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NOTE

Methods of inoculating *Acer* spp., *Populus tremuloides*, and *Fagus grandifolia* logs for commercial spalting applications

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Abstract One of the most promising wood value-added processes currently under development is spalting, where pigment is added to wood via fungal colonization. Previous studies have shown laboratory level spalting to be achievable and highly predictable. However, large-scale spalting for potential commercial applications introduces a substantial number of additional variables which impact the spalting process. To test the potential of commercial-scale spalting, *Acer saccharum*, *Fagus grandifolia*, and *Populus tremuloides* logs were inoculated with multiple known spalting fungi utilizing both liquid spray cultures and live dowel pin cultures. Many of the fungi that successfully produce spalting in small, sterile cultures also produced significant amounts in large logs, with many spalting patterns identical to those found in small-scale testing. Pairings of *Trametes versicolor*/*Scytalidium cuboideum* and *Xylaria polymorpha*/*Xylaria polymorpha* (different isolates) produced significant amounts of zone lines. In addition, the method of inoculation impacted the amount of spalting: more zone lines were produced when fungi were introduced via plugs, while more stain was produced when liquid cultures were sprayed onto the logs. These results indicate that many of the standard spalting fungi are suitable for large-scale applications; however, the inoculation method appears to be a vital component for successful spalting under a restricted time schedule.

Keywords *Acer saccharum* · *Fagus grandifolia* · *Populus tremuloides* · Spalting

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Introduction

Value-added wood products are increasingly relevant to the forest products industry as land managers seek to maximize gains from forested lands. The value-added process is particularly important when applied to low-value or non-commercial wood species, as it can exponentially increase the value of otherwise commercially low-value wood. One of the most promising value-added techniques currently under development is controlled spalting—a process that utilizes fungi to add permanent, penetrating pigments to wood (Fig. 1).

Although spalted wood has been used in artistic endeavors for centuries [1], its use in modern design is only beginning to materialize [2, 3]. However, there is an increasingly growing market demand for spalted wood, and the product is sold at a price premium by speciality lumber retailers. With the growing consumer interest in spalted wood, various attempts have been made to both understand the processes behind spalting and to induce the pigmentation under controlled conditions [3]. It is currently understood that the black line type spalting ('zone lines') is

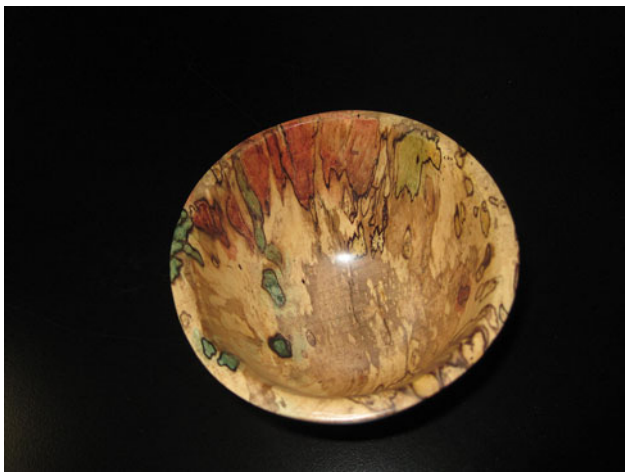

Fig. 1 Spalted bowl showing all three types of spalting—white rot, zone lines, and pigments

generally produced via inter- and/or intra-fungal antagonism, and that many of the bright pigment type spalting is due to extracellular pigments produced by the fungi as mechanisms of resource capture/protection [3]. To date, a small group of fungi have been identified as reliable pigment producers on wood, and several inoculation and incubation methods have been established.

Although there are several well-tested methods for controlled spalting of hardwoods, the methods are specific for jar or bin incubation [4, 5]. This type of incubation is not suitable for commercial spalting production, as large amounts of wood, in larger sizes, need to be spalted in order to make the process economical. Insuring sterility is an additional hurdle with large-scale spalting that is not encountered with jar spalting. Small pieces of wood, and to some extent their incubation containers, can be completely sterilized utilizing an autoclave. Autoclaving is not possible for large numbers of logs, and certainly not possible for the chambers in which these logs are incubated. The lack of sterility affects not only the initial colonization of the target fungi, but also the behavior of the target fungi once inoculated, as other fungi may be present within the log, and will affect the actions of the inoculated fungus.

