ABSTRACT

GILES, RICHARD LEE. Fungal Degradation Properties of Young Small Diameter Genetically Modified Quaking Aspen *(Populus tremuloides*). (Under the direction of Dr. Ilona Peszlen and Dr. Perry Peralta).

Three studies were conducted to evaluate the fungal degradation properties of young transgenic aspen trees (*Populus tremuloides*). The first study focused on development of methods of decaying small diameter tree samples. The second and third studies focused on the mass loss differences and cellulose and lignin degradation between trees with different lignin types and contents.

In the first study, two methods for rapid laboratory fungal decay tests of very young small diameter (5-15 mm) hybrid poplar (Populus nigra x Populus maximowiczii), yellow poplar (Liliodendron tulipifera), and willow (Salix sp.) trees were examined using the white rot fungi *Trametes versicolor* and *Ceriporiopsis subvermispora*. An agar plate method and a modified soil-agar block method were compared using non-standard size stem parts. The agar plate method did not prove to be suitable for testing materials when limited numbers of samples are available because of the extremely high variation of mass loss values. Mass loss using the modified soil-agar block technique was comparable to established methods for small and large blocks using extended colonization periods.

In the second study, one-year old quaking aspen (*Populus tremuloides*) trees including a control wild type aspen and three lines of transgenic trees were analyzed for

resistance to lignin selective white rot fungal decay. The transgenics had reduced lignin content through transfer of an antisense -4CL gene, changed syringly/guaiacyl ratio through insertion of a sense CAld5H gene, and modified lignin content and syringyl/guaiacyl through simultaneous insertion of -4CL and CAld5H genes. Mass loss was used to examine differences between genetic lines. The small diameter transgenic trees were decayed using lignin selective white rot fungus *Ceriporiopsis subvermispora*. A modified soil-agar block method was used with a forty day colonization time. The transgenic lines with higher S/G lignin ratio exhibited a higher mass loss percentage compared to the wild type and other transgenic lines.

In the third study, the transgenic lines were analyzed for resistance to three types of fungal decay. The small diameter transgenic trees were tested using simultaneous white rot fungus *Trametes versicolor*, lignin selective white rot fungus *Ceriporiopsis subvermispora* and a brown rot fungus *Poria placenta*. A modified soil-agar block method was used to decay the samples. Near infrared spectroscopy and chemical analysis determined loss of cellulose and lignin variations between transgenic genotypes. Near infrared transmittance was successful in predicting the cellulose and lignin percentages of the decayed material. The reduced lignin content lines did not affect the rate of lignin decay for all fungi tested. Lignin decay rates were reduced by the increased S/G ratio lines for all fungi tested.

Fungal Degradation Properties of Young Small Diameter Genetically Modified Quaking Aspen (*Populus tremuloides*)

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Master of Science

Wood and Paper Science

Raleigh, North Carolina

August 19, 2008

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DEDICATION

This thesis is dedicated to my loving and encouraging family. To my wife Dare, my mother Jo Ann, and my father Richard, thank you for all your support.

BIOGRAPHY

Richard Giles earned his Bachelor of Science degree in Botany at North Carolina State University in 2004. After graduation he was employed as a mycologist at Environmental Microbiology Laboratory in Union, New Jersey. His experience included indoor air quality analysis, laboratory quality control, and fungal identification. In 2006 he started in the Master of Wood and Paper Science program at North Carolina State University. In 2007, Richard opened North Carolina Microbiology Laboratory, a consultant firm for the indoor air quality and wood products industries. He will be receiving his Master of Science in Wood and Paper Science degree on December 2008.

ACKNOWLEDGMENTS

The author would like to express his appreciation and gratitude to all those who made this thesis possible:

His advisors, Dr. Ilona Peszlen, for making this work possible from research thru publication, Dr. Perry Peralta, for advice on experimental design and academic guidance, Dr. Larry Grand, for continual support and mentoring in the study of mycology, and Dr. Hou-Min Chang for biochemistry advisement and providing staff members to teach me in the laboratory;

To Dr. Roberta Farrell, Dr. Kent Kirk, and Dr. Ellis Cowling for friendly correspondence, analysis of data, and professional advisement;

To Dr. Ewellyn Capanema for chemical analysis support;

The Department of Wood and Paper Science, NCSU for the opportunity for study;

To Laszlo Horvath and Balazs Horvath, for technical and statistical support in the laboratory;

To Dr. Aman Bath and Ann Atkinson for inspiring me to further my education and professional mentoring;

To friends Laura Vance and Lisa Costello for keeping me focused towards my goals;

To friends Seth Pergerson and Justin Powell for support thru humor;

To his parents, Jo Ann and Richard Giles, for continued guidance;

To his wife, Dare, for her love and encouragement.

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CHAPTER 1

Preliminary Study to Develop Decay Testing Methods for Small Diameter Wood Samples

Abstract

Two methods for rapid laboratory fungal decay tests were developed for young non-standard size stem parts with lengths of 10-20 mm and diameters of 5-15 mm. An agar plate method and a modified soil-agar block method were compared using a shortened, 20-day colonization period due to the small size of the specimens. The agar plate method did not prove to be suitable for testing materials when limited numbers of samples are available because of the extremely high variation of mass loss values. The modified soil-agar block technique was comparable to established methods for small and large blocks using extended colonization periods.

Keywords: decay resistance, agar block method, *Ceriporiopsis subvermispora*, *Trametes versicolor*, mass loss

Review of Related Literature

Fungal Decay Methodology

Many methods used in fungal decay research are variations on the ASTM and AWPA soil-jar standard methods for testing wood preservatives. These methods determine the effective amount of preservative in select species of wood in optimum laboratory conditions for 19mm cubical shaped sapwood blocks [ASTM 1413-07, AWPA E14-94]. Although sample size is commonly changed in decay tests, the basic protocol for fungal species, bottle size, and colonization time is used [Otjen et al. 1985, Smith et al. 1996, Clemmons et al. 2002, and Mabicka et al. 2004]. The effect of small sample size has been examined and found that high variation resulted from using the soil-jar decay method [De Groot et al. 1998]. The authors concluded that the soil-jar method must be characterized before it can be accepted as standard protocol for evaluating preservative treated wood.

White Rot Fungi

Fungal degradation is grouped according to the morphology of the wood after decay [Eriksson et al. 1990]. White rot fungi degrade large amounts of lignin, cellulose, and hemicelluloses from wood cell walls. The ratio of decay of each wood component differs between fungal species. Many wood decay fungi belong to the fungal order Polyporacea. Fungal species in this order produce basidiospores in tubes on the basidiocarp as opposed to gills. White rot fungal decay results in a bleached, stringy

appearance in wood. This decay morphology results from the decay of lignin leaving the white cellulose fibrils behind. Simultaneous white rot refers to fungi that simultaneously degrade lignin, cellulose, and hemicelluloses at similar rates. *Trametes versicolor* is an example of a simultaneous white rot fungus. Lignin selective white rot refers to fungi that have a high affinity for lignin decay while a low affinity for carbohydrate decay [Otjen et al. 1985, Eriksson et al. 1990].

Introduction

Substantial research to determine efficacy of wood preservatives and to determine wood degradation have been conducted using established decay test techniques. These techniques include field tests that mimic conditions of wood in service. Field tests use large heartwood samples subjected to natural bacterial, insect, and fungal degradation in uncontrolled outdoor conditions [AWPA E14-94]. Laboratory tests using uniform size samples of wood, allow control of variables to maintain a controlled experiment. Moisture content, temperature, and decay organisms are easily controlled using laboratory decay procedures. ASTM standard soil block technique requires homogenous sized cubic samples of $6.9 cm^3$. Due to the nature of tree improvement programs, sample size is a limiting factor when determining decay characteristics.

Tree improvement programs need determination of properties early in development in order to access tree fitness and wood quality. Investigations of decay characteristics of young age cultivars, hybrid crosses, and transgenic trees provide insight into how chemical composition is linked to wood formation and properties. Testing trees at a young age is important because trees have a slow growth rate; however, the small stem size limits the available material and requires extrapolation of data from tests of small sample sets.

Standard methods for testing decay properties may take up to several months for wood block tests [Cowling 1961, ASTM 1413-07] and require a sample size not available in young tree research. Yet modified methods for smaller square samples do not represent

round diameter young tree sample dimensions. A reduction in size introduces high variability into the mass loss of samples [De Groot et al. 1998]. Procedural analysis performed on smaller 1 cm^3 sapwood samples using smaller bottles than the ASTM soil block technique found the technique resulted in a high mass loss variability between bottles [De Groot et al. 1998]. Wood blocks decay tests require long colonization times to provide homogenous decay among samples. Longer colonization times predetermined for large block samples may not be necessary for small round wood samples.

Standard decay test colonization time, incubation jar size, and sample size are not ideally suited for use in studying small diameter young trees. The ASTM method is intended for use in preservative testing, and decay data is quantified by mass loss. Mass loss is a useful quantitative measure of fungal degradation in wood samples [Cowling 1961, Highley et al. 1970, ASTM 1413-07]. Although mass loss does not give insight into the degraded wood cellular components (cellulose, hemicelluloses, lignin), for preliminary testing it provides easily measured data comparable to larger block tests. Decay resistance is not the objective of decay testing small trees, since the wood is too young to make comparisons to mature tree wood. Due to the nature of early tree testing, wood for testing is limited and leaves little material for replicates making chemical analysis a useful tool. Chemical analysis of post-decay remaining cellulose or lignin contents may give understanding of decay

differences between trees. Samples may not require 12 weeks of decay as used in the soil block method, in order to detect chemical changes within the wood. A shorter colonization period may provide valuable chemical

data while allocating more time for test replication. Development of methods for rapid screening of decay characteristics such as infrared spectroscopy allows further investigation of wood decomposing species [Dorado et al. 1999, Schwanninger et al. 2004, Yeh et al. 2004 and 2005, Kent et al. 2006, Fackler et al. 2007].

Selection of fungal species is significant when designing a wood decay testing technique. Desired decay type is achievable since many fungal species have been characterized on both hardwood and softwoods [Cowling 1961, Blanchette 1991, Hakala et al. 2004]. The increased interest in lignocellulosic materials as glucose sources for bioethanol production presents unique opportunities for white rot fungi research. White rot fungi such as *Trametes versicolor* and *Ceriporiopsis subvermispora* both degrade lignin while increasing enzymatic access to cellulose within wood cell walls. *Ceriporiopsis subvermispora* selectively degrades lignin with little cellulolytic activity thus wood substrate decay characterization is of considerable scientific interest [Dorado et al. 1999, Hakala et al. 2004, Martinez et al. 2005].

Emerging transgenic tree research has created tree lines with reduced lignin and increased cellulose contents [Hu et al. 1999]. Transgenic tree samples decayed with lignin selective white rot fungi would provide valuable chemical and mechanical information for bioethanol or biopulping applications. However, these transgenic trees are less than two-

years-old with small diameter stems. Standard testing methods are not applicable to test small diameter stems. A need to develop new techniques for species and substrate characterization is highly desirable.

Objectives

The objective of this study was to develop a rapid method for testing white rot fungal decay properties of young (1-2 years old) small diameter trees.

Material and Methods

Two new methods were developed to test small diameter (5mm-15mm) young trees. A modified agar plate method was evaluated using small diameter stems and a short colonization time [Flaete 2004, Levi 1969] and a modified soil-agar method combining the standard soil-block method (ASTM 1413-07) and methods used by Oltjen et al. (1985), De Groot et al. (1998), and Worrall et al. (1991) were developed for small diameter tree samples and tested with a short colonization time. Mass loss percentage was used to quantify decay.

Agar Plate Method

Trametes versicolor Mad-697, a simultaneous white rot fungus was obtained from the USDA Forest Products Laboratory (Madison, Wisconsin) culture collection. One-year-old stems cut from lower limbs of a mature flowering *Liriodendron tulipifera* tree were used in the agar plate technique.

Agar Plate Inoculation

Debarked samples were grouped into 3 sizes with 32 samples in each group. The stem sample sizes were (transverse diameter x longitudinal dimension) 5mm x10mm (Group 1), 5mm x 20mm (Group 2), and 10mm x20mm (Group 3). Samples were oven dried at 50° C for 9 days until weights stabilized. Samples weighed to determine dry weight then steam sterilized. Seven 15x100mm Petri plates of Malt Extract Agar with 0.01% Chloramphenicol (Hardy Diagnostics, Santa Maria, CA) were inoculated with

Trametes versicolor. Fungal cultures were grown at room temperature until the plates were fully colonized approximately 7-10 days. 15 samples (5 samples from each size group) were placed standing transversely on the agar around the outer aerial growth of each colonized Petri plate (Fig 1-1). An additional sterile Petri plate was used as a control with 2 samples of each size group. A humidity chamber was lined with 2.5cm of perlite and 1cm of distilled water to increase relative humidity. Plates were placed into the humidity chamber and incubated at room temperature (35° C) in darkness. After 20 days, samples were brushed lightly to remove surface hyphae, dried at 50° C for 9 days and weighed.

Fig 1-1. *Liriodendron tulipifera* wood samples of three size groups transversely placed along the growing edges of colonized Petri plates with *Trametes versicolor.* Arrows point to visible *Trametes versicolor* hyphal mats shared between wood samples.

Modified Soil-Agar Block Method

Trametes versicolor Mad-697 (a simultaneous white rot fungus) and *Ceriporiopsis subvermispora* FP-90031-sp (a lignin selective white rot fungi) were

obtained from the USDA Forest Products Laboratory (Madison, Wisconsin) culture collection. One-year-old *Populus nigra/Populus maximowiczi* hybrid cross and unknown *Salix sp.* trees were harvested for modified soil-agar block testing. The poplar and willow stems were longitudinally designated Upper and Lower portions. Upper designation refers to the middle of the stem towards the direction of vertical growth. Lower designation refers to the middle of the stem towards the base of the tree.

Modified Soil-Agar Block Inoculation

Nineteen debarked *Populus sp*. large diameter lower (ranging 7mm-15mm) and 19 *Populus sp.* small diameter upper (ranging 5-12mm) samples were cut into 20mm lengths. Sixteen *Salix sp*. large diameter lower (ranging 8mm-12mm) and 16 debarked *Salix sp.* small diameter upper (ranging 7-10mm) samples were cut into 20mm lengths. Samples were oven dried for 2 days at 105°C. Samples were weighed to determine dry weight then steam sterilized. Seventy round 2 oz jars were filled with 0.8 grams of vermiculite and 4ml distilled water (Fig 1-2). A needlepoint plastic grid was placed on top of the moist vermiculite in order to prevent saturation of wood samples. Malt Extract Agar with 0.01% Chloramphenicol (Hardy Diagnostics) Petri plates colonized with *Trametes versicolor* and *Ceriporiopsis subvermispora* and cut into 5mm x 20mm agar strips. Agar strips were placed on the plastic grid. *Populus* and *Salix* wood samples were then placed longitudinally perpendicular to the agar strips. The larger diameter lower samples were paired with *T. versicolor* and the smaller diameter upper samples were paired with *C. subvermispora*. Two control sample of each Upper and Lower species

stem group were chosen and placed in jars without agar strips. The mouth of each jar was then sealed with a layer of Parafilm. Sample jars were incubated at room temperature in darkness. After 20 days, samples were brushed lightly to remove surface hyphae, dried at 50° C for 9 days and weighed.

Fig 1-2. *Populus sp.* wood sample fully colonized with *Trametes versicolor*. Hyphae are visible on the transverse section (arrow). Small diameter wood samples were placed longitudinally upon agar strips colonized with white rot fungi. Vermiculite provided a sterile and inert media maintaining high relative humidity within the jar. The agar block and wood sample rested on a small plastic grid to prevent saturation of the sample from the moist vermiculite.

Results

Agar Plate Method Results

Trametes versicolor showed evidence of colonization on all size stem groups as early as the 4th day of incubation. Aerial surface hyphae were present along 100% of the length of Group 1 and 75% of the length of the other two groups by the 4th day. Aerial contamination of the blank plate resulted in the removal of the blank from the humidity chamber. The blank control plate was placed in a separate humidity chamber to prevent further contamination of the experiment, until surface molds overgrew the plate and samples. After colonization weights were compared to the original weights (Fig 1-3). Group 1 mean mass loss was slightly higher than Groups 2 and 3 (Table 1-1).

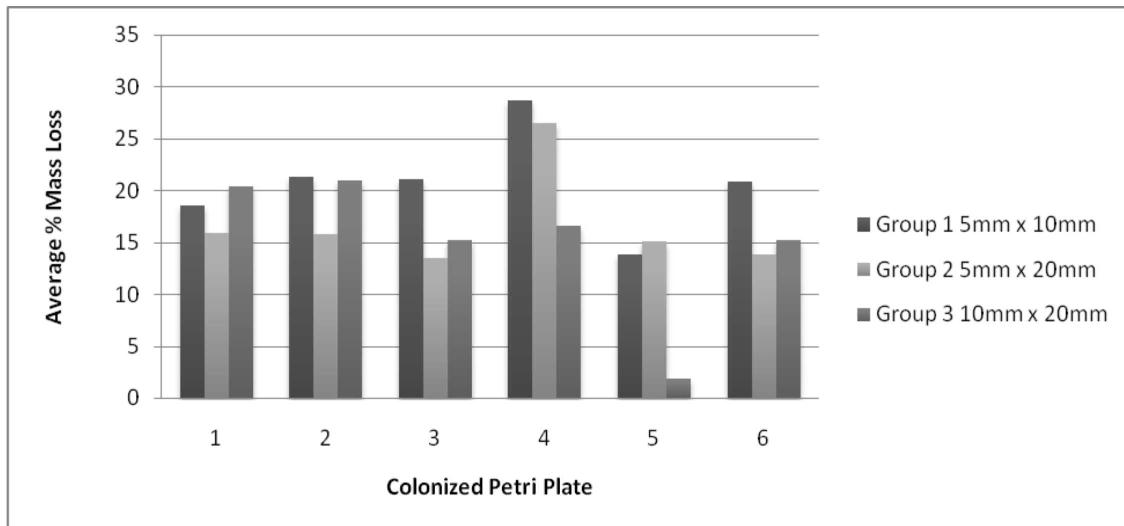

Fig 1-3. *Liriodendron tulipifera* wood samples of three size groups were placed on the transverse face along the actively growing radial edges of fully colonized Petri plates containing *Trametes versicolor.* Average mass loss per size group varied in each Petri plate shown.

Table 1-1. Summary statistics for the three *Liriodendron tulipifera* sample size groups decayed by *Trametes versicolor*.

Sample Group	Fungus	N Obs.	Mean % Mass Loss	Min % Mass Loss	Max % Mass Loss	Coefficient of Variation %
Group 1 5mm x 10mm	*Trametes versicolor*	30	20.76	3.45	35.53	38.94
Group 2 5mm x 20mm	*Trametes versicolor*	30	16.77	0.88	31.90	49.38
Group 3 10mm x 20mm	*Trametes versicolor*	30	15.04	0.21	28.37	50.09

Modified Soil-Agar Block Method Results

The modified soil-agar block sample size was similar to the combined Groups 2 and 3 of the agar plate method. Both *Populus* and *Salix* stem samples exhibited mass loss due to fungal colonization. All samples showed aerial hyphae growth on the wood surface within 7 days. Control samples from both the *Populus* and *Salix* showed some minor mass loss due to repeated cycles of drying and desiccation.

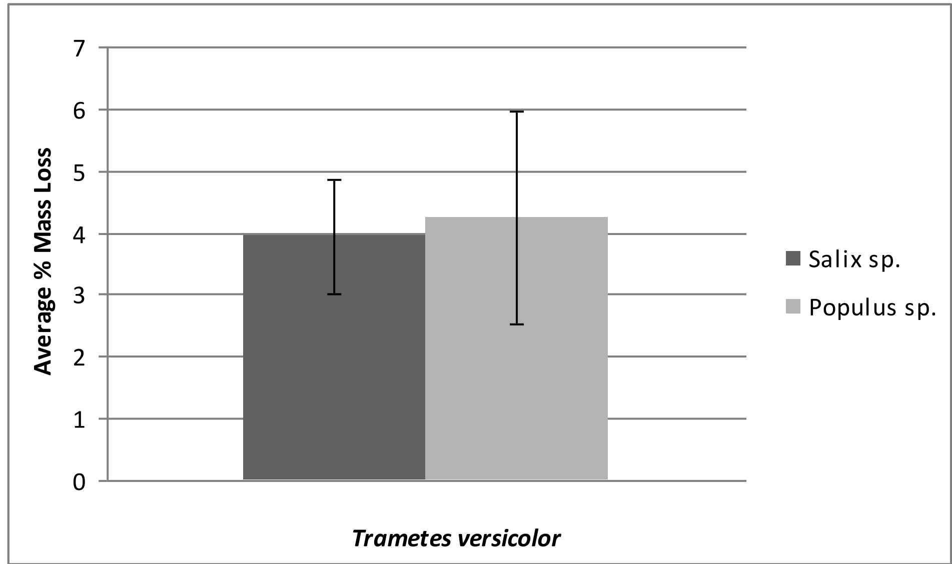

Fig 1-4. Percentage mass loss using a modified soil-agar block technique with fungus *Trametes versicolor.* Error bars denote standard deviation.

