FUNGAL DEGRADATION METHOD DEVELOPMENT FOR SMALL WOOD SAMPLES SUBJECTED TO *CERIPORIOPSIS SUBVERMISPORA*

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Abstract. Genetic modification of commercial tree species has the potential to greatly enhance wood's chemical and physical properties. The effect of lignin biosynthetic pathway modification on biological degradation of these transgenic wood specimens is of great interest. However, genetically modified trees are frequently grown in greenhouses and are young with small-diameter stems. Experimental methods for fungal degradation of such specimens are not well established. The objective of this study was to develop a method that would result in sufficient amount of biomass degradation, measured by weight loss, with low variation among replicates. This study used small-diameter cylindrical and milled wood sweet gum (*Liquidambar styraciflua*) specimens degraded by the white-rot fungus, *Ceriporiopsis subvermispora*. The effects of fungal treatment vs sterile control, shaking vs mechanical homogenization of mycelial slurry, and initial water/wood ratio were analyzed. A method was identified with sufficient weight loss values and significant decrease in variation of weight loss among replicates by direct inoculation of wood with liquid malt extract fungal culture.

Keywords: *Ceriporiopsis subvermispora*, wood degradation, white-rot fungi, fungal degradation.

INTRODUCTION

During the past several years, many research groups around the world have applied genetic modification technologies to forestry in a myriad of applications such as herbicide resistance, pest and disease resistance, abiotic stress tolerance, phytohormone regulation, generation time reduction, flowering suppression, nutrient use efficiency, and cold tolerance (Harfouche et al 2011). These same technological innovations have resulted in transgenic trees with drastic changes in wood chemical composition. North Carolina State University's Forest Biotechnology Group has recently launched the most comprehensive analysis of lignin biosynthesis regulation ever undertaken. That analysis was designed to determine how each of the 33 lignin-producing genes and transcription factors impact the type and amount of lignin in wood (Wang et al 2014). Our research group (as indicated by the following references) has evaluated the anatomical, physical, and mechanical properties of the wood from lignin-modified transgenics (Horvath et al 2010a,b,c,d, 2011a,b, 2012a,b; Li et al 2011; Miller 2014). Of tremendous interest is the effect of lignin genetic modification on cell wall degradation. Investigations are active in the area of chemical degradation such as pulping, acid hydrolysis, and hot water pretreatment. Lignin also plays an important role in biological degradation (Kirk et al 1984; Blanchette 1991). Changes in the type and amount of this chemical component can have the potential to influence wood's interaction with other organisms ranging from the tree's defense against pathogens to ease of biologically based conversion of wood, and eventually to the return of the spent biomass to the earth as part of the carbon cycle.

Several researchers have in the past looked at the impact of lignin composition on the resistance of wood to decay fungi (Obst et al 1994; Choi et al 2006; Skyba et al 2013). To cover a wide range of compositions, frequently the materials used came from different species with different morphologies, ultrastructure, and physical characteristics (Obst et al 1994). The influence of the other complicating factors was difficult to isolate, and thus the results obtained remain inconclusive. Transgenic trees provide a unique opportunity to evaluate the impact of lignin composition on fungal degradation because the phenotype of these transgenics is similar to that of the wild type.

Because of Federal regulations limiting the planting of transgenic trees in the field for biosafety reasons (Sedjo 2004), materials in studies involving transgenic trees typically come from greenhouses. This involves performing wood property evaluations on young trees with stems not exceeding ~20 mm in diameter. Our group has developed a number of techniques to evaluate the mechanical and physical properties of such materials (Kasal et al 2007; Horvath et al 2010b, 2011b). Finding a suitable method of evaluating the fungal degradation of small-diameter transgenics continues to be a major challenge. Results