Hence, there is a larger number of variables to account for when spalting large pieces of wood, especially if spalting on a commercial scale. In order to mass-produce spalted wood to meet growing consumer demands, methods must be developed that allow for quick, mass-scale inoculation of logs to produce penetrating pigments within the wood. The purpose of this research was to investigate methods for the inoculation of sugar maple (*Acer saccharum* Marsh), Norway maple (*Acer platanoides* L.), American beech (*Fagus grandifolia* Ehrh.), and aspen (*Populus tremuloides* Michx.) logs with several well-established spalting fungi, and establish log inoculation methods that (1) minimize external contamination, (2) are easy to apply to large volumes of logs, and (3) produce at least 5 % total area spalting coverage throughout the log sapwood. The applications of this research should enable lumber retailers and log yard owners to mass-induce spalting in their wood, allowing for spalted wood to become a viable value-added product for businesses across the globe.

Materials and methods

Method 1: lateral dowel pin inoculations

Twenty-nine beech (*Fagus grandifolia* Ehrh.) logs measuring approximately 91 cm tall × 20 cm in diameter were inoculated with various zone line and pigment producing fungi, incubated in a humidity (85 ± 5 % RH with steam misting five times per day for 15 min intervals) and temperature (27 ± 4 °C) controlled room for 20 weeks, and then evaluated for spalting coverage. Beech logs with intact bark were taken from a log yard in July and had been felled a week prior to pick-up. Inoculation occurred after 2 weeks storage in the incubation chamber to maintain high moisture content.

For incubation, expandable ribbed hardwood [as either maple (*Acer* spp.) or birch (*Betula* spp.)] dowel pins measuring 0.635 cm × 2.54 cm were submerged in distilled water and autoclaved for 15 min for sterility. The water was then drained from the container, the container was resealed, and the pins were allowed to cool overnight in a laminar flow hood. The sterilized pins were placed in empty Petri plates (65 × 15 mm), with roughly 25 pins per plate, all lying flat with no overlap. A roughly 5 cm^2 of 2 % malt agar with actively growing fungal mycelium [6] was placed on top of the pins, and the plate was then sealed with parafilm. The plates were incubated at room temperature, in the dark, for 1 month to insure fungal colonization before being placed in the logs.

A hand drill was used to drill holes roughly 1 cm wide by 3 cm deep into the logs. Dowels were selected with sterilized tweezers and gently tapped into the holes with a steel hammer. Holes were not sterilized prior to inoculation, and no effort was made to cover the holes once the pin was placed. Drilling was done outside a laminar flow hood.

Fungal inoculation pairings are detailed in Table 1. With the exception of one set, logs were inoculated at two locations within the log—the upper 7.6 cm and the lower 7.6 cm. Two dowel pins were placed in the log at 180° at each location, with each plug within a location having a different fungus (two fungi per log). One set of logs (3 replicates) was inoculated with three fungi: *Xylaria polymorpha* (Pers.) Grev. strain UAMH11518, *Trametes versicolor* (L.) Lloyd strain MAD697, and *Scytalidium*

Table 1 Inoculation assignments and time incubated for log testing

Test	Wood	No. of logs	Fungus 1	Fungus 2	Fungus 3	Incubation period (weeks)
Dowel	Beech	5	*T.v.* UAMH11521	*X.p.* SR015	–	20
		3	*T.v.* UAMH11521	*T.v.* MAD697	–	
		3	*X.p.* SR015	*X.p.* UAMH11518	–	
		3	*T.v.* UAMH11521	*P.b.* FP102389	–	
		3	*S.c.* UAMH11517	*X.p.* SR015	–	
		3	*S.c.* UAMH11517	*T.v.* UAMH11521	–	
		3	*X.p.* UAMH11518	*P.b.* FP102389	–	
		3	*X.p.* UAMH11518	*T.v.* MAD697	*S.c.* UAMH11517	
		3	*X.p.* SR015	–	–	
	SM	3	*T.v.* MAD697	*S.c.* UAMH11517	–	12
		4	*X.p.* UAMH11518	*S.c.* UAMH11517	–	
	NM	4	*P.b.* FP102389	*T.v.* MAD697	–	
		3	*S.c.* UAMH11517	–	–	
Spray	SM	3	*S.c.* UAMH11517 (agar)	–	–	12
		3	*S.c.* UAMH11517 (gelatin)	–	–	
		3	*X.p.* UAMH11518 (agar)	–	–	
		3	*S.c.* UAMH1502 (agar)	–	–	
	NM	3	*S.c.* UAMH11517 (agar)	–	–	
		4	No fungus, no cutting	–	–	
	SM	2	No fungus, cut	–	–	
	Aspen	3	*S.c.* UAMH11517 (agar)	–	–	
		3	*S.c.* UAMH11517 (gelatin)	–	–	
		3	*X.p.* UAMH11518 (agar)	–	–	
		4	No fungus, cut	–	–	
		3	*X.g.* UAMH10320	*S.l.* UAMH1502	–	