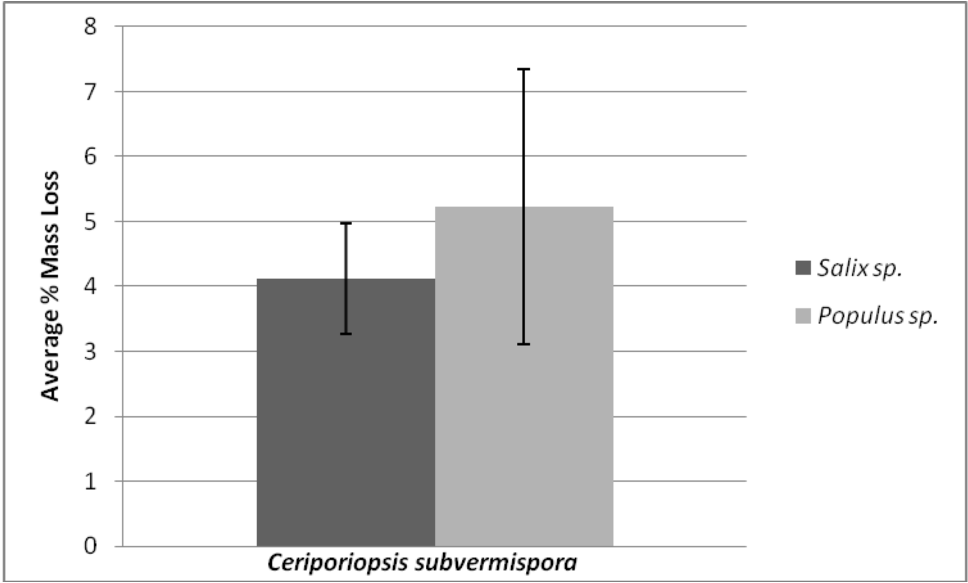

Fig 1-5. Percentage mass loss using a modified soil-agar block technique with fungus *Ceriporiopsis subvermispora* . Error bars denote standard deviation.

Table 1-2. Summary statistics per wood and fungal species.

Wood Species	Zone	Fungus	N Obs	Mean % Mass Loss	Min % Mass Loss	Max % Mass Loss	Coefficient of Variation %
Populus sp.	Upper	*Ceriporiopsis subvermispora*	17	5.23	2.96	9.28	34.07
	Lower	*Trametes versicolor*	16	4.24	2.10	17.10	40.52
Salix sp.	Upper	*Ceriporiopsis subvermispora*	12	3.84	2.77	5.88	13.40
	Lower	*Trametes versicolor*	14	3.94	2.55	5.68	23.68

Discussion

Agar Plate Method

The colonized Petri plate method fully colonized the wood samples with little to no lag time in between the agar to wood substrate. Hyphal mats were present on 100% of Group 1 sample surfaces within 4 days of colonization. At 4 days, hyphal mats covered 75% of Group 2 and 3 sample surfaces. Colonization of the sample surface was slightly faster in the smaller size Group 1, while Group 2 and 3 showed full surface colonization at about the same rate. The small sample size and length contributes to a shorter initial colonization. As expected, the different sizes were all colonized quickly and showed measurable mass loss after the 20 day colonization period. Moisture content change from oven dry to equilibrium could be a contributing factor to the delay of hyphal mats in the larger size sample groups. It was apparent the small sample size did not prevent measurable mass loss (Table 1-1). Even with the high rate of decay, heavily colonized wood samples did not lose structure due to deterioration as expected with white rot fungi.

The sample mass loss was not consistent between each Petri plate (Fig 1-3). Each Petri plate culture showed high mass loss variation. Petri plate 5 (containing samples 21-25 in each size group) had little to no change in sample mass in Group 3. Although each culture was of the same age and subcultured from one original culture, Plate 5/Group 3 limited

sample mass losses may have resulted from lower activity due to possible contamination. Plate 5/Group 3 samples showed signs of dematiaceous hyphae. A direct microscopic examination of plate 5/Group 3 found hyphal growth of a *Cladosporium* species. Competition present in Plate 5/Group 3 between the contamination and *Trametes* versicolor led to a lower mass loss in the affected samples. The susceptibility of uncolonized media areas located on open Petri plates makes maintained sterility key when using this method. Due to the possibility of sample and experiment contamination loss, this method is undesirable for small sample sets or when material for testing is limited. Sample length and size exhibit influence on variation between samples (Table 1-1). Coefficient of variation increased as sample length and size increased, thus evidence of small differences in small stem size contributes to loss variability.

The mean mass loss results of the different sample groups show small sample size in addition to the method, contributes to rapid decay and higher mass loss than other methods [Cowling 1961, Highley et al. 1970, ASTM 1413-07]. This rapid loss in mass results from both the healthy fungal network on the agar surface and inoculation surface area. The heavily colonized Petri plate contained large masses of viable hyphae contributing to the rapid primary colonization of the wood.

Modified Soil-Agar Block Method

The water agar strips seemed suitable inoculums for the small tree samples. All samples were in adequate contact with the agar strips resulting 100% successful colonization. The modified method, including both fungal species, grew hyphal mats on wood sample

transverse surfaces within 7 days. The production of a surface hyphal mat took 2-3 days longer than the agar plate method due to a combination of factors including wood sample inoculation site, fungal hyphal network, and delay of wood moisture content equilibrium. The amount of wood surface area in contact with inoculums is much greater with the agar plate method than the modified soil-agar block method possibly reducing the colonization time by a few days. Alternatively, the transverse face of the stem allows for direct colonization of trachieds and vessel elements upwards through the wood sample. The modified soil-agar block method requires the hypha to penetrate the exterior cell wall of the longitudinal face of the stem before access to the interior of the wood sample. Orientation of the wood sample in the soil-agar block method places the inoculum in the middle of the longitudinal section allowing for growth in two directions. A combination of optimum sample orientation and increased viable inoculum may have led to increased mass loss using the agar plate method.

Wood sample moisture content changes were not measured during the colonization. Differences in relative humidity of each sample jar possibly delayed the wood from reaching suitable moisture content for fungal growth. The samples in the agar plate method were in direct contact with a large surface area of agar leading to an earlier rise in moisture content. Moisture content in small samples using ASTM soil block method has been examined although high variability between results did not allow for interpretation of wood moisture content effects [De Groot et al. 1998]. Future experiments precisely examining the role of

agar strip size, wood sample orientation, and jar relative humidity would give better insight into the affect of sample design

.

The same factors that speed colonization increased mass loss in the agar plate method. *Trametes versicolor* percentage mass loss was significantly lower in the soil-agar block method although the colonization time and wood sample size were consistent with the agar plate method Group 2 and 3 (Tables 1-1 and 1-2). Since the mass loss difference between the *Populus* and *Salix* wood and two different white rot fungi species were minimal, the considerable difference in mass loss between methods is not thought to be due to wood species (Table 1-2). Mass loss difference between fungal species in the soil-agar block method was comparable even though these fungal species degrade selected wood components at different rates [Eriksson 1990]. *T. versicolor* degrades all cell wall components simultaneously while *C. subvermispora* selectively degrades lignin [Choi et al 2006]. The *Populus* species wood mass loss was similar to results from previous studies using a powdered *Populus* wood material and the lignin selective white rot fungi *C. subvermispora* [Choi et al 2006]. The modified soil-agar method exhibited lower coefficient of variation for all sample types except *T. versicolor* on *Populus sp*. wood. The higher COV in the Populus sp. Lower samples was due to one outlier sample with 17% mass loss. Removing this value from statistical analysis shows COV to be closer to values in other wood and fungi combinations tested. The lower variance between tested samples supports higher modified soil-agar method test reproducibility than the agar plate method.

Differences between Upper and Lower groups in the modified soil-agar block method were not found. The short distance between Upper and Lower zones on the tested trees had little diameter variation and compared to the agar plate method's Groups 2 and 3. Evident with the *Populus sp*. samples there was some relationship between tree species and mass loss (Fig 1-5). The relationship conceivably is a result of each tree's genotypic variations of white rot fungi resistance. Coefficient of variation dissimilarity between the Populus and Salix species woods indicates wood species is a factor when assessing suitability of the modified soil-agar block method (Table 1-2). The similarity between fungal species rate of decay should be explored in future experiments using extended colonization periods and chemical analysis of cell wall degradation.

Conclusions

The modified soil-agar block method produced more consistent mass loss in the wood samples than the agar plate method. The homogeneity of the inoculum prevents large variation between sample sets in comparison to the agar plate method tested. The high mass loss variation in the agar plate method is unsuitable for young tree experiments since elevated variation is unwanted in small trial experiments. The small round sample size was found not to adversely affect the results when combined with a shortened colonization time. Therefore, a colonization period of 20 days provides suitable time for quantifiable small tree sample white rot fungi degradation. Optimum colonization times required for statistical purposes needs to be explored in future testing. The modified soil-agar block method performance for small diameter tree samples was similar to decay tests for small and large square wood blocks. The modified soil-agar block method would be appropriate for examining decay properties of small sample sets encountered in young tree testing. Determination of sample number required to reduce sample mass loss variation will require future study. Further chemical analysis of cellular degradation is required to determine suitability for specific analysis techniques.

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Appendices

Appendix A - Raw Agar Plate Method Mass Loss Data

Sample Size	Oven Dry Weight (Grams)	Decayed Oven Dry Weight (Grams)	% Mass Loss
5mmx10mm	0.07	0.05	24.29
5mmx10mm	0.08	0.07	14.10
5mmx10mm	0.05	0.04	18.37
5mmx10mm	0.07	0.06	5.88
5mmx10mm	0.08	0.06	30.38
5mmx10mm	0.07	0.05	20.00
5mmx10mm	0.05	0.04	20.00
5mmx10mm	0.05	0.04	21.15
5mmx10mm	0.06	0.04	21.82
5mmx10mm	0.06	0.04	23.64
5mmx10mm	0.05	0.04	18.75
5mmx10mm	0.04	0.03	14.29
5mmx10mm	0.07	0.05	26.15
5mmx10mm	0.04	0.03	23.26
5mmx10mm	0.07	0.05	23.08
5mmx10mm	0.06	0.04	32.14
5mmx10mm	0.08	0.05	35.53
5mmx10mm	0.07	0.05	27.69
5mmx10mm	0.04	0.03	17.07
5mmx10mm	0.05	0.03	31.25
5mmx10mm	0.06	0.05	18.18
5mmx10mm	0.06	0.06	3.45
5mmx10mm	0.08	0.08	5.00
5mmx10mm	0.04	0.03	27.27
5mmx10mm	0.05	0.04	15.56
5mmx10mm	0.06	0.04	27.12
5mmx10mm	0.06	0.05	13.33
5mmx10mm	0.06	0.05	23.73
5mmx10mm	0.03	0.02	29.41

Sample Size	Oven Dry Weight (Grams)	Decayed Oven Dry Weight (Grams)	% Mass Loss
5mmx10mm	0.04	0.03	10.81
5mmx20mm	0.10	0.08	15.63
5mmx20mm	0.11	0.09	21.93
5mmx20mm	0.10	0.10	6.80
5mmx20mm	0.11	0.09	21.30
5mmx20mm	0.12	0.10	14.05
5mmx20mm	0.14	0.12	17.27
5mmx20mm	0.13	0.10	21.43
5mmx20mm	0.09	0.07	20.00
5mmx20mm	0.11	0.11	0.88
5mmx20mm	0.13	0.10	19.20
5mmx20mm	0.16	0.12	21.29
5mmx20mm	0.09	0.07	24.18
5mmx20mm	0.12	0.10	9.57
5mmx20mm	0.11	0.11	7.08
5mmx20mm	0.12	0.11	5.17
5mmx20mm	0.12	0.09	29.03
5mmx20mm	0.12	0.08	31.90
5mmx20mm	0.10	0.07	26.73
5mmx20mm	0.10	0.08	17.53
5mmx20mm	0.12	0.08	27.59
5mmx20mm	0.09	0.07	28.26
5mmx20mm	0.13	0.11	17.69
5mmx20mm	0.12	0.12	1.61
5mmx20mm	0.11	0.10	11.50
5mmx20mm	0.07	0.06	16.22
5mmx20mm	0.13	0.12	9.02
5mmx20mm	0.11	0.10	8.33
5mmx20mm	0.13	0.10	21.60
5mmx20mm	0.13	0.11	19.70
5mmx20mm	0.08	0.07	10.67
10mmx20mm	0.32	0.24	24.06

Sample Size	Oven Dry Weight (Grams)	Decayed Oven Dry Weight (Grams)	% Mass Loss
10mmx20mm	0.33	0.26	23.19
10mmx20mm	0.34	0.28	18.37
10mmx20mm	0.33	0.27	17.72
10mmx20mm	0.58	0.47	18.92
10mmx20mm	0.35	0.27	21.26
10mmx20mm	0.40	0.33	18.75
10mmx20mm	0.35	0.29	18.13
10mmx20mm	0.58	0.48	18.49
10mmx20mm	0.22	0.15	28.37
10mmx20mm	0.41	0.35	15.05
10mmx20mm	0.52	0.47	9.32
10mmx20mm	0.54	0.48	11.25
10mmx20mm	0.39	0.33	16.33
10mmx20mm	0.37	0.28	24.12
10mmx20mm	0.37	0.30	19.09
10mmx20mm	0.38	0.30	21.73
10mmx20mm	0.29	0.26	11.34
10mmx20mm	0.29	0.27	7.93
10mmx20mm	0.28	0.22	22.70
10mmx20mm	0.42	0.41	2.39
10mmx20mm	0.54	0.53	1.68
10mmx20mm	0.48	0.48	0.21
10mmx20mm	0.27	0.27	2.19
10mmx20mm	0.26	0.25	2.73
10mmx20mm	0.32	0.27	17.65
10mmx20mm	0.27	0.22	17.84
10mmx20mm	0.37	0.32	12.50
10mmx20mm	0.42	0.36	14.35
10mmx20mm	0.41	0.35	13.66

Appendix B - Raw Modified Agar-Block Method Mass Loss Data

Sample/ Tree Number	Wood Species	Location	Fungus	Diameter (mm)	Mass Loss %
1p	Populus	Upper	*C. subvermispora*	5.08	3.95
2p	Populus	Upper	*C. subvermispora*	6.17	4.50
3p	Populus	Upper	*C. subvermispora*	7.94	2.96
4p	Populus	Upper	*C. subvermispora*	7.32	7.05
6p	Populus	Upper	*C. subvermispora*	6.86	7.28
7p	Populus	Upper	*C. subvermispora*	10.56	4.36
8p	Populus	Upper	*C. subvermispora*	8.74	3.40
9p	Populus	Upper	*C. subvermispora*	10.93	4.22
10p	Populus	Upper	*C. subvermispora*	6.94	7.10
11p	Populus	Upper	*C. subvermispora*	7.45	7.36
12p	Populus	Upper	*C. subvermispora*	12.27	4.48
13p	Populus	Upper	*C. subvermispora*	8.75	5.32
14p	Populus	Upper	*C. subvermispora*	10.01	4.51
15p	Populus	Upper	*C. subvermispora*	11.68	3.55
16p	Populus	Upper	*C. subvermispora*	9.73	3.75
17p	Populus	Upper	*C. subvermispora*	5.64	5.84
19p	Populus	Upper	*C. subvermispora*	5.14	9.28
1p	Populus	Lower	*T. versicolor*	7.64	5.46
2p	Populus	Lower	*T. versicolor*	10.18	2.10
3p	Populus	Lower	*T. versicolor*	10.10	2.71
4p	Populus	Lower	*T. versicolor*	11.79	4.66
6p	Populus	Lower	*T. versicolor*	11.62	3.78
7p	Populus	Lower	*T. versicolor*	12.37	4.35
8p	Populus	Lower	*T. versicolor*	10.75	2.24
9p	Populus	Lower	*T. versicolor*	12.68	2.83
10p	Populus	Lower	*T. versicolor*	9.01	6.28
11p	Populus	Lower	*T. versicolor*	8.79	5.78
13p	Populus	Lower	*T. versicolor*	11.70	3.01

Sample/ Tree Number	Wood Species	Location	Fungus	Diameter (mm)	Mass Loss %
14p	Populus	Lower	*T. versicolor*	12.21	5.39
15p	Populus	Lower	*T. versicolor*	13.00	4.00
16p	Populus	Lower	*T. versicolor*	11.89	4.70
17p	Populus	Lower	*T. versicolor*	8.64	2.28
19p	Populus	Lower	*T. versicolor*		8.23
1w	Willow	Upper	*C. subvermispora*	9.80	2.77
2w	Willow	Upper	*C. subvermispora*	11.00	3.15
3w	Willow	Upper	*C. subvermispora*	8.66	4.45
4w	Willow	Upper	*C. subvermispora*	8.36	4.00
5w	Willow	Upper	*C. subvermispora*	8.69	4.37
6w	Willow	Upper	*C. subvermispora*	6.26	3.97
7w	Willow	Upper	*C. subvermispora*	9.36	3.78
8w	Willow	Upper	*C. subvermispora*	6.99	4.09
11w	Willow	Upper	*C. subvermispora*	8.86	3.68
14w	Willow	Upper	*C. subvermispora*	8.45	3.92
15w	Willow	Upper	*C. subvermispora*	10.07	4.45
16w	Willow	Upper	*C. subvermispora*	7.23	3.48
1w	Willow	Lower	*T. versicolor*	11.68	2.89
2w	Willow	Lower	*T. versicolor*	11.71	2.69
3w	Willow	Lower	*T. versicolor*	10.86	2.55
4w	Willow	Lower	*T. versicolor*	9.41	4.36
5w	Willow	Lower	*T. versicolor*	9.62	5.68
6w	Willow	Lower	*T. versicolor*	8.56	4.46
7w	Willow	Lower	*T. versicolor*	10.75	3.12
8w	Willow	Lower	*T. versicolor*	9.69	3.16
10w	Willow	Lower	*T. versicolor*	8.59	4.73
11w	Willow	Lower	*T. versicolor*	9.99	4.48
13w	Willow	Lower	*T. versicolor*	10.73	4.62
14w	Willow	Lower	*T. versicolor*	10.59	3.87
15w	Willow	Lower	*T. versicolor*	11.95	3.92
16w	Willow	Lower	*T. versicolor*	9.94	4.69

CHAPTER 2

Resistance of Genetically Modified Quaking Aspen (*Populus tremuloides*) to Lignin Selective Fungal Decay

Abstract

One-year old quaking aspen (*Populus tremuloides*) trees including a control wild type aspen and three lines of transgenic aspen trees were analyzed for resistance to lignin selective white rot fungal decay. The transgenics had reduced lignin content through transfer of an antisense -4CL gene, changed syringyl/guaiacyl ratio through insertion of a sense CAld5H gene, and modified lignin content and syringyl/guaiacyl through simultaneous insertion of -4CL and CAld5H genes. The small diameter transgenic trees were decayed using lignin selective white rot fungus *Ceriporiopsis subvermispora*. A modified soil-agar block method was used with a forty day colonization time. Mass loss was used to examine differences between genetic lines. The transgenic lines with higher S/G lignin ratio exhibited a higher mass loss percentage compared to the wild type and other transgenic lines.

Keywords: decay resistance, *Ceriporiopsis subvermispora*, transgenic trees, mass loss, biopulping

Review of Related Literature

Characterization of *Ceriporiopsis subvermispora*

Ceriporiopsis subvermispora (Pilat) Gilbertson and Ryvarden is a white rot basidiomycete belonging to the order Polyporacea. *C. subvermispora* produces an annual resupinate fruit body. The hyphae is monomitic, with hyaline generative hyphae containing clamp connections (Fig 2-1). In nature, it is found on decayed angiosperms and gymnosperms [CBS Aphyllophorales Database 2008]. This fungus has been studied extensively for the primary purpose of wood pulp pretreatment. The mechanisms for *C. subvermispora* lignin degradation are not fully characterized; manganese peroxidase is the primary mode of lignin degradation although evidence suggests a lignin peroxidase is produced by the fungus [Srebotnik et al. 1997, Guerra et al. 2004]. *C. subvermispora* lignin selectivity has been examined by several different methods on both hardwoods and softwoods. The pyrolysis and thioacidolysis analysis suggests that *C. subvermispora* selectively degrades syringyl type lignin over guaiacyl lignin [Choi et al. 2006]. Choi and authors also found increased solubility of lignin after four weeks of degradation. Curie-point pyrolysis was determined to be a better technique than gravimetric analysis to determine changes in eucalypt wood lignin when degraded by *C. subvermispora* [Rio et al. 2000].

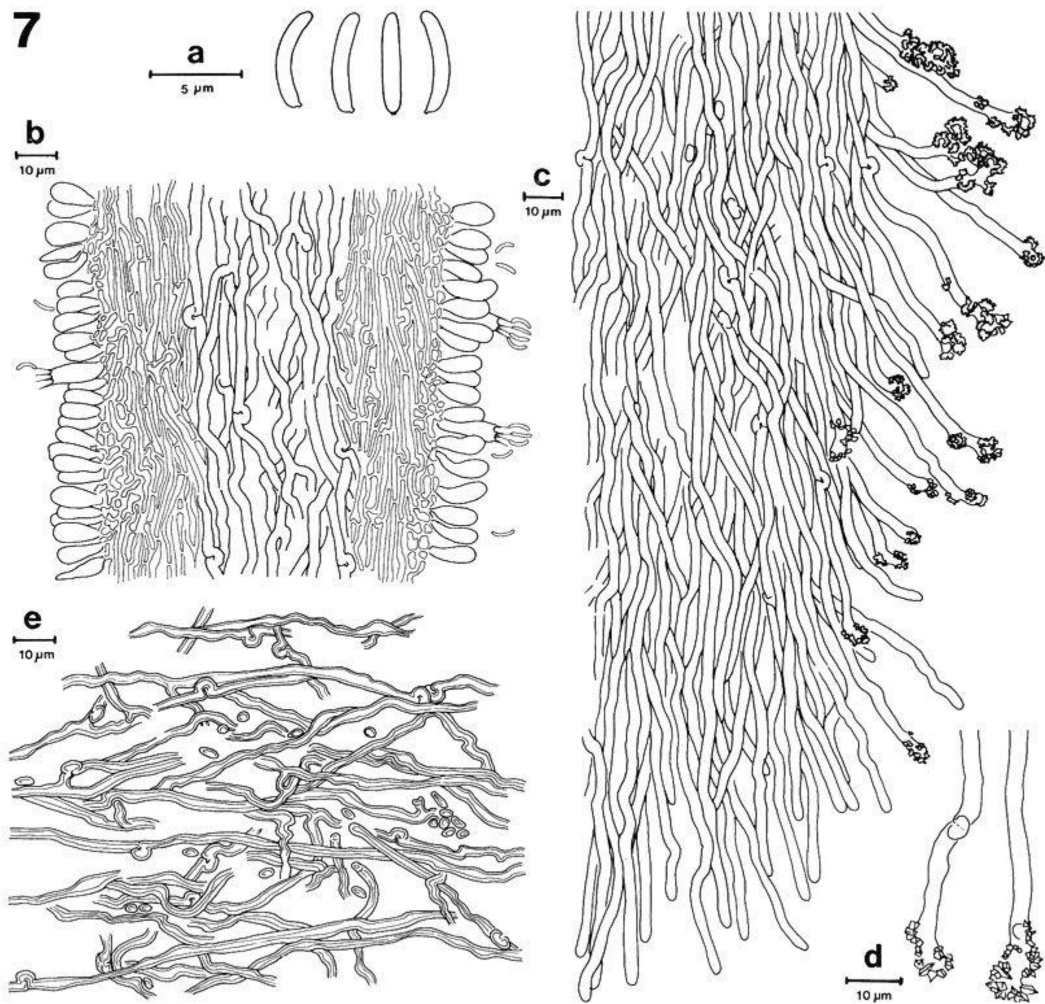

Fig 2-1. a) spores, b) dissepiment in vertical section, showing gelatinized subhymenial layers and spaced medullary hyphae, c) dissepiment edge, d) hyphal tips from the dissepiment edge, partly covered with crystals, e) section through subiculum. [Niemelä 2006, T.N.].