of previous studies on nonstandard specimen size (De Groot et al 1998; Giles et al 2012) indicated relatively large variation in mass loss among replicates. A variety of methods have been evaluated including a modified American Society for Testing and Materials (ASTM) soil-block test (De Groot et al 1998), an agar-plate method (Giles 2008), and a modified soil-agar block method (Giles et al 2012). Different inoculation techniques have also been tested including the addition of fungal spores, addition of mycelium grown in liquid or solid agar medium, and addition of precolonized substrate (Reid 1989a,b; Akhtar et al 1997; Wan and Li 2012). Inoculating wood chips by direct addition of a liquid mycelium slurry has been adopted by many studies investigating lignocellulose biomass treatment and in general showed favorable results (Wan and Li 2012). This technique has the benefits of immediate and direct contact of fungal mycelium to the wood samples, limited addition of excess nutrients, as well as more uniform initial coverage of the fungus across the substrate surface.

This study was conducted in an effort to refine our technique of directly adding liquid mycelium slurry to the biomass substrate. An earlier study with this technique gave very promising results, yielding very small variation although the level of decay was insufficient (Xiang 2011). Developing an acceptable technique dictates using a suitable substrate with very little inherent variation that could serve as a good surrogate for the small-diameter cylindrical transgenic stems to be gathered from the greenhouse. Dowel-sized samples from sweet gum serve this purpose. Sweet gum, as a medium-density hardwood species, is recommended by the ASTM (2008) and the American Wood Protection Association (AWPA 2010) for fungal resistance testing. *Ceriporiopsis subvermispora* served as the fungal probe. It is a lignin-selective white-rot species that preferentially degrades lignin with little loss to cellulose (Otjen et al 1987), making it of interest in studies designed to understand the recalcitrant nature of wood.

The goal of this study was to test several methods for inoculation and incubation using the white-rot fungus *C. subvermispora* to degrade small wood specimens and identify which method resulted in a sufficient amount of biomass degradation (measured by weight loss) and low variation among replicates. Low variation in weight loss values among experimental replicates is an indicator of consistent decay and is important for obtaining reliable results. The method should be suitable for testing young small-diameter stem samples grown in a greenhouse. The effects of fungal treatment vs sterile control, shaking vs mechanical homogenization of mycelial slurry, and initial water/wood ratio were analyzed.

MATERIALS AND METHODS

Wood Samples

To test the inoculation method, sweet gum (*Liquidambar styraciflua*) dowels, which had the closest shape to wood samples of greenhouse-grown, young, small-diameter stems, were obtained from a local lumber mill. In addition, sweet gum was chosen for this study because it was readily available and in abundant supply and also because results of this study could be compared with results of previous publications using wood with similar properties that were subjected to fungal decay. Solid wood samples were prepared by cutting 12.7-mm-diameter dowels into 25.4-mm lengths. In addition, milled wood samples were prepared by grinding with a Wiley mill to pass through a 40-mesh screen. A Wiley mill was used because conventional chipping equipment is designed for larger branches or stems and is therefore impractical for comminution of small-diameter samples.

All samples were dried at 103 $\pm$ 2°C for 24-48 h, and samples were weighed to obtain oven-dry weights. Three solid wood cylinders (a sum of $\sim$2-3 g) or 1 g of milled wood were placed into a 20-mL vial. Samples were then conditioned by adding an appropriate volume of deionized (DI) water to each vial, obtaining a water/wood mass ratio of 1.5 for Methods 1-4. In Method 5, water/wood mass ratios of 1.5 and 1.0 were tested. Aluminum foil was used to cover

the vial opening, and after 24 h, all samples were sterilized by autoclaving at 121°C for 20 min before inoculation.

Fungal Culture

The fungus used for this study was *C. subvermispora*. It was given by the USDA Forest Service, Forest Products Laboratory (Madison, WI). The fungus was first cultured on a petri dish with 5% malt extract agar (MEA) and incubated at 26°C for 7 da. Next, a fungal plug was used to inoculate either 300 mL of malt extract medium (20 g malt extract·L^{-1} in DI water) or a 140-mm-diameter glass petri dish containing 5% MEA. The liquid fungal culture was incubated for 30 da at 26°C, and the inoculated petri dish was incubated for 7 da at 26°C.