T.v., *Trametes versicolor*; *X.p.*, *Xylaria polymorpha*; *P.b.*, *Polyporus brumalis*; *S.c.*, *Scytalidium cuboideum*; *X.g.*, *Xylogone ganodermophtherum*; *S.l.*, *Scytalidium lignicola*; *SM* sugar maple, *NM* Norway maple

cuboideum (Sacc. & Ellis) Sigler strain UAMH11517. In this instance, the upper location contained four dowel pins, placed equidistance apart, alternating between *X. polymorpha* and *T. versicolor*. The lower location contained two equidistant pins of *S. cuboideum*.

Seven control logs each of sugar maple and Norway maple, both known to spalt well with these fungi, were run concurrently to insure there was no malfunction. Results from previous work have indicated that the performance of spalting fungi does not differ between sugar maple and Norway maple [7]. Sugar maple logs were of similar size and shape to beech logs, and were picked up and inoculated at the same time as the beech. Norway maple logs were harvested from a yard in Toronto, Ontario, and sat outside for 1 week before pick-up. The sectioned logs remained in the humidity and temperature controlled chamber for 2 weeks prior to inoculation. Bark remained intact. Logs measured approximately 40 cm in length with a maximum radius of 20 cm at the thickest point. All maple logs were incubated for 12 weeks. Beech logs were initially incubated for 12 weeks; however, when no colonization was apparent, logs were placed back into incubation for a total of 20 weeks.

After incubation, logs were removed from the chamber, checked for approximate moisture content on the top-facing transverse plane with a Protimeter Surveymaster SM (produced by GE, USA), and then allowed to air dry for 1 week. A roughly two and a half centimeter cookie was cut from each end of the log with a chainsaw, as well as a similar sized cookie from the middle of the log. Cookies were dried for 1 week in a forced air dryer (Thermo-Fisher Scientific Isotemp Oven, model 838F) set to 40 °C, and then were sanded to 80 grit on a Delta belt sander (USA) to remove surface inconsistencies. The sanded side of each cookie was scanned on a SHARP MX-M363N photocopier at 800 dpi. Images were then edited in Adobe Photoshop CS3 to remove cracks, background area, and heartwood.

Color analysis was performed using Scion Image software (Scion Corporation, USA) following the protocol established in Robinson et al. [8], where the wood was analyzed for true black, black pigments, and pink pigment utilizing the threshold function for true black and the LUT tool for the pigments. The

amount of zone lines and pink pigment found on each cookie was added together for each cookie from a given log, then divided by three to give an average zone line amount and pigment coverage for each log. As noted above, heartwood was removed from the cookie images before analysis, hence analysis numbers are based on sapwood surface area.

Method 2: liquid media inoculations

Sugar maple, Norway maple, and aspen logs measuring approximately 81–96 cm in length by approximately 15–25 cm diameter were inoculated with liquid media and incubated in a temperature and humidity controlled chamber for 12 weeks. After incubation, logs were removed, air dried for 1 week, cut, and analyzed as described above.

The logs were felled in Spring 2011 and were not debarked prior to inoculation. Logs were stored outside for 1 month, then conditioned in a humidity and temperature controlled chamber for 3 weeks. Pre-inoculation moisture contents for each wood species were as follows: sugar maple (11 replicates): 33.1 ± 10.1 %, aspen (16 replicates): 47.7 ± 13.9 %, Norway maple (7 replicates): 59.9 ± 16.1 %.