Biopulping with *Ceriporiopsis subvermispora*

Ceriporiopsis subvermispora (strain FP-90031-sp) was determined to be a suitable fungus for biomechanical pulping due to its high affinity for lignin and efficacy on both hardwood and softwood species (Table 1-1) [Akhtar et al. 1992].

Table 2-1. Percent loss in total weight, and in specific wood sugars in aspen and loblolly woods after fungal pretreatment for 12 weeks [Akhtar et al 1992].

% loss	Aspen	Loblolly pine
Weight	27	23
Lignin	50	38
Glucose (glucan)	7	14
Xylose (xylan)	32	30
Mannose (mannan)	31	16

Weight loss, energy savings, and physical properties of handsheets were analyzed after four weeks of fungal growth then pulped (Table 1-2). In aspen wood pretreated with *C. subvermispora*, the pulping process took 47% less energy and the burst indices increased 22% while the tear indices increased 119% over sterile controls.

Table 2-2. Properties of handsheets formed from pulp treated with *Ceriporiopsis subvermispora* [Akhtar et al 1992].

Parameters	Aspen Control	Aspen Fungus-treated	Loblolly pine Control	Loblolly pine Fungus-treated
Weight loss (%)	-	6	-	5
Energy savings (%)	-	47	-	37
Burst index (kN/g)	1.01	1.23	0.66	0.93
Tear index (mN.n^2/g)	1.65	3.62	2.18	3.36
Density (kg/m^3)	440	378	404	382
Brightness (%)	61.4	49.9	45.5	36.1
Opacity (%)	96.3	94.6	95.6	94.7
Scattering coefficient (m^2/kg)	65.3	42.6	44.1	32.1

Transgenic Aspen Lignin Biosynthesis

Genetic engineering research has discovered that suppression of the 4-coumarate ligase (4CL) gene with insertion of an antisense 4CL gene, blocks one pathway for lignin monolignol production, therefore reducing lignin production [Hu et al. 1999]. 4CL converts caffeate into caffeoyl-CoA during lignin production. Insertion of a sense coniferyl aldehyde 5-hydrohylase (CAld5H) gene increases the production ratio of syringyl to guaiacyl type lignin [Li et al. 2001]. In addition, combination effects of 4CL and CAld5H gene insertion produces trees with down regulated lignin and higher syringyl/guaiacyl ratio [Li et al. 2003].

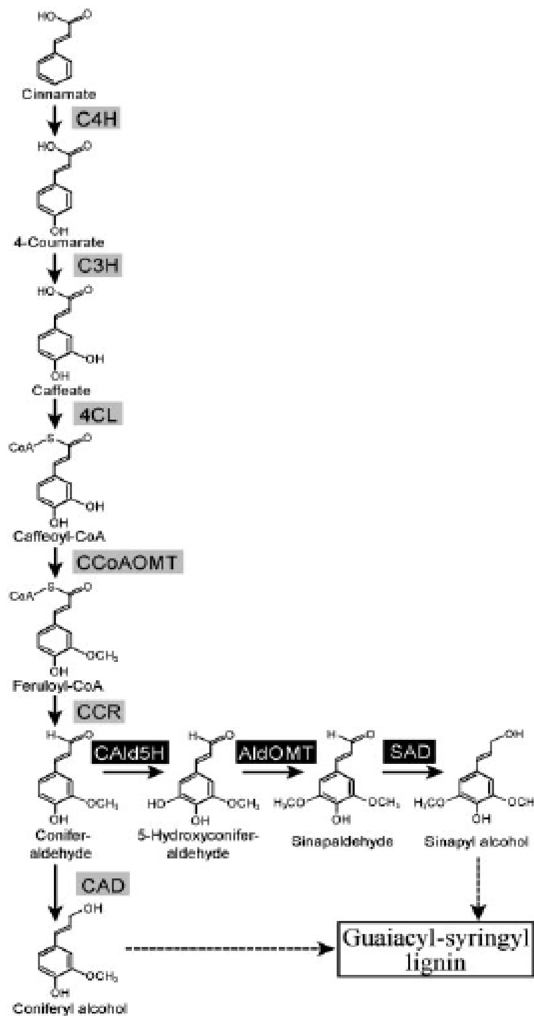

Fig 2-2. Principal biosynthetic pathway to guaiacyl and syringyl monolignols for the formation of guaiacyl:syringyl lignin in woody angiosperms.

[Li et al. 2003].

Fungi and Transgenic Aspen Tree Research

Very few studies have been conducted regarding the transgenic aspen used in this study and fungal interactions. Transgenic aspen effects on soil communities were analyzed

[Bradley et al 2007]. The authors found the interactions between soil type and genetic line made assessment of ecological impacts of transgenic aspen on soil microbial

communities difficult. In a mycorrhizal study of transgenic aspen, the structure of mycorrhizal communities was similar for most transgenic and non transgenic lines [Kaldorf et al. 2002]. Significant differences between treatments' soil and root communities were not established.

Introduction

Current research interest in lower lignin content trees has produced several transgenic lines of aspen [Hu et al. 1999, Li et al. 2003]. These lignin modifications have resulted in tree lines with modified lignin for pulping and biofuel research. The characterization of transgenic trees tailored for industry requires anatomical, mechanical, and chemical analysis to determine the best suitable lines for commercial use.

Future uses for reduced and modified lignin content wood include pulp sources for the paper and biofuel industries. These promising uses for transgenic trees are driving the development of new genetic lines [Hakala et al. 2004]. Lignin is the primary component of the middle lamella that acts as a glue holding fibers together. Reducing the adhesive qualities of lignin may result in easier pulping from the wood in down regulated lignin transgenic trees when compared with wild type wood, due to the weak bonding between the lignin and fiber polyoses. The emerging biofuel industry could also benefit from transgenic tree research. Ethanol yield from wood is low because lignin reduces efficiency of the cellulose and hemicelluloses hydrolysis process. Ethanol produced from low lignin content wood would result in increased hydrolysis of wood sugars, thus higher yield compared with traditional woods. Fungal inoculation could be used as a pretreatment in order to lower the lignin content of pulps before processing. Biopulping has been developed as an alternative method to remove lignin and extractives from pulps before paper processing [Akhtar et al. 1992,

Hakala et al. 2004]. Based on previous work, aspen wood pretreated with lignin selective a white rot fungus produced handsheets with
less energy and improved sheet quality [Akhtar et al. 1992]. A fungal pretreatment that removes lignin could provide processing reductions thus lowering pulping cost when combined with lower lignin content wood [Otjen et al. 1985, Akhtar et al. 1992, Martinez et al. 2005]. Biopulping with a lignin selective white rot fungus has not yet been studied on modified or reduced lignin transgenic trees until now.

Lignin selective white rot fungi are best suited for biopulping applications due to their low affinity for wood sugars. Since many species are wood saprobes, white rot fungi grow well on wood chip and pulp substrates. Although there are many lignin selective white rot fungi, a species of particular interest to biopulping is *Ceriporiopsis subvermispora*. *C. subvermispora* degrades primarily lignin, while using little carbohydrates [Akhtar et al. 1992, Schwanniger et al. 2004]. This characteristic decay is important in that the fungus leaves the cellulose and hemicelluloses behind. *C. subvermispora* has also demonstrated an affinity for syringyl type lignin during wood degradation [Ferraz et al. 2004, Choi et al. 2006]. *C. subvermispora*, in addition to breaking β-O-4 bonds, removes methoxy groups from the syringyl lignin monomer leaving a guaiacyl monomer, in effect lowering the S/G ratio [Ferraz et al. 2004, Martinez et al. 2005, Choi et al. 2006]. In this study, we used *C. subvermispora* to characterize the effect of lignin decay on the reduced lignin and increased S/G ratio transgenic material. Characterizing *C. subvermispora* decay of transgenic wood is an important step in the development of future biopulping procedures.

Objectives

The objective of this study was to determine differences in mass loss between young small diameter transgenic aspen trees with modified lignin concentrations and type when decayed by a lignin selective white rot fungus. The genetic lines provided a novel opportunity to determine decay differences between wide variations of lignin type and content.

Material and Methods

Mass loss is the standard method of analysis to determine the resistance of woods to fungal decay [Cowling 1961, Highley et al. 1970, Smith et al. 1996, De Groot et al. 1998, ASTM 2007]. Mass loss was used in this study to determine the susceptibility of the wild type and transgenic lines to lignin selective white rot fungal decay.

In this experiment, *Ceriporiopsis subvermispora* FP-90031-sp (a lignin selective white rot fungus) was used. One year old quaking aspen trees were cultivated in a greenhouse. A total of forty-five trees were harvested for modified soil-agar block decay testing. Samples along the stem were cut from 9 wild type aspen trees, 16 aspen trees with reduced lignin content, 9 aspen trees with increased syringyl lignin to guaiacyl lignin ratio, and 11 aspen trees with modified lignin content and syringyl/guaiacyl. Based on previous work, several genetic lines demonstrated variation in lignin content and S/G ratio within transgenic groups (Table 2-1.)

Table 2-3. Summary of *Populus tremuloides* lignin contents and S/G ratios per genetic line [Li et al. 2003].

Genetic Treatments	PtrWT (wild type)	Ptr4CL (reduced lignin)			PtrCAld5H (increased S/G ratio)		Ptr4CL/CAld5H (reduced lignin and increased S/G ratio)	
Genetic Lines	271	21	23	37	94	96	72	141
Lignin Content (%)	22.2	16.0	14.4	14.9	20.7	21.1	13.7	10.7
Cellulose Content (%)	41.4	43.1	44.8	ND	43.4	ND	49.2	53.3
S/G Ratio	2.2	2.1	2.2	2.1	4.9	5.5	3.6	2.7

*ND denotes not determined

Modified Soil-Agar Block Inoculation

A total of forty-five trees with diameters ranging from 5 to 13mm were debarked and cut into 20mm lengths. Samples were oven dried for 48 hours at 105°C. Samples were weighed to determine oven dry weight. Two oz jars were filled with 0.8 grams of vermiculite and 4ml distilled water (Fig 2-1). A needlepoint plastic grid was placed on top of the moist vermiculite in order to prevent saturation of wood samples. Wood samples were placed in each jar then steam sterilized. *C. subvermispora* was cultured on Malt Extract Agar with 0.01% chloramphenicol Petri plates and incubated at room temperature for 7-10 days. A plug of colonized MEA was then placed on the center of a Petri plate containing 2% water agar and incubated at room temperature for 7-10 days. Five mm x 20mm strips of mycelium were

cut from the 2% water agar colonized Petri plate and placed on each plastic grid. Wood samples were then placed longitudinally perpendicular to the agar strips [Giles et al. 2008a unpublished]. Additional control samples of each wild type and transgenic line were cut and used to verify sterility and placed in jars without agar strips. The mouth of each jar was sealed with a layer of Parafilm. Sample jars were incubated at room temperature in darkness for 40 days. After colonization, samples were brushed lightly to remove surface hyphae and oven dried for 48 hours at 105°C. Samples were reweighed to determine mass loss.

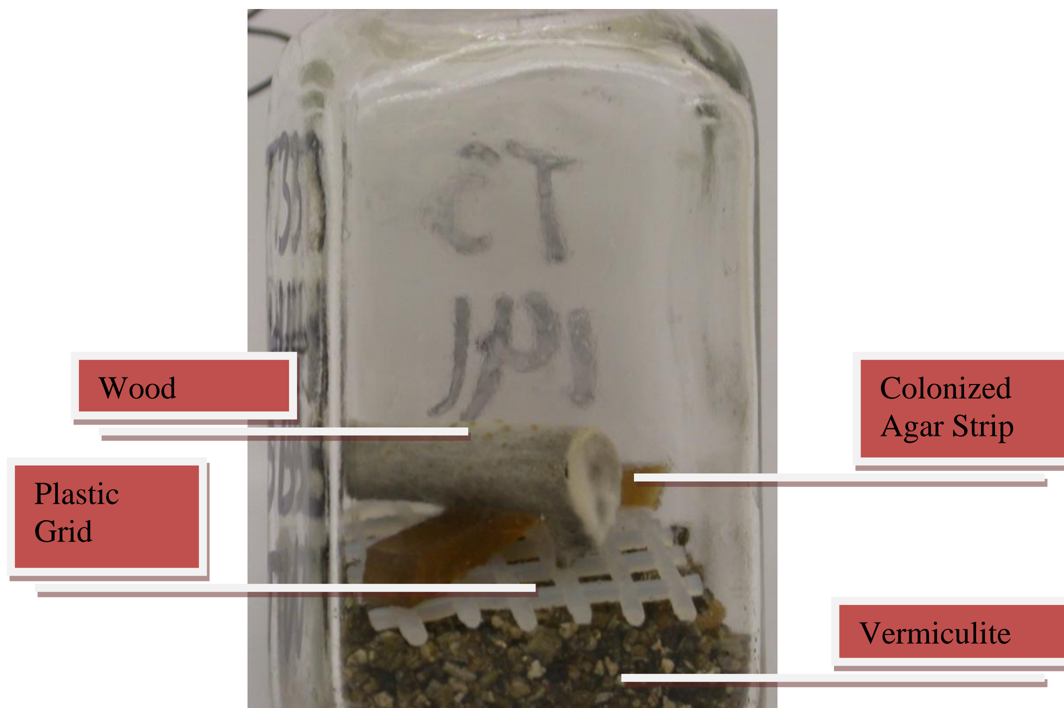

Fig 2-2. Small diameter wood samples were placed longitudinally upon agar strips colonized with white rot fungi. Vermiculite provided a sterile and inert media maintaining high relative humidity within the jar. The agar block and wood sample rested on a small plastic grid to prevent saturation of the sample from the moist vermiculite.

Statistical Analysis

Mass loss of fungal decay was analyzed to test the effect of treatment (wild-type and transgenic groups) and genetic line using descriptive statistics and generalized linear model (GLM) procedure in SAS® Enterprise Guide 4.1 (SAS Institute Inc, 2006). For initial analysis, the following linear model was used:

$$Y_{ijkl} = \mu + TRT_i + L_j + T(L)_k + D_{ijkl} + \varepsilon_{ijkl}$$

Y_{ijkl}	-	mass loss (dependent variable)
μ	-	overall mean
TRT_i	-	effect of ith treatment (wild-type and transgenic groups)
L_j	-	effect of jth genetic line within a treatment
$T(L)_k$	-	effect of kth tree within a genetic line
D_{ijkl}	-	effect of growth (stem diameter) of lth sample of kth tree in j^{th} genetic line of ith treatment
ε_{ijkl}	-	random error

The GLM procedure showed that neither the model nor the effects were significant. Thus, mass loss was not effect by the stem diameter and therefore it was removed from the model. The final linear model was:

$$Y_{ijkl} = \mu + TRT_i + L_j + T(L)_k + \varepsilon_{ijkl}$$

Duncan multiple range tests were used to determine significant differences at. Bartlett`s tests were used to verify the assumption for equal variances. Using the GLM procedure, the effect of the genetic line was not significant at α=0.05. The model showed that there was tree to tree variation in the increased S/G ratio lines.

Microscopy

Verification of sample colonization was determined by surface tape sampling and observed by light microscopy. Lacto-phenol cotton blue was used to stain the hyaline hyphae. Degradation images were obtained on desiccated samples with a JOEL model JSM-6400F scanning electron microscope.

Results

Wood samples exhibited mass loss in all genetic lines inoculated with *C. subvermispora*. The average mass loss within the wild type, reduced lignin, and simultaneous reduced lignin- increased S/G ratio lines was similar within 4% (Table 2-4). The mean mass loss for the lines with increased S/G ratio transgenic lines (94 and 96) was significantly higher than those of the wild type and other transgenic lines (Table 2-4). Comparisons between treatment mass loss means indicate a significantly higher mass loss in the increased S/G ratio lines compared to the other treatments (Table 2-4).

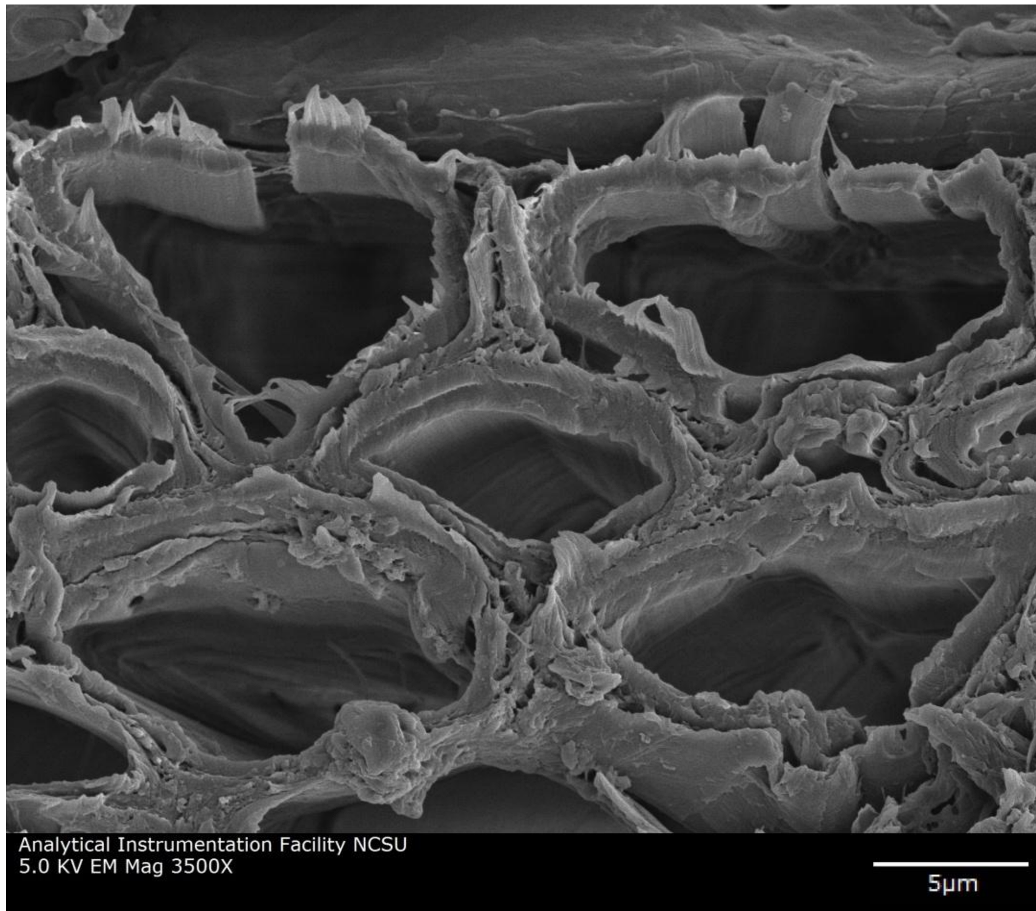

Fig 2-3. SEM transverse section of wild-type (line 271) aspen sample after 40 days inoculation. Selective deligninification by *Ceriporiopsis subvermispora* due to the removal of the middle lamellae between fibers.