Inoculation and Incubation Methods

Our research group has been involved in testing wood from greenhouse-grown genetically modified trees. Previous attempts for fungal treatment testing resulted in large variation of weight loss (Giles 2008) or insufficient decay (Xiang 2011). Building on those experiments, five fungal inoculation techniques were tested (Fig 1).

Method 1 used solid cylindrical wood specimens in 20-mL scintillation vials (Fig 1a). Thirty-da-old liquid fungal culture was aseptically filtered, rinsed with DI water, and suspended in 250 mL of sterile DI water. Then, the mycelium was homogenized by either vigorous manual shaking in the culture bottle for 2 min or by using a mechanical homogenizer to create a mycelial

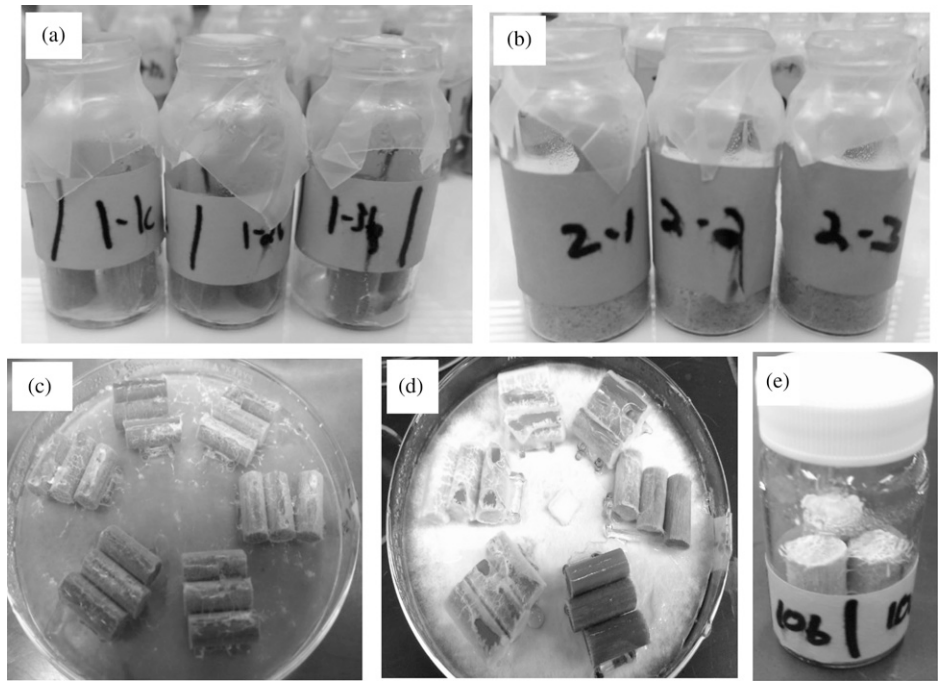

Figure 1. Images of different methods tested for inoculation and incubation of sweet gum samples with *C. subvermispora*; (a) Method 1: solid cylindrical wood specimens incubated in 20-mL vial and inoculated with filtered and rinsed mycelium slurry; (b) Method 2: milled (40 mesh) samples incubated in 20-mL vial and inoculated with filtered and rinsed mycelium slurry; (c) Method 3: six sets of three wood specimens in 140-mm-diameter glass petri dish containing agar inoculated with filtered and rinsed mycelium slurry; (d) Method 4: six sets of three wood specimens in 140-mm-diameter glass petri dish containing preinoculated MEA; and (e) Method 5: solid cylindrical wood specimens incubated in 20-mL vial and inoculated with direct addition of mycelium slurry.

slurry. About 2 mL of mycelial slurry was inoculated into each vial, and the vial openings were covered with parafilm. Fifteen replicates received the shaken mycelial slurry (samples denoted as 1S), and 15 replicates received the mechanically homogenized slurry (denoted as 1H). Five additional vials served as sterile controls in which 2 mL of sterile DI water was added instead of mycelial slurry (denoted as 1SC).