Two types of sprayable media were made: an agar-based and a gelatin-based. The agar-based was mixed in batches containing 0.48 g malt, 0.06 g agar, and 24 mL of distilled water. Each batch was autoclaved in a separate Mason canning jar (Bernardin, Canada) for 15 min, then allowed to cool in a laminar flow hood overnight. After cooling, jars were inoculated with one of the chosen test fungi, and incubated in the dark at room temperature for 10 days. Gelatin jars were mixed in 24 mL batches containing 0.48 g malt and 0.36 g gelatin, and processed as above. Both the gelatin and agar media existed in a semi-solid state, which turned liquid upon agitation.

A total of six inoculation sets were made, and are detailed in Table 1. To prepare for inoculation, logs were cut with a chainsaw to expose an area of sapwood approximately 5 cm wide down the length of the log. For inoculation, a sterilized 100 mL compressed air spray gun (Badger Mini Spray Gun, Badger Air-Brush Co., USA) was used to spray the semi-solid media onto the logs outside a laminar flow hood. Prior to filling the gun, the media was agitated by hand with a sterilized spatula for 35 s, during which time approximately 2 mL of distilled water was mixed into each jar. Exposed areas of the logs were sprayed until heavily coated with media. The entire contents of four jars were used to inoculate each set of six logs, with equal portions of liquid media on each log.

Logs were incubated for 12 weeks, cut, and analyzed as per the procedure described above. Data were analyzed

Table 2 Mean % internal spalting on sapwood of inoculated logs

Test	Wood	Pairing	Mean % ZL	Mean % pink stain
Dowel	Beech	*T.v.* UAMH11521/*X.p.* SR015	4 (8)	0 (0)
		T.v. UAMH11521/*T.v.* MAD697	0 (2)	0 (0)
		X.p. SR015/*X.p.* UAMH11518	12 (6)	0 (0)
		T.v. UAMH11521/*P.b.* FP102389	8 (8)	0 (0)
		S.c./*X.p.* SR015	10 (10)	0 (0)
		S.c./*T.v.* UAMH11521	8 (14)	2 (4)
		X.p. UAMH11518/*P.b.*	2 (2)	0 (0)
		X.p. UAMH11518/*T.v.* MAD697/*S.c.*	4 (6)	0 (0)
		X.p. SR015	4 (4)	0 (0)
	SM	*T.v.* MAD697/*S.c.*	13 (14)	0 (0)
		X.p. UAMH11518/*S.c.*	0 (1)	0 (0)
	NM	*P.b.*/*T.v.* MAD697	1 (1)	0 (0)
		S.c.	3 (2)	0 (0)
Spray	SM	*S.c.* (agar)	1 (1)	0 (1)
		S.c. (gelatin)	1 (1)	0 (0)
		X.p. UAMH11518 (agar)	1 (1)	0 (0)
	NM	*S.c.* (agar)	1 (1)	1 (1)
		No fungus, no cutting	3 (2)	0 (1)
	SM	No fungus, cut	3 (2)	0 (0)
	Aspen	*S.c.* (agar)	0 (0)	0 (1)
		S.c. (gelatin)	1 (1)	0 (1)
		X.p. UAMH11518 (agar)	1 (1)	0 (0)
		No fungus, cut	1 (1)	0 (1)
		X.g. UAMH10320/*S.l.* UAMH1502	1 (1)	0 (1)

Standard deviation is in parentheses. Values indicate mean percent found over both heartwood and sapwood

T.v., *Trametes versicolor*; *X.p.*, *Xylaria polymorpha*; *P.b.*, *Polyporus brumalis*; *S.c.*, *Scytalidium cuboideum*; *X.g.*, *Xylogone ganodermophtherum*; *S.l.*, *Scytalidium lignicola*; *SM* sugar maple, *NM* Norway maple, *ZL* zone lines

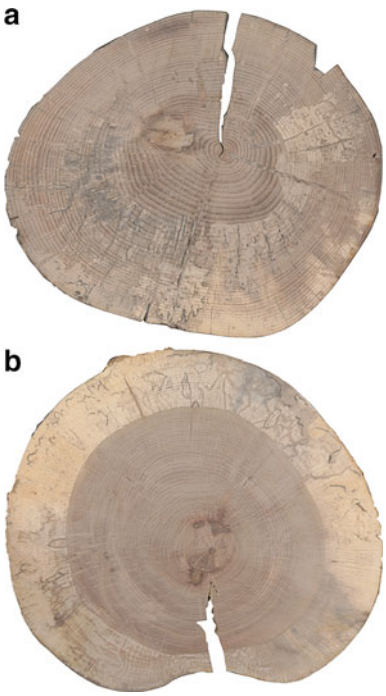

Fig. 2 Log cookies showing typical zone line production. **a** *Trametes versicolor*/*Scytalidium cuboideum* on sugar maple; **b** *Xylaria polymorpha*/*Xylaria polymorpha* on beech