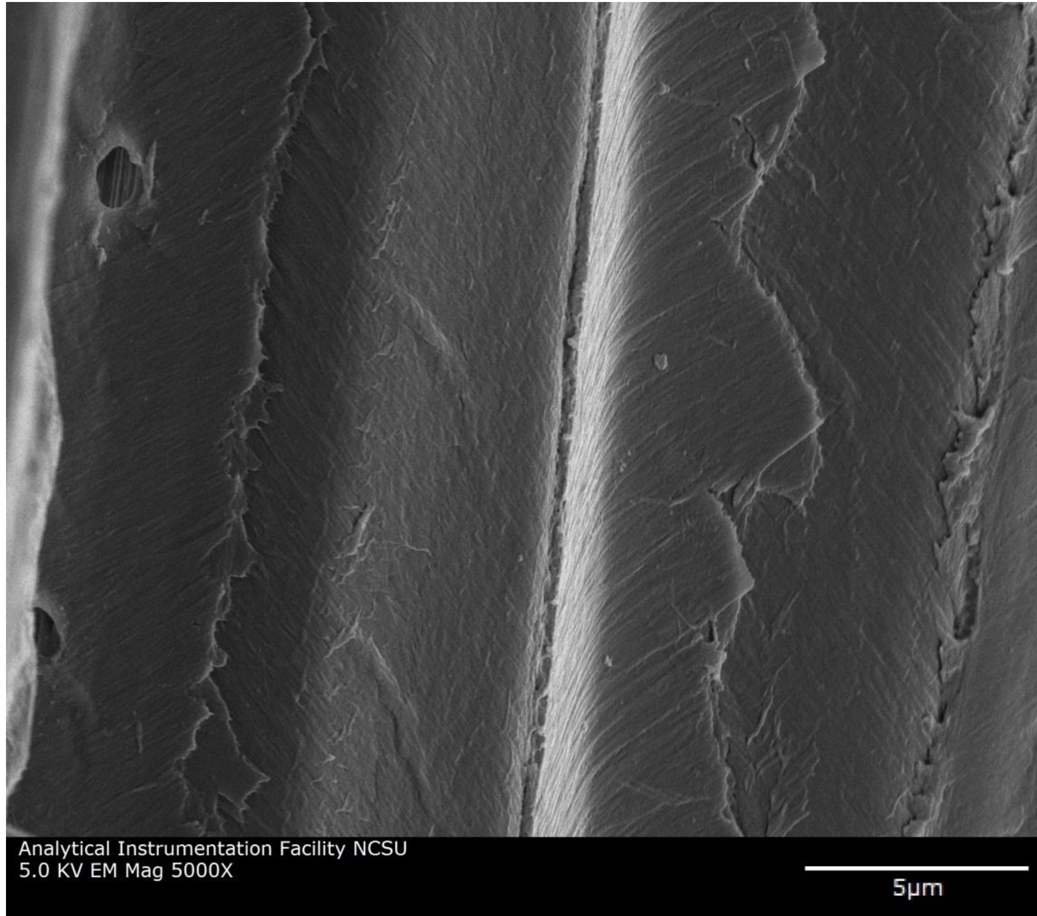

Fig 2-4. SEM radial section of wild-type (line 271) aspen sample after 40 days inoculation. Fiber cell wall erosion due to *Ceriporiopsis subvermispora*.

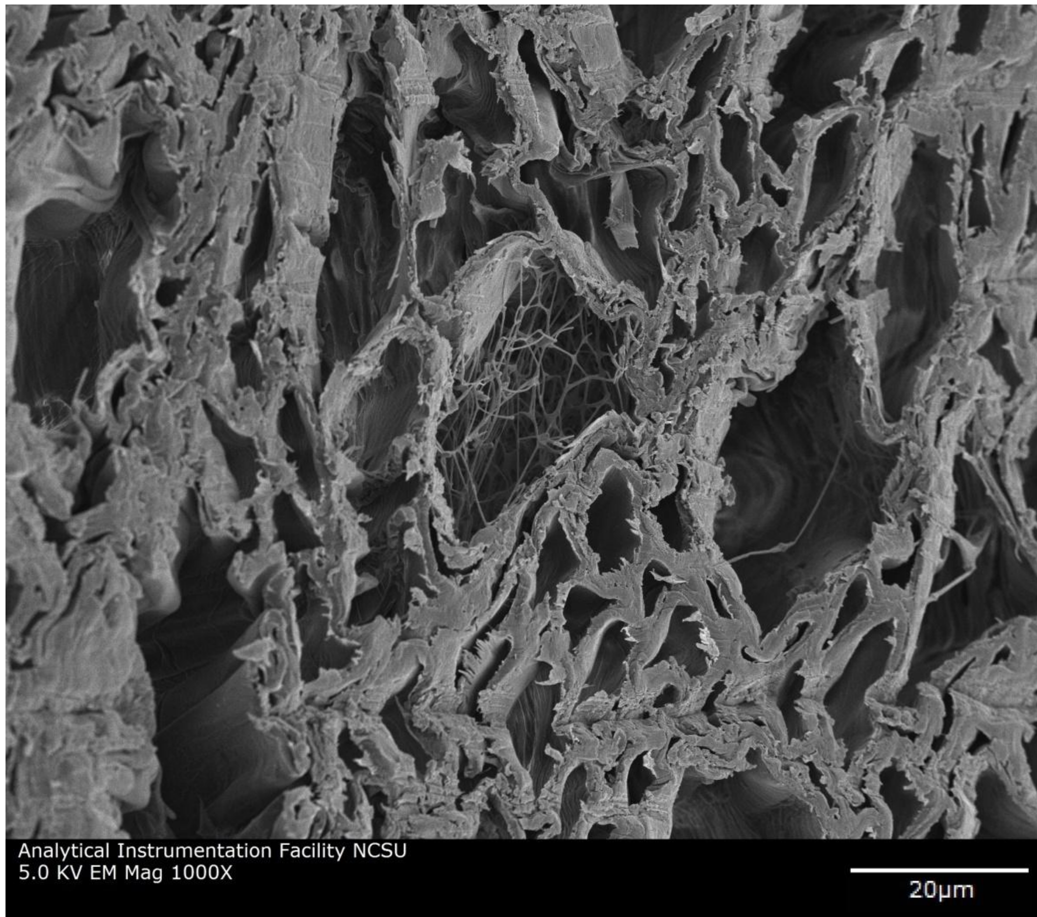

Fig 2-3. SEM transverse section of reduced lignin/increased S/G (line 141) sample after 40 days inoculation. Ceriporiopsis subvermispora hyphae present in the vessel element.

Table 2-4. Summary of *Populus tremuloides* mass loss percentages per genetic treatment and line degraded by *Ceriporiopsis subvermispora*. Table includes Duncan grouping for mean mass loss comparison between genetic treatments. Means with the same letter are not significantly different.

	Mass Loss %			
	Fungus			**N**
	C. subvermispora			
	Mean %	Duncan Grouping	CV %	
Treatment				
T0	11.43	B	34.61	34
T1	12.59	B	26.32	69
T2	15.21	A	27.49	38
T3	11.66	B	30.10	46

Discussion

The lignin selective white rot fungus *C. subvermispora* decayed the aspen wood in both the wild type and transgenic lines. All of the genetic lines tested decayed to some extent. The wild type and transgenic lines mass loss percentage range were within a range of 11-15 % (Table 2-4). The small differences in mass loss suggest the transgenic lines do not exhibit higher mass loss resistance to lignin selective white rot fungal decay. The alteration of lignin concentration and type does not severely impact the resistance of wood to fungal degradation, although significant differences between treatments were observed. Though general observations indicate lignin type contributes to fungal decay there are indications that other wood components may be responsible for mass loss.

Previous research into the affect of lignin concentration and type suggests that lignin type is the limiting determinate of decay. Although the results are ambiguous since different species of wood has been used for comparison of lignin type [Obst et al. 1994]. Only until now has a specific wood species with varying lignin concentrations and types been tested for fungal resistance. *C. subvermispora* has been well characterized on wild type aspen wood [Akhtar et al 1992, Choi et al. 2006]. The mass losses obtained by previous experiments on aspen wood are much lower than obtained with the modified-agar block technique used in this study. The differences in mass loss are explained by the raw material and sample

preparation. In work by Akhtar and Choi, the samples were ground or chips, thus destroying the matrix of vessel elements and fiber lumens needed for quick colonization.

Several factors resulted in the minimal mass loss differences between the wild type and reduced lignin lines. Based on previous experiments, the reduced lignin lines although significantly lower in lignin content, do not adversely affect the rate of lignin degradation by *C. subvermispora* [Giles et al. 2008b unpublished]. Although the lignin concentration is lower in both the Ptr4CL and Ptr4CL/CAld5H genetic lines, the lignin is available for the fungus to degrade during the short forty day colonization.

Since the rate of lignin degradation is evidenced to stay the same, it is likely expected that if the colonization time was increased beyond forty days, the reduced lignin lines would eventually have little accessible lignin as degradation progressed. Therefore, if a longer colonization time were used as degradation progressed, the reduced lignin lines would demonstrate decreasing mass loss percentages and the wild type and increased S/G lines would continue to exhibit mass loss. The expected mass loss increase from additional colonization time is small. In previous work by Choi et al. 2006, aspen wood decayed for 4 weeks compared to 6 weeks exhibited only 1% increase in mass loss.

In previous studies, the lignin selective white rot fungus *C. subvermispora* was demonstrated to preferentially degrade syringyl type lignin over guaiacyl [Ferraz et al 2003, Choi et al 2006]. Both of the increased S/G ratio lines (94 and 96) in this study demonstrated significantly higher mass loss in comparison to other genetic lines (Table 2-4). There are two possibilities of why the increased S/G lines exhibited higher mass loss. One explanation is

the mass loss of the increased S/G ratio line may have resulted from the fungus preferentially degrading the abundant syringyl-type lignin. Since

C. subvermispora degrades primarily syringyl lignin, the plentiful syringyl type lignin in the increased S/G ratio lines may have translated to a moderate increase of mass loss. Although the lines containing both gene insertions have a slightly higher S/G ratio, the CAld5H lines (94 and 96) are considerably higher (Table 2-3). The minimal mass loss percentage variation of the Ptr4CL/CAld5H lines may reflect the degree of lignin type modification needed to increase mass loss by white rot within a short forty day decay period. The other possible reason for increased mass loss may be a hemicelluloses-lignin interaction. The higher proportion of syringyl lignin may result in reduced linkage between hemicelluloses in the cell wall. The reduced linkages to lignin in the cell wall may leave the hemicelluloses unprotected from fungal hydrolysis. The higher mass loss percentage suggests that either additional lignin components (such as methoxy groups) or hemicelluloses were removed in the increased S/G lines compared with the other lines. Chemical analysis suggests that little to no lignin was removed in the increased S/G ratio lines and no increase in cellulose decay [Giles et al. 2008b unpublished]. These results could be further investigated with determination of sugar analysis and S/G ratio post decay. Mass loss is useful indicator of lignin decay if it can be assumed that the components are degrading and leaving the material. Early chemical analysis of the remaining material indicates a possible modification of the lignin in the increased S/G ratio lines. Although there is mass loss, the percentage lignin in the sample does not change [Giles et al. 2008b unpublished]. The particular cell wall

component (cellulose, lignin, etc) losses cannot be determined by mass loss measurement or by microscopy. Future chemical analysis of the lignin by thioacidolysis or nuclear magnetic resonance may reveal modifications of lignin during decay not detectible by mass loss analysis.

Conclusions

All aspen tree genetic lines were degraded by *C. subvermispora* during the forty day colonization period. The mass loss percentages were comparable between the wild type and transgenic lines with the increased S/G ratio line having a slightly higher mass loss percentage. A slightly higher yet significant mass loss percentage of the increased S/G ratio lines may have been due to the fungal degradation preference for syringyl type lignin or reduced linkages between lignin and hemicelluloses. The mass loss percentage differences between transgenic lines due to fungal decay were measurable and evident when using small diameter young tree samples. Therefore, mass loss can be used to measure fungal degradation differences between young small diameter transgenic trees with altered lignin. Future chemical analysis of cellular degradation is required to determine specific lignin modifications. The combination of a high S/G ratio wood and lignin selective white rot fungi could provide more manufacturing cost savings than other genetic lines. The increase in lignin removal during degradation shows the high S/G ratio aspen genetic lines to be most suited for biodegradation of lignin.

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Appendices

Appendix A - Raw Mass Loss Data

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T0	271.00	PtrWT	3.00	c	0.26	6.37	0.22	15.40	*C. subvermispora*	2
T0	271.00	PtrWT	4.00	a	0.36	7.40	0.31	14.11	*C. subvermispora*	2
T0	271.00	PtrWT	4.00	b	0.28	7.06	0.26	7.15	*C. subvermispora*	2
T0	271.00	PtrWT	4.00	c	0.28	6.82	0.22	20.15	*C. subvermispora*	2
T0	271.00	PtrWT	1.00	c	0.38	7.66	0.34	9.98	*C. subvermispora*	2
T0	271.00	PtrWT	2.00	a	0.29	6.69	0.24	16.07	*C. subvermispora*	2
T0	271.00	PtrWT	2.00	b	0.24	6.57	0.19	20.46	*C. subvermispora*	2
T0	271.00	PtrWT	2.00	c	0.26	6.28	0.24	9.08	*C. subvermispora*	2
T0	271.00	PtrWT	3.00	a	0.31	6.62	0.29	7.89	*C. subvermispora*	2
T0	271.00	PtrWT	3.00	b	0.26	6.63	0.23	9.25	*C. subvermispora*	2
T0	271.00	PtrWT	1.00	a	0.45	8.02	0.41	9.55	*C. subvermispora*	2
T0	271.00	PtrWT	1.00	b	0.38	7.93	0.37	3.85	*C. subvermispora*	2
T0	271.00	PtrWT	9.00	a	0.44	8.08	0.38	15.18	*C. subvermispora*	2
T0	271.00	PtrWT	9.00	b	0.33	7.86	0.29	12.59	*C. subvermispora*	2
T0	271.00	PtrWT	1000.00	b	0.34	6.87	0.30	11.57	*C. subvermispora*	2
T0	271.00	PtrWT	14.00	b	0.49	8.45	0.45	7.56	*C. subvermispora*	2
T0	271.00	PtrWT	1000.00	a	0.41	7.76	0.36	11.28	*C. subvermisporaa*	2
T0	271.00	PtrWT	14.00	a	0.58	9.28	0.52	10.36	*C. subvermispora*	2
T0	271.00	PtrWT	16.00	b	0.47	9.09	0.44	5.96	*C. subvermispora*	2
T0	271.00	PtrWT	16.00	a	0.60	8.83	0.52	13.19	*C. subvermispora*	2
T0	271.00	PtrWT	13.00	b	0.46	8.27	0.41	11.84	*C. subvermispora*	2

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T0	271.00	PtrWT	13.00	a	0.38	8.20	0.35	7.18	*C. subvermispora*	2
T0	271.00	PtrWT	1.00	a	0.43	8.38	0.39	8.44	*C. subvermispora*	1
T0	271.00	PtrWT	1.00	b	0.39	8.01	0.35	10.52	*C. subvermispora*	1
T0	271.00	PtrWT	1.00	c	0.38	7.09	0.34	11.21	*C. subvermispora*	1
T0	271.00	PtrWT	2.00	a	0.31	6.71	0.27	10.96	*C. subvermispora*	1
T0	271.00	PtrWT	2.00	b	0.26	6.86	0.22	15.79	*C. subvermispora*	1
T0	271.00	PtrWT	2.00	c	0.23	6.22	0.20	13.57	*C. subvermispora*	1
T0	271.00	PtrWT	5.00	c	0.26	6.80	0.25	4.89	*Sterile*	2
T0	271.00	PtrWT	5.00	a	0.31	6.67	0.30	0.88	*Sterile*	2
T0	271.00	PtrWT	5.00	b	0.29	6.59	0.28	4.04	*Sterile*	2
T0	271.00	PtrWT	19.00	a	0.41	8.08	0.40	4.00	Sterile	2
T0	271.00	PtrWT	19.00	b	0.41	8.14	0.40	4.03	Sterile	2
T0	271.00	PtrWT	5.00	c	0.20	5.75	0.19	1.85	Sterile	1
T1	21.00	Ptr4CL	3.00	c	0.41	7.93	0.37	11.72	*C. subvermispora*	2
T1	21.00	Ptr4CL	2.00	b	0.34	7.12	0.28	17.28	*C. subvermispora*	2
T1	21.00	Ptr4CL	2.00	c	0.24	6.06	0.21	11.01	*C. subvermispora*	2
T1	21.00	Ptr4CL	3.00	a	0.50	8.30	0.44	11.73	*C. subvermispora*	2
T1	21.00	Ptr4CL	4.00	a	0.36	7.36	0.32	11.23	*C. subvermispora*	2
T1	21.00	Ptr4CL	1.00	a	0.32	6.75	0.29	11.46	*C. subvermispora*	2
T1	21.00	Ptr4CL	1.00	b	0.27	6.74	0.23	15.08	*C. subvermispora*	2
T1	21.00	Ptr4CL	2.00	a	0.37	7.22	0.31	16.55	*C. subvermispora*	2
T1	21.00	Ptr4CL	400.00	a	0.71	11.18	0.63	11.20	*C. subvermispora*	2
T1	21.00	Ptr4CL	400.00	b	0.78	10.39	0.68	12.54	*C. subvermispora*	2
T1	21.00	Ptr4CL	401.00	a	0.38	7.42	0.34	9.01	*C. subvermispora*	2
T1	21.00	Ptr4CL	401.00	b	0.40	7.94	0.35	13.39	*C. subvermispora*	2
T1	23.00	Ptr4CL	3.00	b	0.27	6.43	0.25	7.25	*C. subvermispora*	2
T1	23.00	Ptr4CL	3.00	a	0.25	5.83	0.20	20.02	*C. subvermispora*	2

Treatment	Line		Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T1	23.00	Ptr4CL	2.00	a	0.40	7.14	0.36	8.58	*C. subvermispora*	2
T1	23.00	Ptr4CL	4.00	a	0.36	6.53	0.31	13.45	*C. subvermispora*	2
T1	23.00	Ptr4CL	4.00	c	0.38	7.00	0.36	6.43	*C. subvermispora*	2
T1	23.00	Ptr4CL	212.00	a	0.72	9.12	0.69	4.83	*C. subvermispora*	2
T1	23.00	Ptr4CL	1.00	c	0.14	4.46	0.11	18.74	*C. subvermispora*	2
T1	23.00	Ptr4CL	1.00	a	0.34	6.83	0.30	12.67	*C. subvermispora*	2
T1	23.00	Ptr4CL	3.00	c	0.38	7.37	0.34	9.90	*C. subvermispora*	2
T1	23.00	Ptr4CL	2.00	b	0.39	7.23	0.35	10.97	*C. subvermispora*	2
T1	23.00	Ptr4CL	208.00	b	0.49	8.07	0.42	13.43	*C. subvermispora*	2
T1	23.00	Ptr4CL	212.00	b	0.71	9.82	0.63	10.88	*C. subvermispora*	2
T1	23.00	Ptr4CL	213.00	a	0.49	8.39	0.42	14.00	*C. subvermispora*	2
T1	23.00	Ptr4CL	213.00	b	0.47	8.03	0.42	10.09	*C. subvermispora*	2
T1	23.00	Ptr4CL	208.00	a	0.47	7.97	0.41	12.32	*C. subvermispora*	2
T1	23.00	Ptr4CL	211.00	b	0.63	9.42	0.56	10.30	*C. subvermispora*	2
T1	23.00	Ptr4CL	211.00	a	0.64	8.99	0.56	13.00	*C. subvermispora*	2
T1	37.00	Ptr4CL	3.00	c	0.22	5.52	0.18	15.78	*C. subvermispora*	2
T1	37.00	Ptr4CL	4.00	a	0.24	5.84	0.20	15.23	*C. subvermispora*	2
T1	37.00	Ptr4CL	1.00	b	0.21	6.09	0.19	12.20	*C. subvermispora*	2
T1	37.00	Ptr4CL	1.00	a	0.27	6.47	0.24	11.22	*C. subvermispora*	2
T1	37.00	Ptr4CL	2.00	a	0.19	5.68	0.16	14.07	*C. subvermispora*	2
T1	37.00	Ptr4CL	4.00	b	0.22	5.87	0.18	14.94	*C. subvermispora*	2
T1	37.00	Ptr4CL	4.00	c	0.22	5.76	0.18	15.95	*C. subvermispora*	2
T1	37.00	Ptr4CL	1.00	c	0.22	5.67	0.19	16.94	*C. subvermispora*	2
T1	37.00	Ptr4CL	2.00	c	0.17	5.05	0.14	16.46	*C. subvermispora*	2

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T1	37.00	Ptr4CL	2.00	b	0.18	5.48	0.14	18.53	*C. subvermispora*	2
T1	37.00	Ptr4CL	3.00	b	0.29	6.75	0.26	10.38	*C. subvermispora*	2
T1	37.00	Ptr4CL	3.00	a	0.33	6.68	0.27	18.56	*C. subvermispora*	2
T1	37.00	Ptr4CL	310.00	a	0.41	6.88	0.35	13.43	*C. subvermispora*	2
T1	37.00	Ptr4CL	310.00	b	0.34	6.76	0.29	15.62	*C. subvermispora*	2
T1	37.00	Ptr4CL	306.00	a	0.64	9.50	0.60	6.93	*C. subvermispora*	2
T1	37.00	Ptr4CL	306.00	b	0.56	9.48	0.50	11.50	*C. subvermispora*	2
T1	37.00	Ptr4CL	308.00	a	0.31	6.98	0.27	12.91	*C. subvermispora*	2
T1	37.00	Ptr4CL	308.00	b	0.33	6.57	0.29	12.81	*C. subvermispora*	2
T1	37.00	Ptr4CL	309.00	b	0.91	12.03	0.83	9.58	*C. subvermispora*	2
T1	37.00	Ptr4CL	309.00	a	0.93	10.90	0.85	8.17	*C. subvermispora*	2
T1	37.00	Ptr4CL	1.00	a	0.37	6.59	0.35	6.61	*C. subvermispora*	1
T1	37.00	Ptr4CL	1.00	b	0.24	6.17	0.21	13.20	*C. subvermispora*	1
T1	37.00	Ptr4CL	1.00	c	0.22	5.55	0.19	12.10	*C. subvermispora*	1
T1	37.00	Ptr4CL	2.00	a	0.21	5.68	0.18	11.95	*C. subvermispora*	1
T1	37.00	Ptr4CL	2.00	b	0.18	5.09	0.16	13.16	*C. subvermispora*	1
T1	37.00	Ptr4CL	2.00	c	0.17	4.97	0.14	15.03	*C. subvermispora*	1
T1	21.00	Ptr4CL	5.00	c	0.26	6.21	0.25	4.25	*Sterile*	2
T1	21.00	Ptr4CL	5.00	a	0.36	5.64	0.35	3.84	*Sterile*	2
T1	21.00	Ptr4CL	404.00	b	0.82	11.26	0.81	1.10	Sterile	2
T1	21.00	Ptr4CL	404.00	a	0.83	10.99	0.82	0.31	Sterile	2
T1	23.00	Ptr4CL	5.00	b	0.19	5.33	0.18	4.82	*Sterile*	2
T1	23.00	Ptr4CL	5.00	a	0.22	5.51	0.21	3.83	*Sterile*	2
T1	23.00	Ptr4CL	219.00	b	0.59	9.62	0.58	2.17	Sterile	2
T1	23.00	Ptr4CL	219.00	a	0.60	9.59	0.60	0.43	Sterile	2
T1	37.00	Ptr4CL	5.00	a	0.33	7.00	0.31	5.84	*Sterile*	2
T1	37.00	Ptr4CL	5.00	c	0.41	7.74	0.40	3.90	*Sterile*	2
T1	37.00	Ptr4CL	5.00	b	0.39	7.15	0.39	1.17	*Sterile*	2

