocks pretreated with *T. versicolor* for 4 weeks, sterilized, and then inoculated with *A. cuboidea* showed comparable a* values to the sugar maple controls. The effect was particularly striking on internal pink pigmentation (Fig. 3). Both the sterilized and unsterilized sets showed similar colonization patterns of pretreatment, with *T. versicolor* producing white rot on roughly 25% of the internal surface of the blocks. While pink staining occurred throughout all of the sterilized beech blocks, the saturation increases occurred where *A. cuboidea* overlaid the white rot. However, *A. cuboidea* appeared only able to colonize the blocks that had been sterilized, as no pink stain was found on unsterilized blocks.

Although the sterilization treatment was effective in bringing the pink saturation of the beech wood to the same level as the sugar maple control, the use of this procedure on larger pieces of wood, such as logs, may be somewhat limited. Conditions necessary to kill *T. versicolor* would require extended time in a kiln at high temperatures. The logs would also either need to be sprayed regularly to maintain suitable moisture content for inoculation with *A. cuboidea*, or rehydrated after sterilization. Consequently, this procedure may be more economically viable if done on smaller wood pieces, such as those aimed at hobbyists, woodturners, and others who work with small niche market products. Smaller pieces of wood could be sterilized via autoclave, microwave, or even steaming/boiling, all of which are effective, accessible methods of wood sterilization.

The external zone lines present on the unsterilized blocks indicates antagonism of *T. versicolor* towards *A. cuboidea*. Since *T. versicolor* was given 4 weeks to colonize before the introduction of *A. cuboidea*, it appears that *A. cuboidea* was unable to secure enough resources to also colonize the wood, even on areas showing some evidence of white rot. However, *A. cuboidea* was able to inhibit continuing colonization by *T. versicolor*, as weight losses for unsterilized blocks after

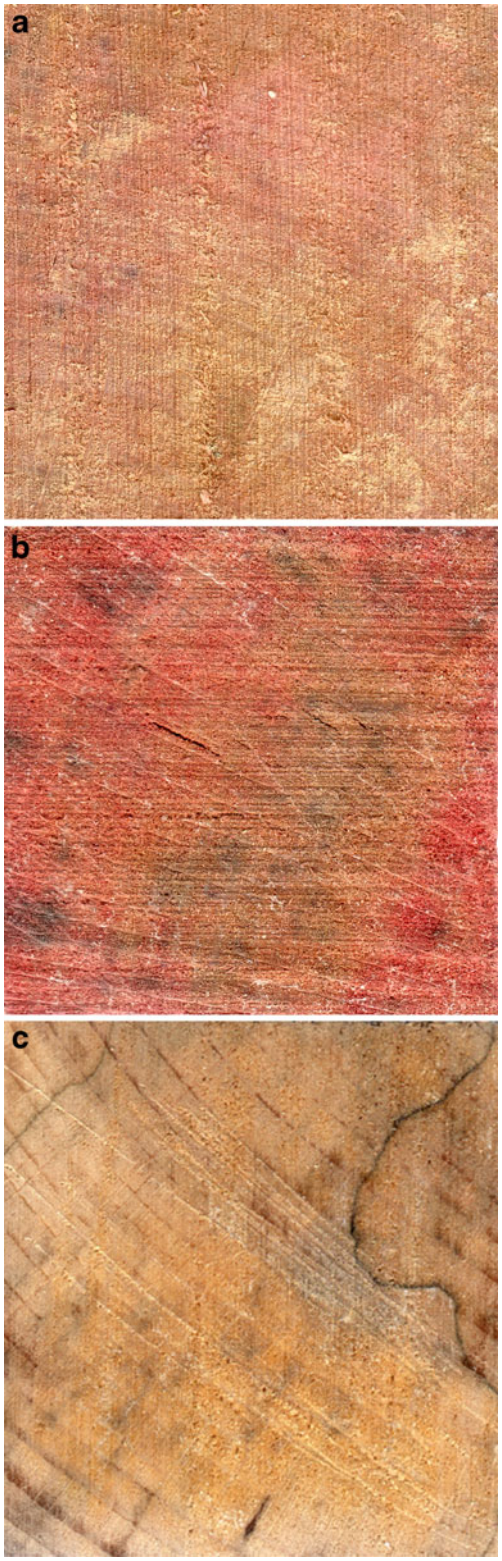

Fig. 2 External zone lines and pigmentation by **a** dual inoculation, **b** pretreatment with sterilization and **c** pretreatment without sterilization