using SAS version 9.2. Three-way ANOVAs were performed for zone line, pink stain, and yellow stain amounts, with wood species, inoculation method (plug or spray), and fungus as the independent variables. Tukey's HSD was utilized to determine the location of differences.

Results

Both agar and gelatin media sprayed easily from the spray gun, with minimal clogging. In addition, none of the tested fungi appeared to prefer one medium over the other, with complete surface colonization occurring in both within 10 days. As there was no difference between the media, data from both types of media were combined for statistical analysis.

Fungal species with multiple isolates did not perform differently regardless of wood species or inoculation method. Hence, separate isolates of the same species were pooled together for statistical analysis.

Table 2 shows the mean percent zone lines and pink stain produced by each fungus/fungal pairing by wood species and inoculation method. Within the three-way ANOVA for zone lines, inoculation method ($P < 0.0001$), fungus ($P < 0.0001$), and the interaction of wood species and fungal species ($P = 0.0002$) were significant. The plugging method of inoculation produced significantly more zone lines than spraying (3 versus 1 %). The combination of *S. cuboideum* and *T. versicolor* produced significantly more zone lines than any other fungus or fungal pairing (7 % average) other than the double *X. polymorpha* pairing (6 % average). For the interaction of wood species and fungus species, the combination of *S. cuboideum* and *T. versicolor* on maple produced the most zone lines (average 10 %; Fig. 2a), although the amount of zone lines produced did not differ significantly from the double *X. polymorpha* pairing on beech (6 %; Fig. 2b) or the *S. cuboideum*/*X. polymorpha* pairing on beech (5 %). It should be noted, however, that beech logs were incubated for 20 weeks, while the hard maple logs were incubated for 12 weeks.

The pink stain of *S. cuboideum*, while present on all logs inoculated with the fungus, only occurred in very small amounts. For the three-way ANOVA for pink stain, only inoculation method was significant ($P = 0.0023$). Spraying the logs with inoculum produced significantly more pink stain than did plugging the logs (1 versus 0 % on average, respectively).

Discussion

Of the fungi tested, only *P. brumalis* failed to produce significant amounts of spalting when paired with another culture. Most of the tested fungi were not capable of producing significant levels of spalting alone, but did so when paired together. The most successful fungal pairing was that of *S. cuboideum* and *T. versicolor* on maple for zone lines, although as noted above, pink stain production by *S. cuboideum* occurred only in very small amounts. Both *S. cuboideum* and *T. versicolor* are known to be aggressive, effective spalting fungi under sterile and semi-sterile conditions [9, 10]. As noted in Robinson et al. [9], when paired together, they are capable of mutual antagonism that results in the creation of zone lines. However, this antagonism appears to mostly prevent the pink pigment production of *S. cuboideum*, not only with *T. versicolor* as noted in the aforementioned paper, but also with the other paired fungi tested herein. The inhibition of pigment production by *S. cuboideum* in the presence of other fungi has been noted under contamination issues [10]. However, when no other fungi are present, this fungus is known to heavily saturate wood test pieces with its pigment. The results of this experiment indicate that *S. cuboideum* reliably antagonizes other spalting fungi, which results in the creation of zone lines. However, mass-scale pink pigmentation by this fungus is feasible only for small-scale inoculation where sterility can be tightly controlled; pink stain production by *S. cuboideum* under non-sterile conditions is not ideal, unless only minimal pink stain is desired.