Method 2 was similar to Method 1, except 1 g of milled wood was used instead of solid wood and 1 mL of mycelium slurry was used for inoculation (Fig 1b). One milliliter of mycelium slurry was added instead of 2 mL, because the 20-mL scintillation vial was able to accommodate only 1 g of milled wood instead of ~2-3 g of cylindrical specimens as in Method 1. Again, 15 vials received shaken slurry (denoted as 2S), 15 vials received mechanically homogenized slurry (denoted as 2H), and 5 sterile control vials (denoted as 2SC) received sterile DI water instead of mycelial slurry. Vials were sealed with parafilm and placed in an incubator.

Method 3 used 140-mm-diameter petri dishes containing 5% agar in DI water as incubation containers (Fig 1c). An agar medium was used to limit the food source for the fungus to just the wood substrate. Four petri dishes were autoclaved, and a 5% agar in DI water solution was added to each petri dish under sterile conditions. Six sets of three cylindrical wood samples previously sterilized in 20-mL vials were aseptically placed on top of glass supporting rods inside of each petri dish. Next, 2 mL of filtered, rinsed, and shaken mycelium slurry was added to each set of three specimens (denoted as 3F). Three petri dishes received the fungal treatment, resulting in 18 samples. Sterile control samples were prepared by aseptically placing six sets of three solid sterilized cylindrical wood specimens on glass supporting rods. No fungal inoculum was added. The petri dishes were then sealed with parafilm and placed in the incubator.

Method 4 also used 140-mm-diameter petri dishes as in Method 3. However, 5% MEA was added to each of three petri dishes under sterile conditions. The three petri dishes were preinoculated with a fungal plug (Fig 1d) and incubated at 26°C for 7 da to allow the fungus to grow. Then, sterile cylindrical wood samples were added on top of glass rod supports on top of the actively growing fungus (denoted as 4F, 18 samples). The sterile control samples prepared for Method 3 served as the control for both Method 3 and 4 because the sample layout and incubation container were the same for both methods, assuming that sterile control samples were not affected by agar medium. All samples from Methods 1 to 4 were incubated in darkness at 29°C for 40 da.

Method 5 used three cylindrical wood specimens in 20-mL vials (similar to Methods 1 and 2). However, the 30-da-old liquid fungal culture was not filtered and rinsed. Instead, it was shaken to homogenize, and 1 mL was directly added to each vial to inoculate the samples (Fig 1e). Additionally, instead of sealing the vial with parafilm, the plastic cap was sterilized with 95% ethanol, tightened on the vial, then loosened one-quarter turn to allow gas exchange. Vials with initial water/wood mass ratios of 1.0 and 1.5 were denoted as 5-WW1-F and 5-WW1.5-F, respectively. For each water/wood mass ratio, five fungal treatment replicates and three sterile control replicates were tested. Sterile control samples were denoted as 5-WW1-SC and 5-WW1.5-SC for 1.0 and 1.5 initial water/wood mass ratio, respectively. Samples were incubated at 29°C in darkness, and an open container of water was placed in the incubator to keep the humidity high. Because of more vigorous fungal growth, Method 5 samples were incubated for 30 da instead of 40 da.

Weight Loss

After the 30- or 40-da incubation period, samples were removed from the incubation containers and surface mycelium was gently removed. Samples were subsequently dried under vacuum at 40°C for 48 h and then weighed. Weight loss was calculated by comparing the

original oven-dry weight to the dry weight after fungal treatment:

$$\text{Weight loss}(\%) = \left(1 - \frac{W_f}{W_i}\right) \times 100$$

where W_f = dry weight of wood sample after fungal treatment and W_i = initial dry weight of wood sample.