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T1	37.00	Ptr4CL	5.00	a	0.35	7.96	0.34	1.26	Sterile	1
T1	37.00	Ptr4CL	5.00	b	0.47	7.78	0.46	2.12	Sterile	1
T1	37.00	Ptr4CL	5.00	c	0.51	7.84	0.50	2.41	Sterile	1
T2	94.00	PtrCAld5H	4.00	c	0.13	4.73	0.11	18.79	*C. subvermispora*	2
T2	94.00	PtrCAld5H	1.00	b	0.17	4.93	0.15	10.17	*C. subvermispora*	2
T2	94.00	PtrCAld5H	2.00	a	0.14	5.01	0.11	20.27	*C. subvermispora*	2
T2	94.00	PtrCAld5H	3.00	a	0.14	4.14	0.12	15.17	*C. subvermispora*	2
T2	94.00	PtrCAld5H	1.00	c	0.16	5.14	0.14	12.20	*C. subvermispora*	2
T2	94.00	PtrCAld5H	4.00	a	0.17	5.05	0.14	15.17	*C. subvermispora*	2
T2	94.00	PtrCAld5H	3.00	b	0.08	3.66	0.07	21.45	*C. subvermispora*	2
T2	94.00	PtrCAld5H	2.00	b	0.15	4.68	0.12	21.77	*C. subvermispora*	2
T2	94.00	PtrCAld5H	4.00	b	0.14	4.99	0.11	18.98	*C. subvermispora*	2
T2	94.00	PtrCAld5H	1.00	a	0.27	5.54	0.23	12.62	*C. subvermispora*	2
T2	94.00	PtrCAld5H	706.00	a	0.36	7.74	0.33	8.16	*C. subvermispora*	2
T2	94.00	PtrCAld5H	706.00	b	0.37	7.23	0.34	9.12	*C. subvermispora*	2
T2	94.00	PtrCAld5H	700.00	b	0.21	5.93	0.18	13.36	*C. subvermispora*	2
T2	94.00	PtrCAld5H	700.00	a	0.22	5.69	0.19	14.04	*C. subvermispora*	2
T2	96.00	PtrCAld5H	4.00	b	0.13	4.72	0.11	15.85	*C. subvermispora*	2
T2	96.00	PtrCAld5H	4.00	c	0.22	5.41	0.18	18.29	*C. subvermispora*	2
T2	96.00	PtrCAld5H	1.00	b	0.17	5.01	0.14	17.37	*C. subvermispora*	2
T2	96.00	PtrCAld5H	2.00	a	0.20	5.00	0.17	13.50	*C. subvermispora*	2
T2	96.00	PtrCAld5H	3.00	a	0.16	4.83	0.14	16.07	*C. subvermispora*	2
T2	96.00	PtrCAld5H	3.00	b	0.16	4.90	0.13	20.67	*C. subvermispora*	2
T2	96.00	PtrCAld5H	3.00	c	0.20	5.10	0.16	19.44	*C. subvermispora*	2
T2	96.00	PtrCAld5H	4.00	a	0.15	4.71	0.11	22.89	*C. subvermispora*	2
T2	96.00	PtrCAld5H	1.00	a	0.20	4.97	0.17	16.93	*C. subvermispora*	2

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T2	96.00	PtrCAld5H	603.00	a	0.25	5.67	0.23	9.12	*C. subvermispora*	2
T2	96.00	PtrCAld5H	603.00	b	0.22	5.49	0.19	11.22	*C. subvermispora*	2
T2	96.00	PtrCAld5H	600.00	b	0.28	6.27	0.25	8.68	*C. subvermispora*	2
T2	96.00	PtrCAld5H	600.00	a	0.21	5.80	0.19	11.41	*C. subvermispora*	2
T2	94.00	PtrCAld5H	1.00	a	0.17	4.85	0.14	16.58	*C. subvermispora*	1
T2	94.00	PtrCAld5H	1.00	b	0.15	4.90	0.13	16.61	*C. subvermispora*	1
T2	94.00	PtrCAld5H	1.00	c	0.21	5.65	0.19	9.54	*C. subvermispora*	1
T2	94.00	PtrCAld5H	2.00	a	0.23	4.82	0.19	15.70	*C. subvermispora*	1
T2	94.00	PtrCAld5H	2.00	b	0.08	3.37	0.06	15.46	*C. subvermispora*	1
T2	94.00	PtrCAld5H	707.00	b	0.32	6.39	0.31	2.15	Sterile	2
T2	94.00	PtrCAld5H	707.00	a	0.33	6.64	0.31	3.53	Sterile	2
T2	96.00	PtrCAld5H	5.00	a	0.15	4.83	0.14	6.05	*Sterile*	2
T2	96.00	PtrCAld5H	5.00	b	0.14	4.47	0.14	3.60	*Sterile*	2
T2	96.00	PtrCAld5H	607.00	a	0.25	6.12	0.24	1.38	Sterile	2
T2	96.00	PtrCAld5H	607.00	b	0.24	5.92	0.23	2.85	Sterile	2
T3	72.00	Ptr4CL/CAld5H	3.00	c	0.10	3.86	0.08	17.03	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	4.00	a	0.32	6.38	0.27	16.83	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	4.00	b	0.33	6.70	0.29	13.70	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	5.00	b	0.24	6.14	0.22	6.86	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	1.00	a	0.26	5.46	0.23	14.12	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	1.00	b	0.29	5.39	0.25	15.11	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	2.00	b	0.37	6.65	0.34	8.71	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	3.00	a	0.25	6.09	0.22	10.05	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	3.00	b	0.20	6.07	0.17	18.54	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	2.00	a	0.32	6.09	0.27	15.27	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	504.00	a	0.47	7.72	0.42	9.22	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	504.00	b	0.41	7.26	0.37	9.85	*C. subvermispora*	2

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T3	72.00	Ptr4CL/CAld5H	506.00	b	0.45	7.60	0.43	6.01	*C. subvermispora*	2
T3	72.00	Ptr4CL/CAld5H	506.00	a	0.43	7.61	0.39	10.49	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	3.00	a	0.45	7.75	0.42	5.28	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	3.00	b	0.35	7.20	0.32	10.07	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	3.00	c	0.29	6.97	0.24	15.80	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	4.00	a	0.36	6.99	0.30	15.61	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	4.00	b	0.42	7.01	0.35	16.92	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	4.00	c	0.26	6.47	0.24	7.75	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	1.00	a	0.32	6.51	0.28	14.12	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	1.00	b	0.34	6.36	0.30	13.23	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	2.00	a	0.59	8.20	0.53	9.74	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	2.00	b	0.29	6.98	0.26	11.48	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	2.00	c	0.33	7.13	0.30	6.63	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	114.00	a	0.42	7.60	0.36	14.92	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	114.00	b	0.34	7.47	0.32	6.80	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	107.00	b	0.39	7.19	0.36	8.36	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	107.00	a	0.43	7.73	0.40	8.25	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	104.00	b	0.52	8.52	0.46	11.65	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	115.00	a	0.56	9.06	0.50	11.13	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	104.00	a	0.58	8.86	0.52	10.12	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	115.00	b	0.59	8.93	0.52	11.37	*C. subvermispora*	2
T3	141.00	Ptr4CL/CAld5H	1.00	a	0.29	6.10	0.24	15.09	*C. subvermispora*	1
T3	141.00	Ptr4CL/CAld5H	1.00	b	0.23	5.88	0.20	13.82	*C. subvermispora*	1
T3	141.00	Ptr4CL/CAld5H	2.00	a	0.30	7.08	0.26	12.64	*C. subvermispora*	1
T3	141.00	Ptr4CL/CAld5H	2.00	b	0.30	7.41	0.27	11.32	*C. subvermispora*	1

Treatment	Line	Gene	Tree	Sample Location	OD weight (g)	Diameter (mm)	Decayed weight (g)	Mass Loss %	Fungus	Trial
T3	141.00	Ptr4CL/CAld5H	2.00	c	0.30	6.97	0.27	9.01	*C. subvermispora*	1
T3	72.00	Ptr4CL/CAld5H	5.00	a	0.26	5.78	0.24	5.56	*Sterile*	2
T3	72.00	Ptr4CL/CAld5H	507.00	b	0.49	7.90	0.47	3.84	Sterile	2
T3	72.00	Ptr4CL/CAld5H	507.00	a	0.47	7.81	0.45	3.07	Sterile	2
T3	141.00	Ptr4CL/CAld5H	5.00	b	0.25	6.76	0.25	2.19	*Sterile*	2
T3	141.00	Ptr4CL/CAld5H	5.00	a	0.33	7.28	0.33	1.39	*Sterile*	2
T3	141.00	Ptr4CL/CAld5H	116.00	b	0.51	8.41	0.50	1.35	Sterile	2
T3	141.00	Ptr4CL/CAld5H	116.00	a	0.71	8.99	0.70	2.17	Sterile	2
T3	141.00	Ptr4CL/CAld5H	4.00	b	0.33	6.76	0.33	2.11	Sterile	1

Appendix B – Stem Diameter Effect

The GLM Procedure
Fungus=C. subvermispora

Class Level Information		
Class	**Levels**	**Values**
Treatment	4	T0 T1 T2 T3
Line	8	21 23 37 72 94 96 141 271
Tree	30	1 1000 104 107 114 115 13 14 16 2 208 211 212 213 3 306 308 309 310 4 400 401 5 504 506 600 603 700 706 9
Diameter	136	3.37 3.66 3.86 4.14 4.46 4.68 4.71 4.72 4.73 4.82 4.83 4.85 4.9 4.93 4.97 4.99 5 5.01 5.05 5.09 5.1 5.14 5.39 5.41 5.46 5.48 5.49 5.52 5.54 5.55 5.65 5.67 5.68 5.69 5.76 5.8 5.83 5.84 5.87 5.88 5.93 6.06 6.07 6.09 6.1 6.14 6.17 6.22 6.27 6.28 6.36 6.37 6.38 6.43 6.47 6.51 6.53 6.57 6.59 6.62 6.63 6.65 6.68 6.69 6.7 6.71 6.74 6.75 6.76 6.82 6.83 6.86 6.87 6.88 6.97 6.98 6.99 7 7.01 7.06 7.08 7.09 7.12 7.13 7.14 7.19 7.2 7.22 7.23 7.26 7.36 7.37 7.4 7.41 7.42 7.47 7.6 7.61 7.66 7.72 7.73 7.74 7.75 7.76 7.86 7.93 7.94 7.97 8.01 8.02 8.03 8.07 8.08 8.2 8.27 8.3 8.38 8.39 8.45 8.52 8.83 8.86 8.93 8.99 9.06 9.09 9.12 9.28 9.42 9.48 9.5 9.82 10.39 10.9 11.18 12.03

Number of Observations Read	**153**
Number of Observations Used	153

Linear Models

The GLM Procedure
Dependent Variable: Mass Loss % Mass Loss %
Fungus=C. subvermispora

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	151	2298.574964	15.222351	6.78	0.2985
Error	1	2.245259	2.245259		
Corrected Total	152	2300.820223			

R-Square	Coeff Var	Root MSE	Mass Loss % Mean
0.999024	11.80536	1.498419	12.69270

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	287.990744	95.996915	42.76	0.1118
Line	4	37.447383	9.361846	4.17	0.3500
Tree(Line)	50	964.714851	19.294297	8.59	0.2656
Diameter	94	1008.421985	10.727893	4.78	0.3516

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	0	0.000000	.	.	.
Line	4	17.263019	4.315755	1.92	0.4894
Tree(Line)	9	147.179918	16.353324	7.28	0.2804
Diameter	94	1008.421985	10.727893	4.78	0.3516

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Intercept	16.92307692	B	1.49841871	11.29	0.0562
Treatment T0	-1.74289674	B	2.11908406	-0.82	0.5618
Treatment T1	-1.97842748	B	2.11908406	-0.93	0.5218
Treatment T2	-4.34390406	B	2.99683742	-1.45	0.3845
Treatment T3	0.00000000	B	.	.	.
Line 21	-1.55961067	B	2.11908406	-0.74	0.5961
Line 23	-8.51420325	B	2.11908406	-4.02	0.1553
Line 37	0.00000000	B	.	.	.
Line 72	-6.43437245	B	2.11908406	-3.04	0.2025
Line 94	-4.42334327	B	3.67036126	-1.21	0.4409
Line 96	0.00000000	B	.	.	.

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Line 141	**0.00000000**	**B**	**.**	**.**	**.**
Line 271	0.00000000	B	.	.	.
Tree(Line) 1 21	-1.92896924	B	2.11908406	-0.91	0.5299
Tree(Line) 2 21	3.16762880	B	2.11908406	1.49	0.3754
Tree(Line) 3 21	2.91712750	B	2.99683742	0.97	0.5086
Tree(Line) 4 21	-2.15716267	B	2.11908406	-1.02	0.4943
Tree(Line) 400 21	-2.18043914	B	2.11908406	-1.03	0.4909
Tree(Line) 401 21	0.00000000	B	.	.	.
Tree(Line) 1 23	6.23977287	B	2.11908406	2.94	0.2084
Tree(Line) 2 23	3.57012925	B	2.99683742	1.19	0.4446
Tree(Line) 208 23	6.99747895	B	2.11908406	3.30	0.1872
Tree(Line) 211 23	3.86765679	B	2.11908406	1.83	0.3191
Tree(Line) 212 23	4.44462415	B	2.11908406	2.10	0.2832
Tree(Line) 213 23	7.57361885	B	2.11908406	3.57	0.1737
Tree(Line) 3 23	3.46689521	B	2.11908406	1.64	0.3493
Tree(Line) 4 23	0.00000000	B	.	.	.
Tree(Line) 1 37	5.45637326	B	2.99683742	1.82	0.3197
Tree(Line) 2 37	-1.93756006	B	1.83518063	-1.06	0.4827
Tree(Line) 3 37	-4.56891694	B	2.11908406	-2.16	0.2765
Tree(Line) 306 37	-8.01380252	B	2.11908406	-3.78	0.1646
Tree(Line) 308 37	0.88799660	B	3.67036126	0.24	0.8489
Tree(Line) 309 37	-5.36145286	B	2.11908406	-2.53	0.2396
Tree(Line) 310 37	-1.51438823	B	2.11908406	-0.71	0.6050
Tree(Line) 4 37	0.00000000	B	.	.	.
Tree(Line) 1 72	3.63343293	B	2.11908406	1.71	0.3361
Tree(Line) 2 72	12.98291385	B	3.67036126	3.54	0.1754
Tree(Line) 3 72	7.76102700	B	3.67036126	2.11	0.2812
Tree(Line) 4 72	3.20786386	B	2.11908406	1.51	0.3716
Tree(Line) 5 72	-3.63011006	B	2.11908406	-1.71	0.3364
Tree(Line) 504 72	-1.26488164	B	2.11908406	-0.60	0.6574
Tree(Line) 506 72	0.00000000	B	.	.	.
Tree(Line) 1 94	1.38395947	B	2.11908406	0.65	0.6317

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Tree(Line) 2 94	9.65769209	B	3.51410336	2.75	0.2222
Tree(Line) 3 94	7.01353712	B	2.11908406	3.31	0.1868
Tree(Line) 4 94	3.56266904	B	2.80328471	1.27	0.4244
Tree(Line) 700 94	5.20665826	B	2.11908406	2.46	0.2461
Tree(Line) 706 94	0.00000000	B	.	.	.
Tree(Line) 1 96	2.32678722	B	4.10358863	0.57	0.6716
Tree(Line) 2 96	0.91806748	B	3.67036126	0.25	0.8440
Tree(Line) 3 96	1.01693137	B	4.23816812	0.24	0.8501
Tree(Line) 4 96	5.71296197	B	3.67036126	1.56	0.3635
Tree(Line) 600 96	-3.89457984	B	3.67036126	-1.06	0.4811
Tree(Line) 603 96	0.00000000	B	.	.	.
Tree(Line) 1 141	-2.80105772	B	2.11908406	-1.32	0.4123
Tree(Line) 104 141	-6.79868162	B	2.11908406	-3.21	0.1924
Tree(Line) 107 141	-8.67422900	B	2.11908406	-4.09	0.1525
Tree(Line) 114 141	2.47574607	B	2.99683742	0.83	0.5604
Tree(Line) 115 141	-5.79611544	B	2.11908406	-2.74	0.2231
Tree(Line) 2 141	-2.51998993	B	2.99683742	-0.84	0.5549
Tree(Line) 3 141	4.26689588	B	3.67036126	1.16	0.4522
Tree(Line) 4 141	0.00000000	B	.	.	.
Tree(Line) 1 271	-6.74028248	B	2.11908406	-3.18	0.1939
Tree(Line) 1000 271	-3.90458186	B	2.11908406	-1.84	0.3165
Tree(Line) 13 271	-3.34149848	B	2.11908406	-1.58	0.3598
Tree(Line) 14 271	-4.82346813	B	2.11908406	-2.28	0.2635
Tree(Line) 16 271	-9.21864172	B	2.11908406	-4.35	0.1438
Tree(Line) 2 271	8.30610794	B	4.23816812	1.96	0.3004
Tree(Line) 3 271	-5.92959470	B	2.11908406	-2.80	0.2185
Tree(Line) 4 271	-1.06906907	B	2.11908406	-0.50	0.7025
Tree(Line) 9 271	0.00000000	B	.	.	.

Note:	**The X'X matrix has been found to be singular, and a generalized inverse was used to solve the normal equations. Terms whose estimates are followed by the letter 'B' are not uniquely estimable.**

Linear Models

The GLM Procedure
Fungus=Sterile

Class Level Information		
Class	**Levels**	**Values**
Treatment	4	T0 T1 T2 T3
Line	8	21 23 37 72 94 96 141 271
Tree	9	116 19 219 4 404 5 507 607 707
Diameter	33	4.47 4.83 5.33 5.51 5.64 5.75 5.78 5.92 6.12 6.21 6.39 6.59 6.64 6.67 6.76 6.8 7 7.15 7.28 7.74 7.78 7.81 7.84 7.9 7.96 8.08 8.14 8.41 8.99 9.59 9.62 10.99 11.26

Number of Observations Read	34
Number of Observations Used	34

Linear Models

The GLM Procedure

Dependent Variable: Mass Loss % Mass Loss %

Fungus=Sterile

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	33	80.89249808	2.45128782	.	.
Error	0	0.00000000	.		
Corrected Total	33	80.89249808			

R-Square	Coeff Var	Root MSE	Mass Loss % Mean
1.000000	.	.	2.893378

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	2.62973431	0.87657810	.	.
Line	4	11.07341653	2.76835413	.	.
Tree(Line)	7	32.29645579	4.61377940	.	.
Diameter	19	34.89289145	1.83646797	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	0	0.00000000	.	.	.
Line	0	0.00000000	.	.	.
Tree(Line)	1	0.00304018	0.00304018	.	.
Diameter	19	34.89289145	1.83646797	.	.