The pairing of different isolates of *X. polymorpha* also created a significant number of zone lines. This result is somewhat surprising, as previous research indicated that isolates of *X. polymorpha* seldom manage to zone line wood internally, although it is completely capable of colonizing the wood [8, 11, 12]. There are several possible explanations for this effect. The addition of other fungi, whether those already inside the log, or simply the other isolate of *X. polymorpha*, may have stimulated antagonism and therefore also stimulated zone line production. It is also possible that the dowel pin inoculation forced internal zone line formation, as the fungus was not allowed to first colonize the external face before proceeding into the wood. Based upon the previous literature [3], this is the first instance in which *X. polymorpha* has produced a significant number of internal zone lines. As this fungus degrades wood at a much slower rate than *T. versicolor* or *P. brumalis* under controlled conditions [6, 13], its potential use in large-scale spalting will allow for similar zone line production to *T. versicolor*/*S. cuboideum*, without the potential for higher levels of white-rot decay.

The method of inoculation significantly impacted how the fungi performed within the logs, and fungi with different types of pigmentation appeared to prefer different methods of inoculation. Zone line producing fungi produced more zone lines when introduced into the logs in sterile plugs, while pink staining fungi performed better when sprayed onto the logs. Both methods are based in past literature, which includes pressing fungal mats against log ends [14, 15], spraying a mixture of distilled water and fungi on the logs [16–18], packing inoculated sawdust into drilled holes in the log [19], and using inoculated plugs to deliver active cultures directly into the wood [20]. Although spray inoculation was more effective with *S. cuboidea*, it is possible that the opening of the sapwood for spray inoculation also partially inhibited the pink stain production. As noted by Leatham [20], inoculum generally performs better when the bark is retained, as the bark helps maintain moisture content, insulates against temperature changes, and can inhibit the colonization by airborne contaminants. An inoculation method which enables entire surface coverage without exposing large portions of the internal wood would be ideal, but would be exceptionally difficult to achieve.

The literature indicates that red pine is commonly used for fungal cultivation in log form [14], but poplar species, maple, and oak [20] are also utilized. Of the wood species tested in this research, the maple species performed well with their associated inoculated fungi, while the beech logs performed well only after extended incubation. The aspen logs did not perform well, and showed very little spalting. The maples performed well with the zone line producing fungi in particular, while *S. cuboideum* did not appear to have a preference among any of the wood species, nor did the separate isolate pairing of *X. polymorpha*. Maple logs showed the most spalting in terms of wood species when inoculated with *S. cuboideum*/*T. versicolor*, although the amount of spalting was not significantly different than for several other pairings in beech. However, beech logs took almost twice the length of time to achieve the same level of spalting. This is very likely a similar effect as noted in Robinson et al. [8], where the higher amounts of beech heartwood either inhibited or obfuscated spalting, unless a white-rot fungus was utilized to first lighten the wood. As the fungal pairings that did produce spalting on beech all involved at least one white-rot capable fungus, it is probable that the extra time required by the beech logs to show spalting was taken by *X. polymorpha* lightening the wood, before other pigments could be seen.

The purposeful inoculation of logs with fungi is usually conducted either for biological control of other fungi or insects, or for the purpose of edible mushroom cultivation. The inoculation of logs with fungi to produce varying amounts of color, however, is not represented in the literature. There is a growing body of literature on the inoculation of smaller, sterile and semi-sterile sections of wood for spalting, such as boards and test blocks [3]. However, this research represents the first attempt to methodically introduce fungi into non-sterile green logs for the purpose of coloration. Based upon the results of this research, it does not appear practical to utilize beech or aspen logs for larger-scale spalting. In addition, the utilization of *S. cuboideum* for the production of pink pigment, unless desired in only very small quantities, does not appear to be a viable option. It is possible that other fungi would perform better on these wood species. *Chlorociboria* sp., for example, appears to readily pigment aspen wood [21, 22] and might be a better genus for inoculation.

If the appropriate inoculation procedures are followed, the results of this research indicate that *S. cuboideum*, *T. versicolor*, and *X. polymorpha* can successfully spalt larger-scale logs. The use of a dual inoculation of *T. versicolor*/*S. cuboideum* or different strains of *X. polymorpha* in maple appears to be viable options for spalting logs within a 12-week timeframe. The reliability and speed with which these pairs of fungi colonize the maple logs is potentially conducive to commercial production. If incubation time is not a concern, then beech could also be utilized, albeit with different fungal pairings. Although maple already has substantial value in the lumber market, the addition of value through spalting could potentially interest consumers who would otherwise purchase exotic woods. In addition, the use of *X. polymorpha*/*X. polymorpha* or *X. polymorpha*/*S. cuboideum* to American beech has the potential to create a market demand for an otherwise commercially insignificant wood species.

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