Statistical Analysis

One-way analysis of variance (ANOVA) was applied using SAS version 9.4 statistical software (SAS Institute, Cary, NC). A general linear model (Proc GLM) was used to test for significance ($\alpha = 0.05$) of the fungal treatment vs sterile control within each method. In addition, the significance ($\alpha = 0.05$) of shaking vs mechanical homogenization of mycelial slurry was tested within Methods 1 and 2, and the significance of the initial water/wood mass ratio within Method 5 was tested. Standard deviation and coefficient of variation (CV) were calculated for the weight loss for each method.

RESULTS AND DISCUSSION

Table 1 and Fig 2 show the means, standard deviations, and CV for weight loss values for each method tested. One-way ANOVA indicated that there was a significant difference between the weight loss values of the sterile control samples and those of samples treated with white-rot fungus in all methods except Method 4. In Method 4, two out of the three petri dishes receiving the fungal treatment showed little fungal degradation and minimal weight loss. Methods 1 and 2 showed that the mean weight loss for samples receiving manually shaken vs mechanically homogenized mycelium slurry was not significantly different. Reid (1989a) previously tested the effect of mycelium inoculation. Reid (1989a) showed that the observed lag time and fungal respiration rate after liquid mycelium inoculation was similar to inoculating with precolonized wood chips and there was no apparent damage from homogenization of mycelium slurry (Reid 1989a).

Methods 1S and 1H using solid wood samples in 20-mL vials, with shaken (1S) and mechanically homogenized (1H) mycelium slurry for inoculation, had the smallest average weight losses of 1.25% and 0.98%, respectively. In addition, the CV values were high at 111.9% and 99.4% for 1S and 1H, respectively. This agrees with visual inspection in which very little fungal growth was observed. Aguiar et al (2006) reported weight loss of ~4% in *Pinus taeda* chips treated for 30 da using a similar method of inoculating with filtered and rinsed mycelium slurry from *C. subvermispora*. It is possible that the greater weight loss reported by Aguiar et al (2006) was caused by the fact that forced aeration was used in the bioreactor. In this study, sealing the incubation vials with parafilm may not have allowed enough gas exchange to allow the fungus to grow well.

Methods 2S and 2H, with 3.38% and 4.09% weight loss, respectively, had greater weight loss values than Method 1 samples. However, the weight loss measured for the sterile control (2SC) was high compared with that of sterile control samples of the other methods tested. This was most likely because of the smaller particle size of the wood, which underwent greater degradation during the sterilization procedure. In addition, the CV values were high, ranging from 53.5% to 62.0%.

Method 3 was tested based on similarities with a technique used to test the decay resistance of wood or woody biomass as outlined by Schilling and Jacobson (2011), whereas Method 4 was tested based on similarities with the British standard (BSI 2004). Although Methods 3 and 4 were based on techniques that were designed to test the decay resistance of treated wood, they serve to understand the effect of using a larger incubation container and using precolonized MEA in the case of Method 4 as opposed to the liquid mycelium slurry used in the other methods. In addition, because of the larger area in the petri dish, multiple wood samples can be placed in a

Table 1. Overview of incubation and inoculation methods tested using the white-rot fungus *C. subvermispora* on sweet gum samples and mean weight loss measured after 30 or 40 da of treatment.