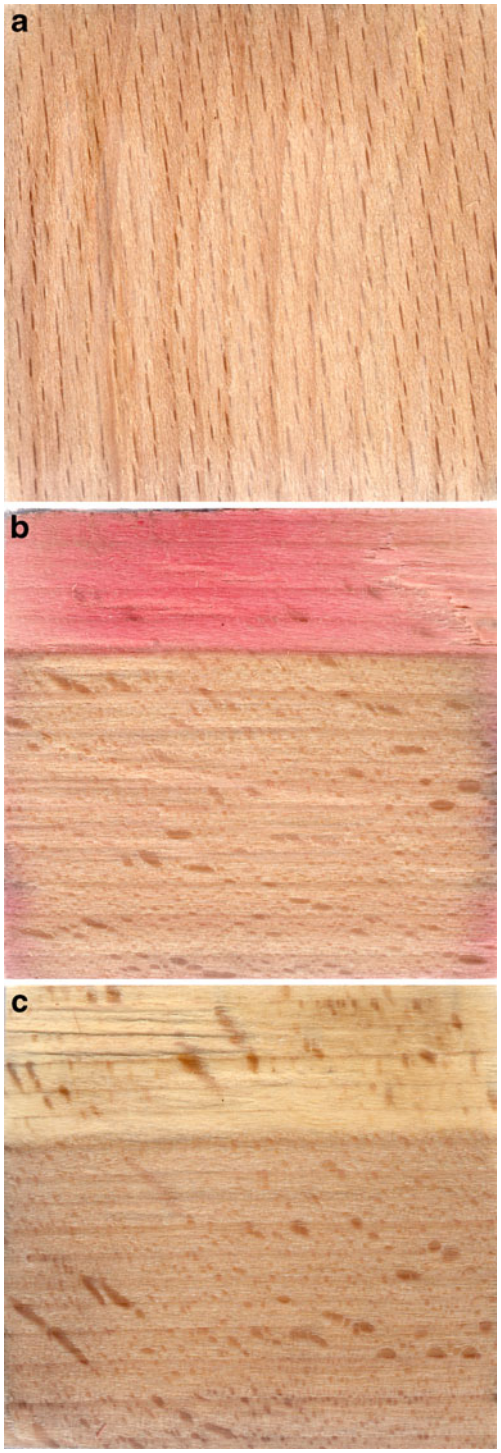

Fig. 3 Internal pigmentation by **a** dual inoculation, **b** pretreatment with sterilization and **c** pretreatment without sterilization

14 weeks of colonization were minimal. *T. versicolor* is capable of inhibiting growth of other fungi in wood systems, probably due to production of secondary metabolites as growth inhibitors (Heilmann-Clausen and Boddy 2005); hence, the presence of zone lines and lack of pink stain is not surprising in unsterilized blocks. The external zone lines indicate fungal antagonism, which is not uncommon for either *T. versicolor* (Rayner and Boddy 1978; Rayner 1977) or *A. cuboidea* (Robinson and Laks 2011). Although the

objective was to develop pink coloration, the production of zone lines when *A. cuboidea* was paired with *T. versicolor* was also beneficial, as zone lines are the most commonly marketed type of spalting (Bell Forest Products 2011; Loyalist Forest Products 2011).

Results from this research indicate that American beech may not be an ideal wood for zone line production, but is suitable for certain types of staining. Although the pink pigment produced by *A. cuboidea* can be hard to see due to the large amount and dark color of beech heartwood, pretreatment of beech with *T. versicolor*, followed by sterilization and subsequent inoculation of *A. cuboidea* can bring the pink saturation up to similar levels found on sugar maple. This process may be applicable to darker wood species as well, such as black walnut (*Juglans nigra* L.) and cherry (*Prunus serotina* Ehrh.), which, due to their darker color, would also fail to show a high saturation of fungal color. A pretreatment method, such as fungal 'bleaching' through white rot degradation, has the potential to open most wood species to pigment spalting, and thus allow for this value-added process to be applied to a much broader range of wood species.

It is important to recognize that this procedure may not be viable for large-scale spalting due to the necessity of sterilization. Due to these factors, it is recommended that beech be utilized for controlled spalting when surface pigmentation is required, when inoculated pieces are small enough to adequately sterilize, or when facilities exist (such as kilns equipped with steam) with appropriate machinery for large-scale sterilization. The process of inducing stain in sound beech wood for decorative purposes should help create a market for American beech, potentially increasing its value and use in the North American hardwood market.

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