Appendix C – Final Statistical Model

Linear Models

The GLM Procedure
Fungus=C. subvermispora

Class Level Information		
Class	**Levels**	**Values**
Treatment	4	T0 T1 T2 T3
Line	8	21 23 37 72 94 96 141 271
Tree	30	1 1000 104 107 114 115 13 14 16 2 208 211 212 213 3 306 308 309 310 4 400 401 5 504 506 600 603 700 706 9

Number of Observations Read	153
Number of Observations Used	153

Linear Models

The GLM Procedure

Dependent Variable: Mass Loss % Mass Loss %
Fungus=C. subvermispora

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	57	1290.152979	22.634263	2.13	0.0006
Error	95	1010.667244	10.638603		
Corrected Total	152	2300.820223			

R-Square	Coeff Var	Root MSE	Mass Loss % Mean
0.560736	25.69735	3.261687	12.69270

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	287.9907445	95.9969148	9.02	<.0001
Line(Treatment)	4	37.4473832	9.3618458	0.88	0.4790
Tree(Line)	50	964.7148515	19.2942970	1.81	0.0065

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	0	0.0000000	.	.	.
Line(Treatment)	0	0.0000000	.	.	.
Tree(Line)	50	964.7148515	19.2942970	1.81	0.0065

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Intercept	13.42589467	B	1.88313591	7.13	<.0001
Treatment T0	0.45748058	B	2.97749931	0.15	0.8782
Treatment T1	1.95073819	B	2.66315634	0.73	0.4657
Treatment T2	-3.25637853	B	2.97749931	-1.09	0.2769
Treatment T3	0.00000000	B	.	.	.
Line(Treatment) 271 T0	0.00000000	B	.	.	.
Line(Treatment) 21 T1	-4.17948995	B	2.97749931	-1.40	0.1637
Line(Treatment) 23 T1	-5.43650682	B	2.97749931	-1.83	0.0710
Line(Treatment) 37 T1	0.00000000	B	.	.	.
Line(Treatment) 94 T2	-1.52911807	B	3.26168707	-0.47	0.6403
Line(Treatment) 96 T2	0.00000000	B	.	.	.
Line(Treatment) 72 T3	-5.17427211	B	2.97749931	-1.74	0.0855
Line(Treatment) 141 T3	0.00000000	B	.	.	.
Tree(Line) 1 21	2.07132060	B	3.26168707	0.64	0.5269
Tree(Line) 2 21	3.75138532	B	2.97749931	1.26	0.2108
Tree(Line) 3 21	0.52585368	B	3.26168707	0.16	0.8723
Tree(Line) 4 21	0.03073320	B	3.99473452	0.01	0.9939
Tree(Line) 400 21	0.67753543	B	3.26168707	0.21	0.8359
Tree(Line) 401 21	0.00000000	B	.	.	.
Tree(Line) 1 23	5.76309518	B	3.26168707	1.77	0.0805
Tree(Line) 2 23	-0.16342435	B	3.26168707	-0.05	0.9601
Tree(Line) 208 23	2.93472183	B	3.26168707	0.90	0.3705
Tree(Line) 211 23	1.71095734	B	3.26168707	0.52	0.6011
Tree(Line) 212 23	-2.08779854	B	3.26168707	-0.64	0.5236
Tree(Line) 213 23	2.10670171	B	3.26168707	0.65	0.5199
Tree(Line) 3 23	2.44970460	B	2.97749931	0.82	0.4127
Tree(Line) 4 23	0.00000000	B	.	.	.
Tree(Line) 1 37	-3.33021066	B	2.30636105	-1.44	0.1521
Tree(Line) 2 37	-0.51200259	B	2.30636105	-0.22	0.8248
Tree(Line) 3 37	-0.47043235	B	2.66315634	-0.18	0.8602
Tree(Line) 306 37	-6.16152139	B	2.97749931	-2.07	0.0412
Tree(Line) 308 37	-2.51624043	B	2.97749931	-0.85	0.4002

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Tree(Line) 310 37	-0.85081700	B	2.97749931	-0.29	0.7757
Tree(Line) 4 37	0.00000000	B	.	.	.
Tree(Line) 1 72	**6.36477118**	**B**	**3.26168707**	**1.95**	**0.0540**
Tree(Line) 2 72	3.73954362	B	3.26168707	1.15	0.2545
Tree(Line) 3 72	6.95677772	B	2.97749931	2.34	0.0216
Tree(Line) 4 72	7.01265665	B	3.26168707	2.15	0.0341
Tree(Line) 5 72	-1.39302815	B	3.99473452	-0.35	0.7281
Tree(Line) 504 72	1.28621478	B	3.26168707	0.39	0.6942
Tree(Line) 506 72	0.00000000	B	.	.	.
Tree(Line) 1 94	4.31290366	B	2.66315634	1.62	0.1087
Tree(Line) 2 94	9.66132141	B	2.82470386	3.42	0.0009
Tree(Line) 3 94	9.66703410	B	3.26168707	2.96	0.0038
Tree(Line) 4 94	9.00563489	B	2.97749931	3.02	0.0032
Tree(Line) 700 94	5.06202449	B	3.26168707	1.55	0.1240
Tree(Line) 706 94	0.00000000	B	.	.	.
Tree(Line) 1 96	6.97598948	B	3.26168707	2.14	0.0350
Tree(Line) 2 96	3.32772420	B	3.99473452	0.83	0.4069
Tree(Line) 3 96	8.55821211	B	2.97749931	2.87	0.0050
Tree(Line) 4 96	8.83906906	B	2.97749931	2.97	0.0038
Tree(Line) 600 96	-0.12379757	B	3.26168707	-0.04	0.9698
Tree(Line) 603 96	0.00000000	B	.	.	.
Tree(Line) 1 141	0.63813981	B	2.49115465	0.26	0.7984
Tree(Line) 104 141	-2.54118200	B	2.97749931	-0.85	0.3956
Tree(Line) 107 141	-5.12319773	B	2.97749931	-1.72	0.0886
Tree(Line) 114 141	-2.56119822	B	2.97749931	-0.86	0.3919
Tree(Line) 115 141	-2.17673843	B	2.97749931	-0.73	0.4665
Tree(Line) 2 141	-3.28848968	B	2.30636105	-1.43	0.1572
Tree(Line) 3 141	-3.04372382	B	2.66315634	-1.14	0.2560
Tree(Line) 4 141	0.00000000	B	.	.	.
Tree(Line) 1 271	-4.95827787	B	2.66315634	-1.86	0.0657
Tree(Line) 1000 271	-2.45874408	B	3.26168707	-0.75	0.4528
Tree(Line) 13 271	-4.37374928	B	3.26168707	-1.34	0.1831
Tree(Line) 2 271	0.43970226	B	2.66315634	0.17	0.8692
Tree(Line) 3 271	-3.03828002	B	2.97749931	-1.02	0.3101
Tree(Line) 4 271	-0.07912090	B	2.97749931	-0.03	0.9789
Tree(Line) 9 271	0.00000000	B	.	.	.

Linear Models

The GLM Procedure
Fungus=Sterile

Class Level Information		
Class	**Levels**	**Values**
Treatment	4	T0 T1 T2 T3
Line	8	21 23 37 72 94 96 141 271
Tree	9	116 19 219 4 404 5 507 607 707

Number of Observations Read	34
Number of Observations Used	34

Linear Models

The GLM Procedure

Dependent Variable: Mass Loss % Mass Loss %
Fungus=Sterile

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	45.99960663	3.28568619	1.79	0.1181
Error	19	34.89289145	1.83646797		
Corrected Total	33	80.89249808			

R-Square	Coeff Var	Root MSE	Mass Loss % Mean
0.568651	46.83672	1.355163	2.893378

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treatment	3	2.62973431	0.87657810	0.48	0.7018
Line(Treatment)	4	11.07341653	2.76835413	1.51	0.2398
Tree(Line)	7	32.29645579	4.61377940	2.51	0.0522

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	0	0.00000000	.	.	.
Line(Treatment)	0	0.00000000	.	.	.
Tree(Line)	7	32.29645579	4.61377940	2.51	0.0522

Parameter	Estimate		Standard Error	t Value	Pr > |t|
Intercept	1.786805353	B	0.95824526	1.86	0.0778
Treatment T0	1.126623497	B	1.17360597	0.96	0.3491
Treatment T1	0.998355401	B	1.10648632	0.90	0.3782
Treatment T2	0.324960816	B	1.35516345	0.24	0.8131
Treatment T3	0.000000000	B	.	.	.
Line(Treatment) 271 T0	0.000000000	B	.	.	.
Line(Treatment) 21 T1	1.261653393	B	1.10648632	1.14	0.2684
Line(Treatment) 23 T1	1.540380211	B	1.10648632	1.39	0.1800
Line(Treatment) 37 T1	0.000000000	B	.	.	.
Line(Treatment) 94 T2	0.731239129	B	1.35516345	0.54	0.5957
Line(Treatment) 96 T2	0.000000000	B	.	.	.
Line(Treatment) 72 T3	1.668139446	B	1.35516345	1.23	0.2334
Line(Treatment) 141 T3	0.000000000	B	.	.	.
Tree(Line) 404 21	-3.340543036	B	1.35516345	-2.47	0.0234
Tree(Line) 5 21	0.000000000	B	.	.	.
Tree(Line) 219 23	-3.023164314	B	1.35516345	-2.23	0.0379
Tree(Line) 5 23	0.000000000	B	.	.	.
Tree(Line) 5 37	0.000000000	B	.	.	.
Tree(Line) 5 72	2.100610757	B	1.65972948	1.27	0.2209
Tree(Line) 507 72	0.000000000	B	.	.	.
Tree(Line) 707 94	0.000000000	B	.	.	.
Tree(Line) 5 96	2.713451798	B	1.35516345	2.00	0.0597
Tree(Line) 607 96	0.000000000	B	.	.	.
Tree(Line) 116 141	-0.025930465	B	1.35516345	-0.02	0.9849
Tree(Line) 4 141	0.320359006	B	1.65972948	0.19	0.8490
Tree(Line) 5 141	0.000000000	B	.	.	.
Tree(Line) 19 271	1.096682984	B	1.17360597	0.93	0.3618
Tree(Line) 5 271	0.000000000	B	.	.	.

Appendix D – Summary Mass Loss Table

	Mass Loss %						**N**
	Fungus						
	C. subvermispora			Sterile			
	Mean	StdDev	CV	Mean	StdDev	CV	
Treatment T0	11.43	3.96	34.61	3.28	1.55	47.30	34
T1	12.59	3.31	26.32	2.68	1.74	64.96	69
T2	15.21	4.18	27.49	3.26	1.61	49.28	38
T3	11.66	3.51	30.10	2.71	1.42	52.27	46

CHAPTER 3

Chemical Degradation Properties of Young Small Diameter Genetically Modified Quaking Aspen *(Populus tremuloides)*

Abstract

In this study, one-year old quaking aspen (*Populus tremuloides*) trees including a control wild type aspen and three lines of transgenic trees were analyzed for resistance to fungal decay. The transgenics had reduced lignin content through transfer of an antisense - 4CL gene, changed syringyl/guaiacyl ratio through insertion of a sense CAld5H gene, and modified lignin content and syringyl/guaiacyl through simultaneous insertion of 4-CL and CAld5H genes. The small diameter transgenic trees were tested using simultaneous white rot fungus *Trametes versicolor*, lignin selective white rot fungus *Ceriporiopsis subvermispora* and a brown rot fungus *Poria placenta*. A modified soil-agar block method was used with a 40 day colonization time. Near infrared spectroscopy and chemical analysis determined loss of cellulose and lignin variations between transgenic genotypes. Near infrared transmittance was successful in predicting the cellulose and lignin percentages of the decayed material. The reduced lignin content lines did not affect the rate of lignin decay for all fungi tested. Lignin decay rates were reduced by the increased S/G ratio lines for all fungi tested. The reduced lignin lines did not affect the rate of cellulose decay for all fungi except Trametes versicolor. The increased S/G ratio lines did not affect the rate of cellulose decay.

Keywords: *Ceriporiopsis subvermispora*, *Trametes versicolor*, *Poria placenta*, lignin selective white rot, biopulping, transgenic aspen, NIR

Review of Related Literature

Near Infrared Spectroscopy Characterization of Transgenic Aspen Wood

NIR has been developed to characterize the lignin and cellulose contents of solid and ground wood (Table 3-1) [Yeh et al. 2005]. Strong correlations between traditional chemical analysis methods and NIR spectra have been obtained [Yeh et al. 2004]. The authors were successful in estimating α-cellulose and lignin contents in loblolly pine and aspen increment cores.

Table 3-1. Summary statistics for α-celluloses and total lignin contents for the calibration and prediction sets [Yeh et al. 2005].

chemical compositions (%)[a]	calibration set					prediction set				
	n	min	max	avg	std[b]	*n*	min	max	avg	std
pine α-cellulose										
stacked-wafer model	38	35.6	47.3	42.4	2.4	12	38.2	46.4	42.0	2.5
single-wafer model	38	35.6	47.3	42.4	2.4	12	38.2	43.8	41.0	2.0
pine total lignin	39	28.0	32.0	30.0	0.9	14	28.5	32.0	29.8	0.9
aspen total lignin	39	20.9	28.6	25.3	2.3	14	21.4	27.1	24.6	1.9

[a] On the basis of extractive-free, OD wood weight. [b] Standard deviation.

In other methods, such as diffuse reflectance NIR, the analysis requires large samples not available in transgenic tree studies. In a study by Yamada et al. 2006, the lignin content, S/G ratio, cellulose, and xylose contents were successfully predicted by near infrared transmittance with 75mg pellets of ground material. Correlations between traditional chemical analysis methods and NIR spectra of transgenic aspen lines used in this study have been explored. The authors suggested that NIR transmittance is a suitable tool for determining chemical properties of transgenic trees.

Near Infrared Spectroscopy Characterization of *Ceriporiopsis subvermispora*

Several strains of *C. subvermispora* have been characterized by near and mid infrared spectroscopy on degraded spruce wood [Schwanninger et al. 2004]. The authors determined it was possible to compare and differentiate between strains using near infrared spectroscopy. The ability to determine strain lignin selectivity by NIR resulted of the study. Near infrared spectroscopy has been evaluated to determine correlations between NIR spectra and mass loss [Ferraz et al. 2004]. The authors were unable to correlate mass loss, but were successful in reflecting the α-cellulose degree of polymerization and the linkage contents of lignin. It was concluded that NIR was an adequate process control technique for biopulping.

Introduction

Lignin is an aromatic polymer found in the cell wall of woody plant cells. Lignin is also a major component of wood comprising approximately 15-36% of its dry weight [Eriksson et al. 1990]. Lignin's complex bonding of three monolignols (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) provides compressive strength to wood, a linkage between hemicelluloses and celluloses in the cell wall, and a physical barrier to pathogen enzymes. Lignin prevents water penetration into the cell wall thus increasing the wood's resistance to attack by microorganisms [Eriksson et al. 1990]. Since lignin is resistant to enzymes, it is difficult to develop the cellulose and sugars from wood for use in production of paper and ethanol.

Present research interest in lower lignin content trees has produced several transgenic lines of aspen with down regulated lignin [Hu et al. 1999, Li et al 2000, Li et al. 2003]. Suppression of lignin biosynthesis with insertion of an antisense 4CL gene, blocks one pathway for lignin monolignol production, therefore reducing pathways for lignin production and reducing lignin concentration [Hu et al 1999]. The type of lignin produced by biosynthetic pathway has also been altered by gene insertion. Insertion of a sense coniferyl aldehyde 5-hydrohylase (CAld5H) gene increases the production ratio of syringyl to guaiacyl type lignin thus increasing the syringyl/guaiacyl type lignin ratio [Li et al. 2001].

Combination effects of both gene insertions produce trees with reduced lignin and higher syringyl/guaiacyl ratios [Li et al. 2003].

Fungal natural wood decay processes could be harnessed to reduce the pulping energy input and combined with reduced or modified lignin tree technology. Biopulping has demonstrated that energy reductions are possible when using a fungal pretreatment before paper processing [Akhtar et al. 1992]. Fungal species that can reduce manufacturing energy belong to the white rot decay classification. White rot fungi degrade lignin, hemicelluloses, and celluloses in wood at similar rates. In addition, there are a few species of white rot fungi that selectively decay lignin and leave carbohydrates intact. These lignin selective white rot fungi are suitable for use in commercial pretreatment biodegradation [Akhtar et al. 1992]. Wood decay fungi that selectively degrade lignin decay the middle lamella between fibers thus making the pulp easier to process. Lignin type may also influence how well a fungus performs as a biopulping agent. Lignin selective fungi have been found to selectively degrade syringyl lignin over guaiacyl [Choi et al. 2006].The emerging field of biofuel research would also benefit from better access to wood carbohydrates due to reduced lignin. Ethanol production from wood is difficult since lignin prevents interactions between enzymes with carbohydrates. A fungal pretreatment to reduce lignin before introduction of cellulolytic enzymes or yeasts could increase yield. The next step in biopulping is the merging of highly lignin selective white rot fungi with transgenic trees with altered lignin. Combining transgenic trees and fungal pretreatment is a novel solution to the production problems

associated with lignin present in wood. Transgenic trees with reduced or modified lignin may be the key to reducing time and cost for manufacture of wood based products. Since there have been many advances in genetic modification, the development of transgenic trees requires testing to determine the best suitable lines for these commercial uses. Analysis of transgenic tree degradative properties allows characterization of the role of lignin in protection from fungal decay and its potential uses.

In previous fungal decay studies, comparisons of different tree cultivars or species represented narrow varying percentages of lignin content and S/G ratio [Obst et al.1994]. The narrow range of lignin contents makes it difficult to distinguish differences between degradative properties. With the advent of transgenics, it is now possible to examine the effects of decay by a large range of lignin percentages and types of lignin within the same tree species.

Mass loss is the standard method of analysis to determine the resistance of woods to fungal decay [Cowling 1961, Highley et al. 1970, De Groot et al. 1996, Smith et al. 1996, De Groot et al. 1998, ASTM 2007]. Although mass loss results suggest fungal resistance, it does not give insight into the chemical changes within wood following degradation. Determination of the effect of reduced or modified lignin is important to understanding the characteristics of transgenic tree lignin. Chemical analytical methods such as the Klason lignin method and α-cellulose extractions provide more insight into the degradative processes of fungi. While standard chemical analyses methods work well, it is time consuming when numerous samples are analyzed. Development of methods such as infrared spectroscopy allows quick

analysis of sample properties [Levi et al. 1969, Dorado et al. 1999 Schwanninger et al. 2004, Yeh et al. 2004, Yeh et al. 2005, Kent et al. 2006, Yamada et al. 2006, Fackler et al. 2007]. Near infrared transmittance technology allows rapid analysis of chemical characteristics. NIR has recently been used in determining mechanical properties and decay resistance of fungal treated wood with success [Kelley et al. 2002, Pandey et al. 2003, Flaete et al. 2004]. A rapid determination of decay properties is critical to reduce the amount of time spent in choosing transgenic lines for further research. Calibration of the spectra for lignin and cellulose allows unknown wood sample values to be determined. NIR transmittance can then be used to quickly scan a sample, giving spectra that are correlated to known sample chemical values. Near infrared spectroscopy, although not as sensitive as standard analytical chemistry, is a potentially useful tool for use with degradation studies. Assessment of fungal degraded wood using standard analytical chemistry and near infrared spectroscopy methods is valuable to determine the most suitable analysis method.

Objectives

The objective of this study was to determine differences in remaining cellulose and lignin between young small diameter transgenic aspen trees with modified lignin concentrations and types when decayed by a lignin selective white rot, simultaneous white rot, and brown rot fungi. The wide variations of lignin type and content provided a novel opportunity to determine decay differences between genetic lines. Comparisons between traditional analytical chemistry and near infrared spectroscopy analytical methods were performed to determine differences between methods.

Material and Methods

Due to the nature of young tree testing, wood is limited and leaves little material for replicates making chemical analysis a useful tool. Chemical analysis of post-decay remaining cellulose or lignin contents provides important decay differences between tree genetic lines. Samples may not require 12 weeks of decay as used in the soil block method, in order to detect chemical changes within the wood. A shorter forty day colonization period provides valuable chemical data while allocating more time for test replication [Giles et al. 2008a unpublished].

Ceriporiopsis subvermispora FP-90031-sp (a lignin selective white rot fungus), *Trametes versicolor* Mad-697 (a simultaneous white rot fungus), and *Poria placenta* Mad-698-R (a brown rot fungus) were used. Cultures were obtained from the USDA Forest Products Laboratory (Madison, Wisconsin) culture collection.

One-year-old quaking aspen young trees were harvested for modified soil-agar block decay testing. Samples along the stem were cut from 5 wild type aspen trees, 5 aspen trees with reduced lignin content, 5 aspen trees with changed syringyl/guaiacyl ratio, and 5 aspen trees with both modified lignin content and increased syringyl/guaiacyl ratio. Transgenic groups demonstrated variation in lignin content and S/G ratio (Table 3-1).

Table 3-2. Summary of aspen wood lignin content and S/G ratio per genetic line.

Genetic Line	PtrWT (wild type)	Ptr4CL (reduced lignin)	PtrCAld5H (increased S/G ratio)	Ptr4CL/CAld5H (reduced lignin and increased S/G ratio)
	271	37	94	141
Lignin Content (%)	22.2	14.9	20.7	10.7
Cellulose Content (%)	41.4	ND	43.4	53.3
S/G Ratio	2.2	2.1	4.9	2.7

[Li et al. 2003]

Modified Soil-Agar Block Inoculation

Twenty debarked young wild type and transgenic trees were cut into 20mm samples. Five sample trees as replicates from each genetic line were used for the wild type and three transgenic lines. Within each genetic line, two trees were used for *Ceriporiopsis subvermispora*, 1 tree for *Trametes versicolor*, 1 tree for *Postia placenta*, and 1 tree used as a sterile control. Within each tree 3 specimens were cut into 5-15 mm diameter x 20 mm lengths. Samples were oven dried for 48 hours at 105°C. Samples were weighed to determine dry weight. Two oz jars were filled with 0.8 grams of vermiculite and 4ml distilled water (Fig 3-1). A needlepoint plastic grid was placed on top of the moist vermiculite to prevent saturation of wood samples. Wood samples were placed in each jar

then steam sterilized. *C. subvermispora*, *T. versicolor*, and *P. placenta* was cultured on Malt Extract Agar with 0.01% chloramphenicol Petri plates and incubated at room temperature for 7-10 days. A plug of colonized MEA was then placed on the center of a Petri plate containing 2% water agar and incubated at room temperature for 7-10 days. Five mm x 20mm strips of mycelium were cut from the 2% water agar colonized Petri plate and placed on the plastic grid. Aspen wood samples were then placed longitudinally perpendicular to the agar strips [Giles et al 2008a unpublished]. Control samples of each wild type and transgenic line were chosen to verify sterility and placed in jars without agar strips. The mouth of each jar was sealed with a layer of Parafilm. Sample jars were incubated at room temperature in darkness for 40 days. After colonization, samples were brushed lightly to remove surface hyphae and oven dried for 48 hours at 105°C. Samples were reweighed to determine mass loss.

Chemical Analysis Methods

Analysis of the sterile controls and decayed samples were performed using techniques outlined in Yokoyama et al 2002. Samples were microtomed for holocellulose and α-cellulose analysis. Samples were ground for Klason lignin and NIR analysis. Sample extractives were removed post colonization from debarked microtomed and ground samples. Each sample was placed in a capped tube and acetone was added and allowed to sit at room temperature. The samples were drained and new acetone replaced every 48 hours for a total of six rinses [Yokoyama et al 2002].

Klason Lignin

Sterile and decayed wood samples were combined and ground in a Wiley mill using 40-80 mesh screens. Fifty milligrams of oven dried sample were placed into 10 ml beakers into which 1.5 ml of sulfuric acid 72% was added. Each sample beaker was then stirred every fifteen minutes for two hours to ensure uniform hydrolysis. The samples were then washed with 56 ml deionized water and placed into serum bottles. The bottles were closed with rubber stoppers and aluminum caps. The bottles were autoclaved for ninety minutes at 121° C and cooled overnight. The hydrolysis solution was then vacuum filtered through a medium pore 15 ml previously weighed crucible. Deionized water was used to rinse the solids until a neutral pH was reached. The filtrate containing crucibles were oven dried at 105° C for 24 hours then weighed to determine acid insoluble filtrate. Acid soluble lignin in the filtrate was determined by UV spectroscopy and measured at 205 μm. Replicate analysis of each sample was performed and the values averaged.