		Treatment description			% Weight loss				
Method	Sample name	Inoculation medium	Sample/container	N[a]	Minimum	Maximum	Mean	Standard deviation	%CV
1	1S	Shaken, filtered, rinsed mycelium slurry	Three solid wood specimens, 20-mL vial sealed with parafilm	15	0.11	4.26	1.25	1.39	111.9
	1H	Mechanically homogenized, filtered, rinsed mycelium slurry		13[b]	0.07	2.86	0.98	0.97	99.4
	1SC	Sterile control		5	−0.54	−0.31	−0.44	0.09	20.1
2	2S	Shaken, filtered, rinsed mycelium slurry	Milled wood (40 mesh, 1 g), 20-mL vial sealed with parafilm	15	0.45	6.32	3.38	2.10	62.0
	2H	Mechanically homogenized, filtered, rinsed mycelium slurry		15	0.90	7.15	4.09	2.32	56.8
	2SC	Sterile control		5	0.81	2.65	1.35	0.75	55.3
3	3F	Shaken, filtered, rinsed mycelium slurry	Six sets of three solid wood specimens, 140-mm petri dish containing agar	18	0.91	7.25	4.37	1.76	40.2
	3SC[c]	Sterile control		6	−0.44	0.78	0.32	0.41	128.6
4	4F	Malt extract agar was preinoculated with fungus	Six sets of three solid wood specimens, 140-mm petri dish containing MEA	18	0.31	5.98	1.36	1.78	130.4
	4SC[c]	Sterile control		6	−0.44	0.78	0.32	0.41	128.6
5	5-WW1-F	Shaken, unfiltered mycelium slurry	Three solid wood specimens, 20-mL vial sealed with loose cap	5	3.80	4.39	4.17	0.24	5.67
	5-WW1.5-F			5	3.76	4.87	4.38	0.43	9.91
	5-WW1-SC	Sterile control		3	0.16	0.28	0.23	0.06	27.52
	5-WW1.5-SC			3	0.28	0.48	0.39	0.10	26.50

[a] Sample size.
[b] Two samples were removed as outliers.
[c] A single sterile control sample set was used as the control for both Method 3 and 4.

single container. This may provide a more uniform environment for fungal decay and could yield better results for comparing different wood types or wood treatments.

Method 3 had an overall weight loss of 4.37%. The variation in mean weight loss was lower than Methods 1 and 2 with a CV of 40.2%. Giles (2008) reported CV values of 40-50% using a similar method (modified agar-plate method). Method 4 yielded very inconsistent results with a CV of 130.4%, which could have been caused by the lack of control in the volume of MEA in each of the petri dishes. Giles (2008) reported a similar finding of large variation using a modified agar-block method with small cylindrical *Liriodendron tulipifera* wood samples degraded by white-rot *Trametes versicolor*, with a CV ranging from 38.94% to 50.09%.

Method 5 had the greatest observed weight losses at 4.17% and 4.38% for initial water/wood mass ratios of 1.0 and 1.5, respectively. In addition, Method 5 exhibited lower variation among replicates with CV values of 5.67% and 9.91% for 5-WW1-F and 5-WW1.5-F, respectively. The