α-cellulose

Wood samples were microtomed into 0.2 mm slices while avoiding the pith and oven dried at 105° C for 24 hours. Approximately 100 mg of each oven dried sample was weighed then placed in a 10 ml round bottom flask with 4 ml deionized water. The samples were then soaked for 5 minutes before adding 200 mg of 80% sodium chloride and 0.8 ml of glacial acetic acid in the flask. The flasks were then stoppered, clamped, and placed into a 90° C water bath for one hour. The flasks were then cooled in a cold

water bath for one hour. The samples were then filtered into previously weighed 30ml coarse crucibles and washed with deionized water. The crucibles were oven dried at 105° C for 20 hours. The oven dry crucibles were then weighed to determine holocellulose content. Hollocelulose from each sample was then used to determine α-cellulose. Approximately 50 mg of oven dry holocellulose and 4 ml of 17.5% sodium hydroxide was placed into a 10 ml beaker and stirred with a glass rod for one minute. The beaker was left at room temperature for an additional 29 minutes. Four milliliters of deionized water was then added to the beaker and stirred for one minute. The samples were then left to react for an additional 29 minutes. The suspension was then filtered through a previously weighed coarse crucible and washed with 30 ml deionized water three times then soaked in 1.0 M acetic acid for five minutes. The α-cellulose was washed again with 30 ml deionized water three times. The crucibles were then oven dried at 105° C for 20 hours to determine sample α-cellulose content. Replicate analysis of each sample was performed and the values averaged.

NIR Analysis Methods

Near infrared spectroscopic analysis of the sterile controls and decayed samples was performed using techniques outlined in Yamada et al. (2006). Wood samples were combined and ground in a Wiley mill using 40-80 mesh screens for NIR analysis. Sample

extractives were removed post colonization from debarked ground samples. A correlation determined by Yamada et al. (2006) and Horvath et al. (2008) between NIR data and analytical chemistry analysis was used. The correlations of NIR spectra for the

transgenic material allow analysis of lignin, α-cellulose, and xylan contents of the decayed transgenic material.

Milled powder from Wiley mill passed through an 80 mesh screen was used to prepare 1.3 cm diameter pellets. Pellets were created from 75 mg of oven dried sample pressed using a pellet press at 4000 psi. The pellets were then placed into a desiccators containing P_2O_5 for forty-eight hours. The pellets were analyzed on a Foss NIRSystems NIR spectrometer equipped with an InTact Single Tablet Module (NR-1650) and a monochromator (NR-6500-V/H). Absorbance spectra were collected over the range 600-1900 nm. Spectra was analyzed as described previously [Yeh et al 2004, Yeh et al 2005, Yamada et al 2006, Horvath et al unpublished].

The NIR calibration and analytical chemistry methods were designed for sterile samples, thus a sample weight calculation was used to prorate the approximate amount of lignin and cellulose in the sterile and decayed samples (Fig 3-1). Prorating the analytical chemistry values provides lignin and cellulose contents based on original sample weight.

Fig 3-1. The NIR prediction and analytical chemistry values were prorated according to mass loss during colonization.

$$\frac{((\text{Predicted or Measured Content} / 100) * (\text{Final Sample Weight})}{(\text{Original Sample Weight} / 100)} = \text{Sample Content}$$

Results

Analytical Chemistry

Each wood sample decayed demonstrated evidence of colonization on all genetic lines except the sterile control samples that were free of fungal contamination. In the samples inoculated by lignin selective *C. subvermispora,* the rate of cellulose decay in wild type and transgenic lines were similar between 2-3% (Fig 3-2). The rate of lignin decay by *C. subvermispora* was similar between 5-6% in the wild type and reduced lignin lines and the rate of lignin decay decreased 0-1% in the increased S/G lines (Fig 3-3). In samples inoculated with the simultaneous white rot fungus *T. versicolor*, the rate of cellulose decay was similar between 1-3% in the wild type and reduced lignin/increased S/G line and increased 6-14% in the reduced lignin and increased S/G lines (Fig 3-4). Due to limited remaining material, only the wild type, reduced lignin content, and the reduced lignin/increased S/G lines decayed by *T. versicolor* were analyzed for lignin. The rate of lignin decay was similar between 0-1% in the wild type and reduced lignin/increased S/G line and slightly higher in the reduced lignin line with 4% (Fig 3-5). In the samples inoculated with brown rot fungus *P. placenta,* the decay rate of cellulose was smaller between 0-5% in the transgenic lines samples than the wild type (11%) (Fig 3-6). The rate of lignin decay by *P. placenta* was similar between 1% in the wild type and increased S/G lignin lines and the rate of lignin decay increased 3% in the increased S/G lines (Fig 3-7).

Analytical Chemistry and NIR Comparison

NIR cellulose prediction of sterile samples was within 2% of analytical chemistry results (Fig 3-2, Fig 3-4, Fig 3-6). The NIR predicted cellulose contents of the decayed samples were within 1-16% of the analytical chemistry results. NIR lignin prediction of sterile samples was within 1-3% of the Klason lignin results (Fig 3-3, Fig 3-5, Fig 3-7). The NIR predicted lignin contents of the decayed samples were within 0-5% of the Klason lignin results.

Mass Loss Analysis

Mean mass losses and coefficient of variations were calculated (Table 3-4). The limited number of samples prevented standard methods of statistical analysis. *Ceriporiopsis subvermispora* exhibited the most consistent COV of all three fungi tested with a range of approximately 19-24% (Table 3-4). *Postia placenta* exhibited the widest range of mass loss COV with a range of 12-46% (Table 3-4).

Table 3-4. Summary of *Populus tremuloides* mass loss percentages per genetic treatment degraded by *Ceriporiopsis subvermispora*, *Trametes versicolor*, and *Postia placenta*. Table includes Coefficient of Variation for each genetic line per fungus.

Genetic Line	Fungus	Mean Mass Loss %	N	Coefficient of Variation %
Control Wild Type	*Ceriporiopsis subvermispora*	11.75	6	21.88
	Postia placenta	16.45	3	32.58
	Trametes versicolor	23.77	3	42.56
Reduced Lignin Content	*Ceriporiopsis subvermispora*	12.01	6	23.85
	Postia placenta	8.41	3	12.03
	Trametes versicolor	18.43	3	34.02
Increased S/G Ratio	*Ceriporiopsis subvermispora*	14.78	5	20.12
	Postia placenta	9.29	3	12.21
	Trametes versicolor	17.25	2	6.19
Reduced Lignin and Increased S/G Ratio	*Ceriporiopsis subvermispora*	12.38	5	18.93
	Postia placenta	25.65	3	45.89
	Trametes versicolor	14.13	2	24.23

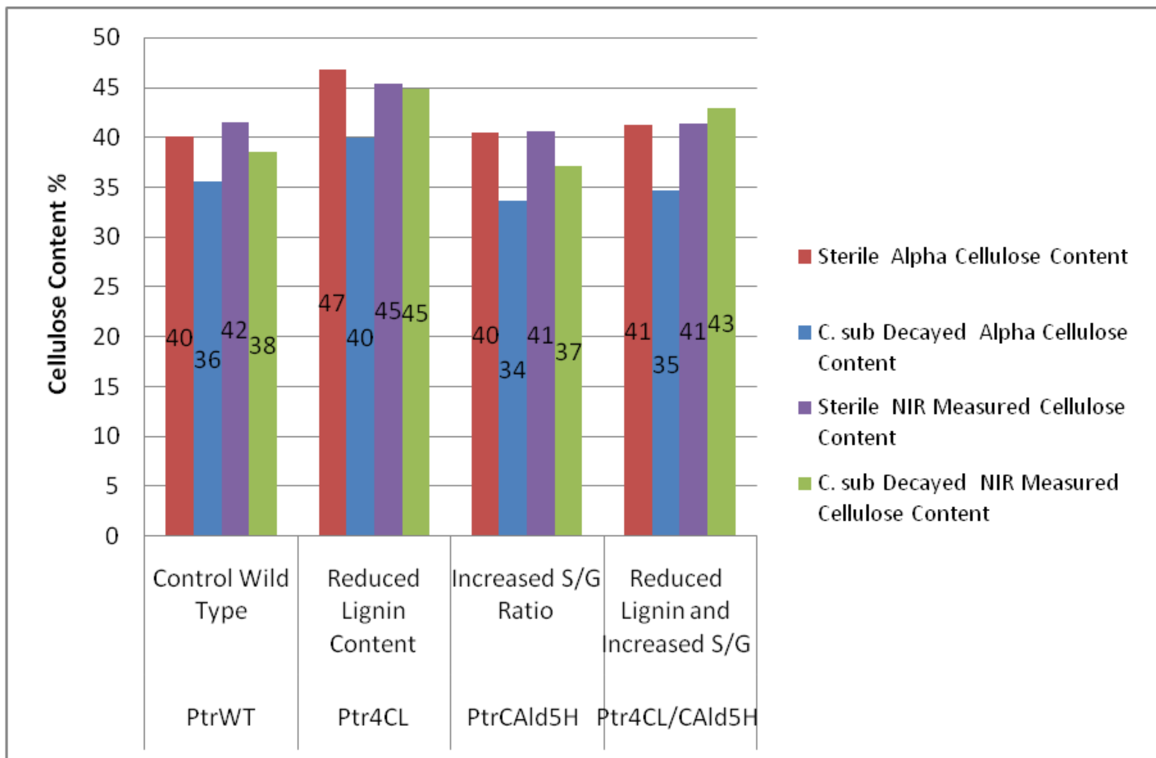

Fig 3-2. Comparison of analytical chemistry and NIR α-cellulose analysis results. *Populus* species wood samples after colonization with *Ceriporiopsis subvermispora.*

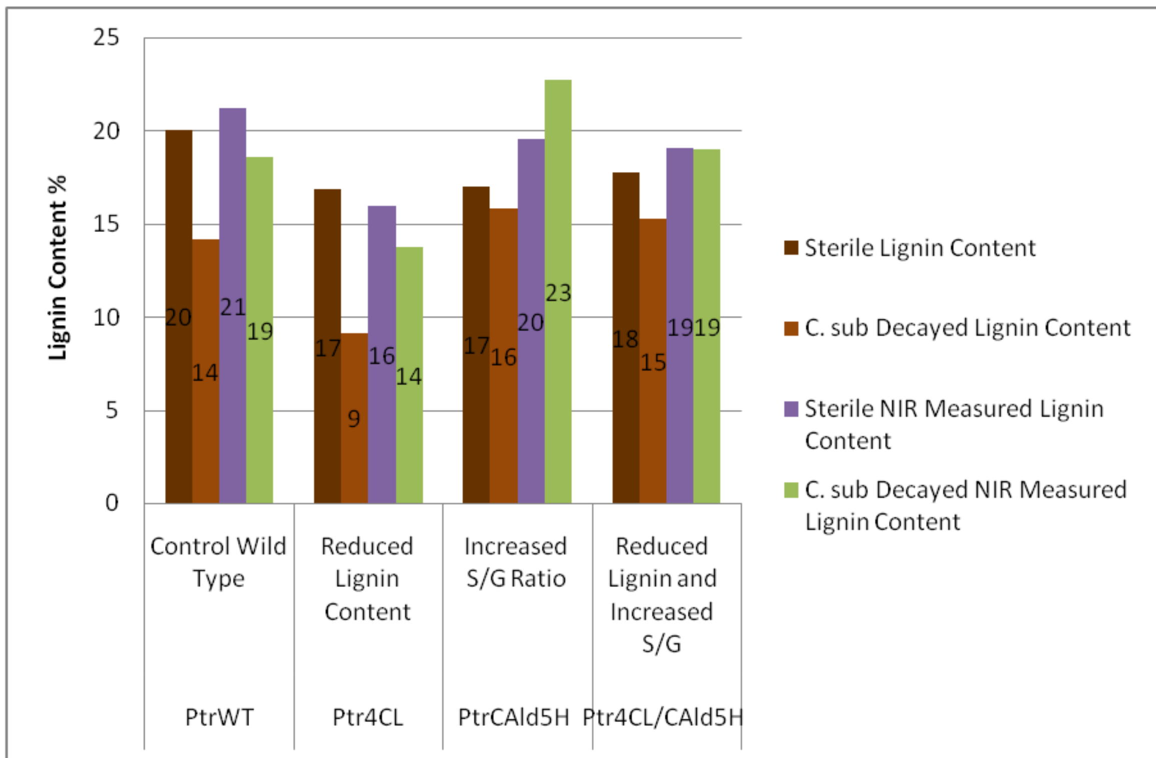

Fig 3-3. Comparison of analytical chemistry and NIR lignin analysis results. *Populus* species wood samples after colonization with *Ceriporiopsis subvermispora.*

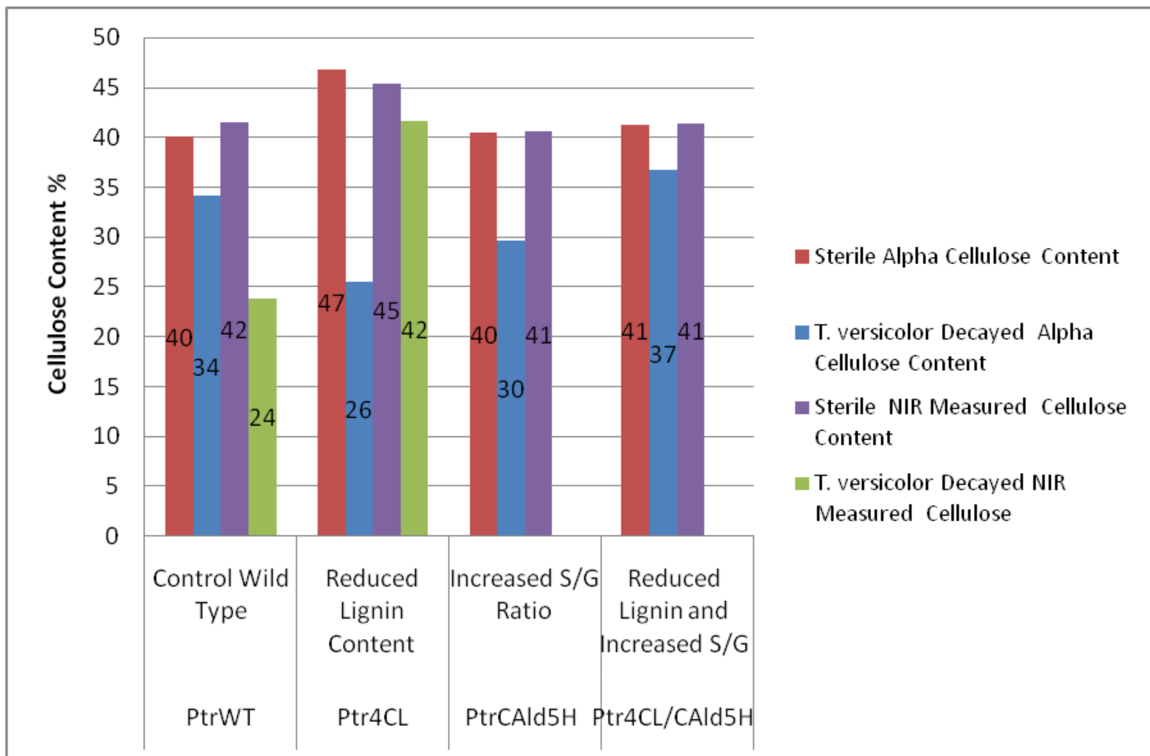

Fig 3-4. Comparison of analytical chemistry and NIR α-cellulose analysis results. *Populus* species wood samples after colonization with *Trametes versicolor.* Due to limited material, only the wild type and reduced lignin content lines were analyzed by NIR.

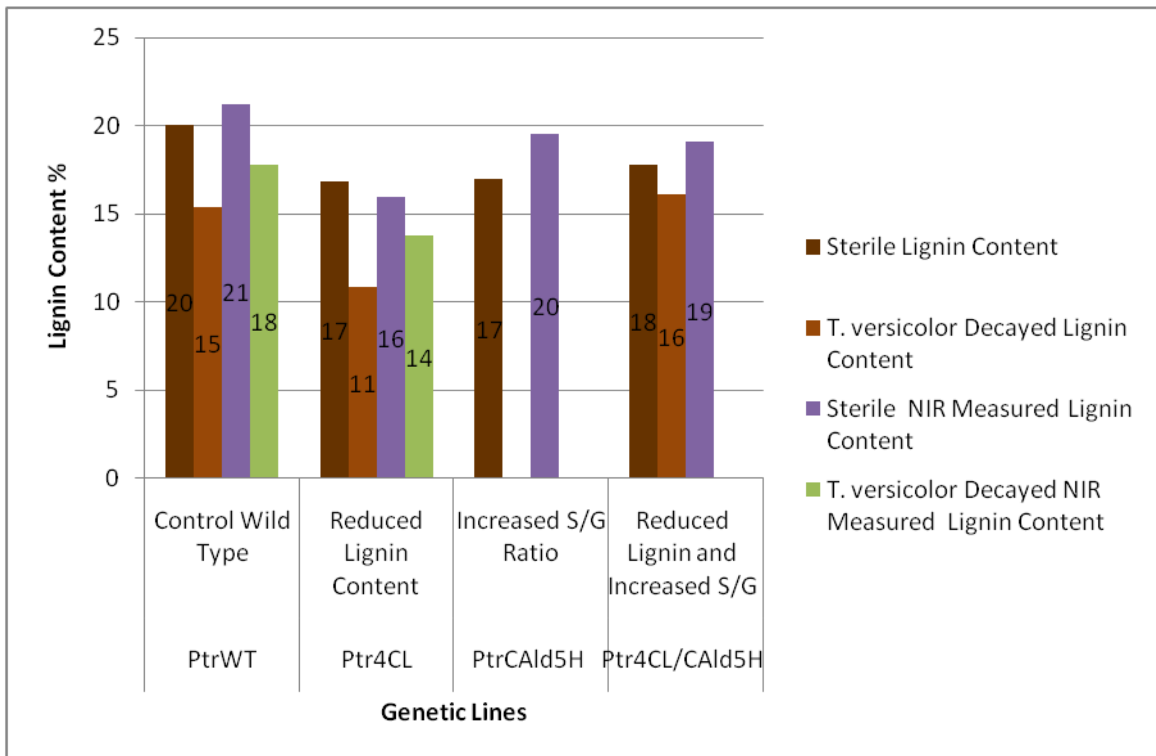

Fig 3-5. Comparison of analytical chemistry and NIR lignin analysis results. *Populus* species wood samples after colonization with *Trametes versicolor.* Due to limited material, only the wild type and reduced lignin content lines were analyzed by NIR.

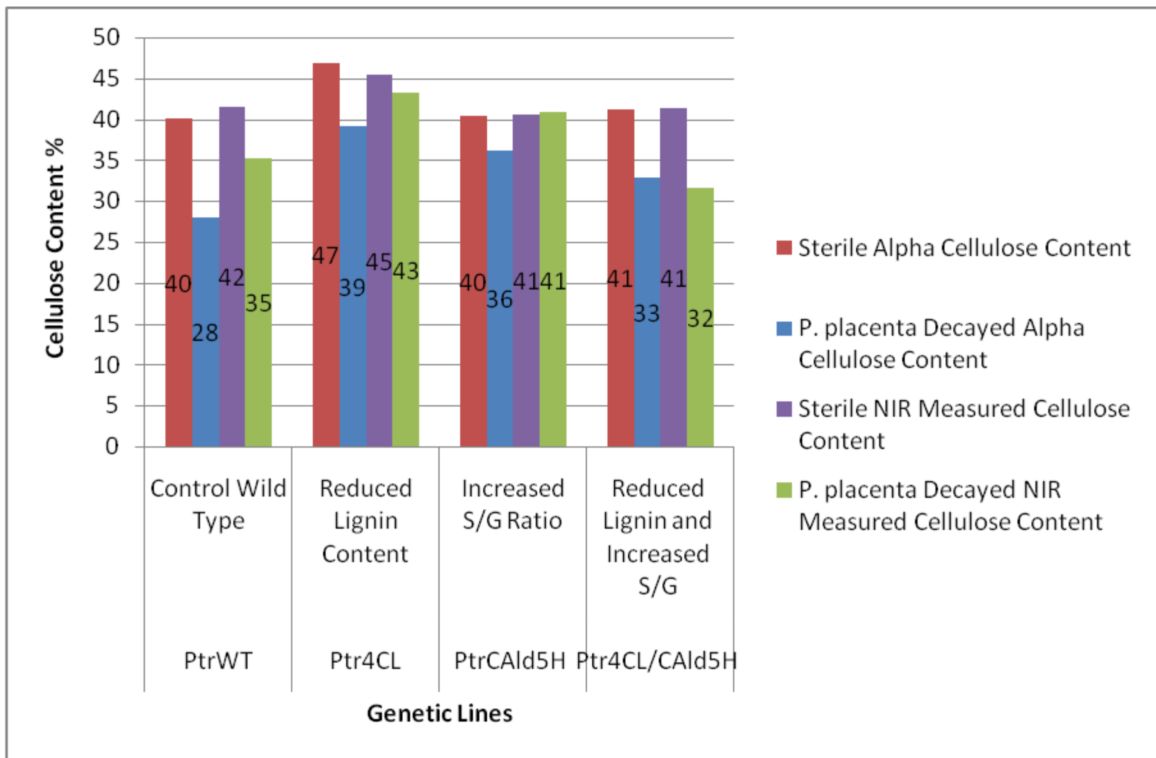

Fig 3-6. Comparison of analytical chemistry and NIR α-cellulose analysis results. *Populus* species wood samples after colonization with *Postia placenta.*

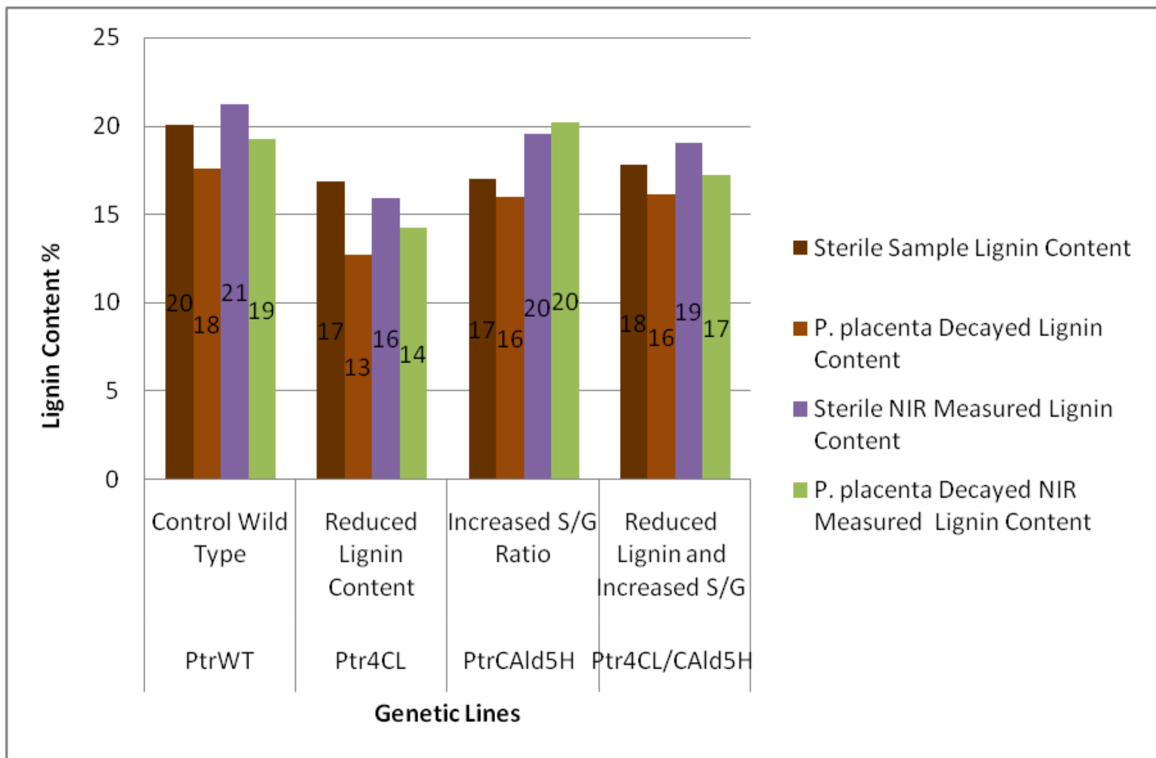

Fig 3-7. Comparison of analytical chemistry and NIR lignin analysis results. *Populus* species wood samples after colonization with *Postia placenta.*

Discussion

Unexpectedly the transgenic lines decayed by the brown rot fungus *Postia placenta* exhibited less cellulose decay than the wild type (Fig 3-6). Brown rot fungi primarily decay cellulose with peroxides which rapidly depolymerizing the cellulose polymer chains [Eriksson et al. 1990]. These small molecules easily diffuse thru the wood and are not affected by the lignin matrix during degradation. Further chemical analysis along with additional decay testing, could elucidate the cause of this reduction in cellulose decay rate. The reduced lignin line decayed by the brown rot fungus exhibited a higher percentage lignin loss than the other lines (Fig 3-7). The slight increase in lignin loss suggests that the low lignin concentration reduces the wood's resistance to fungal decay by brown rot fungi.

The reduced lignin line decayed by the simultaneous white rot fungus *Trametes versicolor* exhibited more cellulose decay than the wild type or other transgenic lines (Fig. 3-4). The cellulose decay results from the simultaneous white rot fungus *T. versicolor* suggest that carbohydrate decay rate is increased when lignin concentration is decreased, although this is not the case with the other fungi tested. The reduced lignin line decayed by the simultaneous white rot fungus exhibited a slightly higher percentage lignin loss compared with the other lines (Fig 3-5).

Although brown rot and simultaneous white rot fungi are not currently used for biopulping, it is significant that these types of decay are reduced by an increase in syringyl lignin content. Resistance to fungal decay is an important feature for woods used

in a structural capacity. The inclusion of the brown and simultaneous white rot fungi into this study allows comparisons to be made between different decay mechanisms. Brown rot fungi have the ability to rapidly depolymerize cellulose far from the hyphae using peroxides. Depolymerization using this mechanism may lead to future biopulping techniques. The results are also useful in future transgenic forest management and forest pathology diagnosis.

The transgenic lines decayed by the lignin selective white rot fungus *Ceriporiopsis subvermispora* exhibited similar rates of cellulose decay as the wild type (Fig. 3-2). These results suggest that modifications of lignin concentration and type do not increase the rate of carbohydrate decay by *C. subvermispora*. The increased S/G ratio lines exhibited little to no lignin loss (Fig 3-3). The lignin analysis results suggest that the lines with increased syringyl lignin content were more resistant to decay.

Ceriporiopsis subvermispora in this study provides important details into the type of lignin removed during selective degradation. The lignin selective white rot fungus *C. subvermispora* degrades primarily lignin, leaving most sugars intact [Schwanniger et al 2004)]. *C. subvermispora* has demonstrated an affinity for syringyl type lignin during wood degradation [Ferraz et al 2004, Choi et al 2006]. The lignin selective white rot fungus *C. subvermispora*, in addition to breaking β-O-4 bonds, removes methoxy groups from the syringyl lignin monomer leaving a guaiacyl monomer [Ferraz et al 2004, Martinez et al 2005, Choi et al 2006]. In previous mass loss testing, the increase of S-type lignin translated to a moderate increase of mass loss [Giles et al. 2008b unpublished]. Although there is mass loss,

the percentage lignin in the sample does not change [Giles et al. 2008b unpublished]. The additional mass loss may be attributed to an increase in hemicellulose degradation.

The fungal mechanisms for wood degradation rely on diffusion of enzymes and peroxides within short distances of the hyphae [Eriksson et al 1990, Blanchette et al 1991]. Although the lignin percentage is lower in both the Ptr4CL and Ptr4CL/CAld5H genetic lines, the lignin is available for the fungus to degrade during the short forty day colonization. Since the rate of lignin degradation is evidenced to stay the same, it is expected that during an extended colonization the reduced lignin lines would eventually have little accessible lignin as degradation progressed. The reduced lignin content lines during an extended colonization therefore would show decreasing mass loss percentages as the wild type and increased S/G lines would continue to exhibit mass loss.

The negligible difference between the remaining lignin in the reduced lignin lines indicates the rate of degradation is not affected by the reduced availability of lignin. The reduced availability of the lignin may not be significant enough in a short forty day decay period. Extending the colonization time may produce an eventual decline of lignin loss in the reduced lignin lines, although any commercial use for fungal pretreatment of transgenic pulps would not extend past forty days used in this study [Akhtar et al. 1992].

All three fungi exhibited little to no lignin loss in the increased S/G lignin lines (Fig 3-3, Fig 3-5, and Fig 3-7). This indicates that the slightly higher S/G ratio increased the wood's resistance to all types of decay regardless of fungal decay mechanism. These findings support previous studies, in which the increase of syringyl type lignin suggested an increased

resistance to fungal decay enzymatic processes, thru additional polymer bonds and additional functional groups on the monolignol's aromatic ring [Obst et al. 1994, Choi et al. 2006]. This study supports earlier findings in which Klason lignin with higher S/G lignin ratios are removed slowly during white rot degradation [Obst et al. 1994].

The near infrared spectroscopic analysis and calibration model used to determine chemical properties worked well for determining α-cellulose content of the decayed samples. The NIR prediction for lignin produced similar results although a higher rate of difference between Klason determined values was observed. The slight differences between the analytical chemistry and NIR predicted values verified that NIR can be used to roughly determine chemical composition of decayed material. The accuracy of the decayed sample prediction is heavily influenced by the NIR calibration model. The prediction model was designed for determining chemical characteristics of sound wood samples. A comparison between decayed and sterile samples gives insight into the cause of the prediction error rate. In the case of predicted lignin, the higher percentage of difference between methods in the decayed samples suggests that the presence of hyphae or lignin polymerization effects the estimation of lignin using NIR transmittance and a sound wood calibration model. Since the Klason lignin analysis is not designed for decayed samples, there is also possibility of hyphal fragment accumulation during the Klason method according to comparisons between sterile and colonized samples (Fig 3-3, Fig 3-5, Fig 3-7) [Martinez et al. 2005].

The analytical chemical and NIR analysis methods used in this study are unable to detect the possible modification of the lignin, even though analysis suggests there is a

degradation of lignin structure. Based on previous experiments, the little to no change in lignin percentage after colonization may be attributed to the modification of the lignin present in the sample [Giles et al. 2008b unpublished]. The minimal S/G ratio differences between the modified S/G ratio lines may reflect the degree of lignin type modification needed to alter lignin decay by fungi within a short decay period (Table 3-10. Although these lines contain a slightly higher S/G ratio, the CAld5H lines S/G lignin ratios are considerably higher (Table 3-1).

Conclusions

All of the wild type and transgenic lines inoculated were not resistant to fungal decay. The rate of cellulose decay with the lignin selective white rot fungus *C. subvermispora* was similar between the wild type and transgenic lines. All three fungi exhibited little to no lignin loss in the increased S/G lignin lines suggesting that the slightly higher S/G ratio increased the wood's resistance to all types of decay regardless of fungal decay mechanism. Notably, for brown rot and lignin selective white rot, the reduced lignin content lines did not increase the rate of carbohydrate decay. Within all the fungi tested, the increased S/G ratio lines lower lignin decay rates. This observation gives insight into the particular lignin modifications suited for use with a lignin selective white rot fungal pretreatment. The wood chemical content and composition significantly affected degradation across fungal decay types. The use of near infrared transmittance was successful in predicting the cellulose and lignin percentages of the decayed material although calibration with both sound and decayed material may reduce the error rate.

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Appendices

Appendix A - Holocellulose Content of Samples

Treatment	Fungus	Line	Sample ID	% Holocellulose of Decayed Sample
T0	C.sub	271.00	1A	49.31
T0	C.sub	271.00	2A	70.95
T0	P. pla	271.00	3A	34.36
T0	T.vers	271.00	4A	68.15
T0	None	271.00	5A	76.06
T0	P. pla	271.00	3A	77.96
T0	C.sub	271.00	2A	75.19
T0	C.sub	271.00	1A	76.94
T0	T.vers	271.00	4A	78.52
T1	C.sub	37.00	1A	67.97
T1	P. pla	37.00	3A	65.58
T1	T.vers	37.00	4A	58.31
T1	C.sub	37.00	2A	75.41
T1	None	37.00	5A	73.36
T1	P. pla	37.00	3A	76.65
T2	C.sub	94.00	1A	66.40
T2	C.sub	94.00	2A	78.98
T2	T.vers	94.00	3A	70.44
T2	P. pla	94.00	4A	77.91
T2	None	94.00	5A	73.91
T3	C.sub	141.00	1A	67.32
T3	C.sub	141.00	2A	48.42
T3	P. pla	141.00	3A	77.18
T3	T.vers	141.00	5A	70.64
T3	C.sub	141.00	2A	70.23
T3	P. pla	141.00	3A	75.51
T3	C.sub	141.00	1A	71.01
T3	None	141.00	4A	75.61

Appendix B - α-cellulose Content of Samples

Treatment	Clone	Fungus	Tree	Position	% Alpha Cellulose	% Alpha Cellulose Average
T0	271.00	*Ceriporiopsis subvermispora*	1.00	A	39.84	39.36
T0	271.00	*Ceriporiopsis subvermispora*	1.00	A	39.99	
T0	271.00	*Ceriporiopsis subvermispora*	2.00	A	39.17	
T0	271.00	*Ceriporiopsis subvermispora*	2.00	A	38.45	
T0	271.00	*Postia placenta*	3.00	A	31.55	31.32
T0	271.00	*Postia placenta*	3.00	A	31.09	
T0	271.00	*Trametes versicolor*	4.00	A	37.69	39.38
T0	271.00	*Trametes versicolor*	4.00	A	41.07	
T0	271.00	Sterile	5.00	A	43.48	42.28
T0	271.00	Sterile	5.00	A	41.08	
T1	37.00	*Ceriporiopsis subvermispora*	1.00	A	42.90	43.66
T1	37.00	*Ceriporiopsis subvermispora*	1.00	A	41.76	
T1	37.00	*Ceriporiopsis subvermispora*	2.00	A	46.32	
T1	37.00	*Postia placenta*	3.00	A	41.28	42.30
T1	37.00	*Postia placenta*	3.00	A	43.32	
T1	37.00	*Trametes versicolor*	4.00	A	33.15	33.15
T1	37.00	Sterile	5.00	A	49.59	47.43
T1	37.00	Sterile	5.00	A	45.27	
T2	94.00	*Ceriporiopsis subvermispora*	1.00	A	35.12	40.03
T2	94.00	*Ceriporiopsis subvermispora*	2.00	A	44.95	
T2	94.00	*Trametes versicolor*	3.00	A	35.54	35.54
T2	94.00	*Postia placenta*	4.00	A	40.27	40.27
T2	94.00	Sterile	5.00	A	41.81	41.81
T3	141.00	*Ceriporiopsis subvermispora*	1.00	A	40.22	40.18
T3	141.00	*Ceriporiopsis subvermispora*	1.00	A	41.43	
T3	141.00	*Ceriporiopsis subvermispora*	2.00	A	38.42	
T3	141.00	*Ceriporiopsis subvermispora*	2.00	A	40.65	
T3	141.00	*Postia placenta*	3.00	A	42.76	42.08
T3	141.00	*Postia placenta*	3.00	A	41.40	
T3	141.00	Sterile	4.00	A	43.57	43.13
T3	141.00	Sterile	4.00	A	42.69	
T3	141.00	*Trametes versicolor*	5.00	A	41.57	41.57

Appendix C - Klason Lignin Data

Sample	Gene	Fungus	Lignin Content %	Replicate Lignin Content %	Average Lignin Content %
1BC	PtrWT	*Ceriporiopsis subvermispora*	18.45	16.46	17.45
2BC	PtrWT	*Ceriporiopsis subvermispora*	15.29	11.72	13.51
3BC	PtrWT	*Postia placenta*	27.51*	21.84	21.84
4BC	PtrWT	*Trametes versicolor*	25.95*	21.42	21.42
5BC	PtrWT	Sterile	22.21	19.30	20.76
1BC	Ptr4CL	*Ceriporiopsis subvermispora*	12.50	11.91	12.21
2BC	Ptr4CL	*Ceriporiopsis subvermispora*	8.21	10.15	9.18
3BC	Ptr4CL	*Postia placenta*	13.60	14.30	13.95
4BC	Ptr4CL	*Trametes versicolor*	13.44	12.59	13.02
5BC	Ptr4CL	Sterile	16.28	18.21	17.24
1BC	PtrCAld5H	*Ceriporiopsis subvermispora*	18.10	5.18	11.64
4BC	PtrCAld5H	*Postia placenta*	16.60	18.39	17.49
5BC	PtrCAld5H	Sterile	16.68	19.25	17.97
2BC	Ptr4CL/CAld5H	*Ceriporiopsis subvermispora*	16.58	18.26	17.42
3BC	Ptr4CL/CAld5H	*Postia placenta*	21.75	22.80	22.28
4BC	Ptr4CL/CAld5H	Sterile	18.29	18.45	18.37
5BC	Ptr4CL/CAld5H	*Trametes versicolor*	19.30	25.99*	19.30

Samples denoted with * abnormally high and removed from average calculation.

Appendix D – NIR Determined Values

Sample	Fungus	Lignin % (Vision Generated)	Adjusted Calculated Lignin %	Cellulose % (Vision Generated)	Adjusted Calculated Cellulose %
T0 271 1BC	Lignin Selective White Rot	21.35	19.03	39.81	35.49
T0 271 2BC	Lignin Selective White Rot	18.17	15.49	43.62	37.18
T0 271 3BC	Brown Rot	23.87	19.23	41.19	33.18
T0 271 4BC	Simultaneous White Rot	24.81	17.80	33.30	23.89
T0 271 5BC	Control	21.99	21.23	43.03	41.53
T1 37 1BC	Lignin Selective White Rot	16.20	14.14	46.98	41.02
T1 37 2BC	Lignin Selective White Rot	13.36	11.48	49.57	42.60
T1 37 3BC	Brown Rot	15.61	14.25	48.64	44.39
T1 37 4BC	Simultaneous White Rot	16.46	13.75	49.79	41.59
T1 37 5BC	Control	16.31	15.94	46.44	45.39
T2 94 5BC	Control	20.67	19.56	42.87	40.56
T2 94 1BC	Lignin Selective White Rot	22.70	19.85	37.12	32.47
T2 94 4BC	Brown Rot	22.12	20.18	42.26	38.55
T3 141 2BC	Lignin Selective White Rot	19.01	17.07	42.87	38.51
T3 141 3BC	Brown Rot	23.77	17.19	39.96	28.90
T3 141 4BC	Control	19.70	19.06	42.75	41.36

Appendix E – Raw Mass Loss Data

Mass Loss %	Treatment/Genetic Line	Fungus	Diameter (mm)	Initial (g)	Post Decay (g)
8.44	T0	*Ceriporiopsis subvermispora*	8.38	0.4301	0.3938
10.52	T0	*Ceriporiopsis subvermispora*	8.01	0.3861	0.3455
11.21	T0	*Ceriporiopsis subvermispora*	7.09	0.3826	0.3397
10.96	T0	*Ceriporiopsis subvermispora*	6.71	0.3066	0.273
15.79	T0	*Ceriporiopsis subvermispora*	6.86	0.2628	0.2213
13.57	T0	*Ceriporiopsis subvermispora*	6.22	0.227	0.1962
10.46	T0	*Postia placenta*	6.74	0.2869	0.2569
20.78	T0	*Postia placenta*	6.69	0.2743	0.2173
18.12	T0	*Postia placenta*	6.49	0.2748	0.225
13.44	T0	*Trametes versicolor*	7.37	0.3676	0.3182
24.22	T0	*Trametes versicolor*	7.11	0.3584	0.2716
33.66	T0	*Trametes versicolor*	6.7	0.2671	0.1772
4.61	T0	Sterile	6.68	0.2862	0.273
1.85	T0	Sterile	5.75	0.195	0.1914
6.61	T1	*Ceriporiopsis subvermispora*	6.59	0.3721	0.3475
13.20	T1	*Ceriporiopsis subvermispora*	6.17	0.2394	0.2078
12.10	T1	*Ceriporiopsis subvermispora*	5.55	0.2173	0.191
11.95	T1	*Ceriporiopsis subvermispora*	5.68	0.2059	0.1813
13.16	T1	*Ceriporiopsis subvermispora*	5.09	0.1808	0.157
15.03	T1	*Ceriporiopsis subvermispora*	4.97	0.1697	0.1442
7.45	T1	*Postia placenta*	6.5	0.3116	0.2884

Mass Loss %	Treatment/Genetic Line	Fungus	Diameter (mm)	Initial (g)	Post Decay (g)
8.32	T1	*Postia placenta*	6.54	0.3208	0.2941
9.46	T1	*Postia placenta*	5.32	0.1828	0.1655
22.92	T1	*Trametes versicolor*	5.92	0.2107	0.1624
11.27	T1	*Trametes versicolor*	5.71	0.1917	0.1701
21.11	T1	*Trametes versicolor*	5.79	0.216	0.1704
2.12	T1	Sterile	7.78	0.4672	0.4573
2.41	T1	Sterile	7.84	0.5136	0.5012
16.58	T2	*Ceriporiopsis subvermispora*	4.85	0.1677	0.1399
16.61	T2	*Ceriporiopsis subvermispora*	4.9	0.1535	0.128
9.54	T2	*Ceriporiopsis subvermispora*	5.65	0.2086	0.1887
15.70	T2	*Ceriporiopsis subvermispora*	4.82	0.2286	0.1927
15.46	T2	*Ceriporiopsis subvermispora*	3.37	0.0757	0.064
16.50	T2	*Trametes versicolor*	3.63	0.0788	0.0658
18.01	T2	*Trametes versicolor*	3.72	0.0894	0.0733
10.04	T2	*Postia placenta*	5.2	0.1842	0.1657
7.99	T2	*Postia placenta*	5.01	0.1903	0.1751
9.85	T2	*Postia placenta*	4.74	0.1422	0.1282
5.00	T2	Sterile	4.78	0.1641	0.1559
5.99	T2	Sterile	3.86	0.1119	0.1052
15.09	T3	*Ceriporiopsis subvermispora*	6.1	0.2876	0.2442
13.82	T3	*Ceriporiopsis subvermispora*	5.88	0.2294	0.1977
12.64	T3	*Ceriporiopsis subvermispora*	7.08	0.2959	0.2585
11.32	T3	*Ceriporiopsis subvermispora*	7.41	0.3047	0.2702
9.01	T3	*Ceriporiopsis subvermispora*	6.97	0.2985	0.2716
21.98	T3	*Postia placenta*	7.94	0.5483	0.4278
38.82	T3	*Postia placenta*	7.52	0.3715	0.2273
16.15	T3	*Postia placenta*	6.92	0.3591	0.3011

Mass Loss %	Treatment/Genetic Line	Fungus	Diameter (mm)	Initial (g)	Post Decay (g)
2.11	T3	Sterile	6.76	0.3322	0.3252
4.54	T3	Sterile	6.72	0.2861	0.2731
11.70	T3	*Trametes versicolor*	7.26	0.3154	0.2785
16.54	T3	*Trametes versicolor*	5.8	0.2068	0.1726