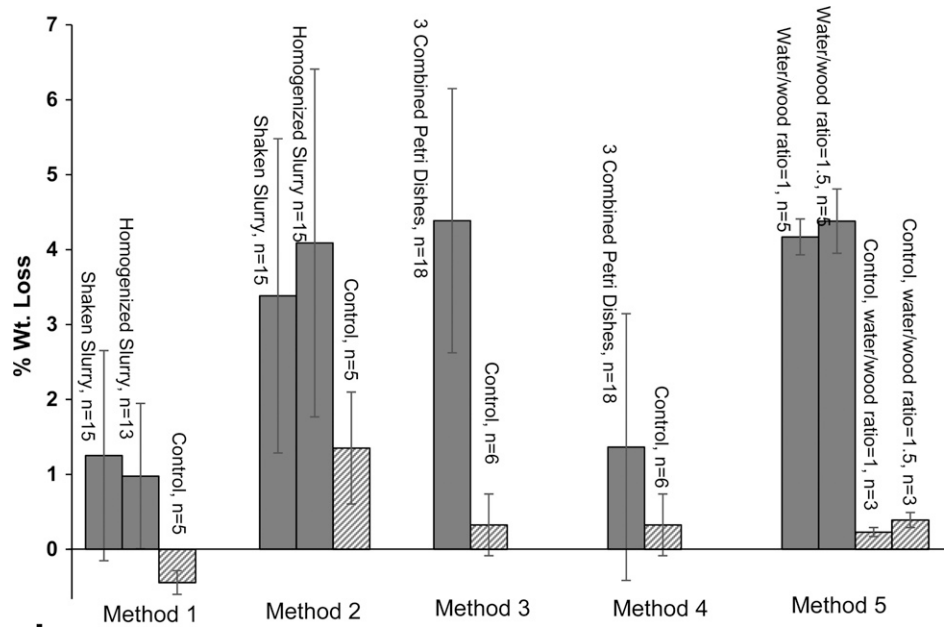

Figure 2. Mean weight loss (%) of sweet gum wood samples after 40 da (Methods 1-4) or 30 da (Method 5) of treatment with *C. subvermispora*; error bars represent mean ± 1 standard deviation.

initial water/wood mass ratio in the range tested (1.0 and 1.5) had little effect on the weight loss and was not statistically significant. It is possible that if a larger range of water/wood mass ratios had been tested, significant differences may have been observed.

There was a considerable amount of variation among the five methods tested. The weight loss observed for Method 5 was the greatest among all the methods and fell within the range observed by others for solid-state degradation of hardwoods with white-rot fungi. For example, Reid (1989a) reported weight losses of 3.4-6.5% after 6 wk of exposure of aspen to *Phlebia tremellosa* using preinoculated wood chips, and Ferraz et al (2003) reported ~5% weight loss of *Eucalyptus grandis* treated with *C. subvermispora* for 30 da using a modified agar-plate inoculation method. Conversely, the weight losses reported for Method 5 were relatively low compared with results observed by others using similar methods of liquid mycelium slurry inoculation. For example, Choi et al (2006) reported 6.2% weight loss after 4 wk of treatment of *Populus tremuloides* wood chips with *C. subvermispora*, and Wan and Li (2011) reported ~10% weight loss of hardwood chips after 18 da of treatment with *C. subvermispora*.

Based on the results of all methods tested, Method 5, which used liquid mycelium slurry that was not filtered, rinsed, and suspended in water, had among the greatest weight loss values and significantly decreased variation among replicates. In addition, more rapid fungal growth and wood degradation were observed based on the shorter incubation period of 30 da as opposed to 40 da, which was used for the other methods. The process of filtering, rinsing, and suspending in water may have induced stresses on the fungal mycelium such as quickly changing temperature, osmotic potential, and pH. These factors may have been responsible for the poor and inconsistent fungal growth and degradation that was observed in Methods 1-3. Additionally, the use of loose caps on the vials in Method 5 may have allowed greater air

exchange than sealing the vials with parafilm, allowing the fungus to grow more vigorously. Method 5, regardless of the initial water/wood mass ratio, yielded the best results and is recommended for future fungal decay experiments with the aim of comparison testing of young small-diameter stem samples for fungal treatment. Early results from decay experiments on wild type and transgenic cottonwood support this finding (Edmunds 2015).

CONCLUSIONS

The results from this study will help guide future experimentation of fungal treatments on young small-diameter stem samples. The objective of this study was to examine variables in the inoculation and incubation methods using white-rot fungi *C. subvermispora* on small wood specimens to develop a method that produces a sufficient amount of biomass degradation with low variation among replicates. The lack of significant difference between manual shaking and mechanical homogenization of mycelium slurry suggests that the method of homogenization is not a critical factor. The most significant finding was the decreased variation in weight loss among replicates and sufficient weight loss that were observed in Method 5 compared with the other methods tested. Therefore, Method 5 is recommended for future fungal treatment testing with young small-diameter stem samples. The method developed here will usher further research that will improve our understanding of the role played by lignin composition on the fungal degradation of wood. This may lead to the development of technologies that improve decay resistance of wood structures or decrease wood recalcitrance in conversion processes such as pulping and biofuel–biochemical manufacturing.

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