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# MOLECULAR APPROACHES FOR CHARACTERIZATION OF BIODEGRADATION GENES EXPRESSED DURING MICROBIAL COLONIZATION ON DECAY-RESISTANT AND NON-RESISTANT WOODS IN FOREST SOIL

By

Youngmin Kang

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Forest Resources
in the Department of Forest Products

Mississippi State, Mississippi

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Youngmin Kang

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MOLECULAR APPROACHES FOR CHARACTERIZATION OF

BIODEGRADATION GENES EXPRESSED DURING

MICROBIAL COLONIZATION ON DECAY-

RESISTANT AND NON-RESISTANT

WOODS IN FOREST SOIL

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White and brown-rot fungi damage wood by production of enzymes that attack the structural components. The objective of this study was to characterize decay related genes and proteins that are expressed on three different wood types undergoing decay over 18 months. Variation in gene expression, presence of decay enzymes and proteins were determined for untreated pine (non-resistant), western red cedar (naturally durable), and alkaline copper quaternary (ACQ) treated pine (chemically resistant) exposed in a soil decay bed test. Decay was assessed by visual decay ratings, dynamic modulus of elasticity (MOE), and microscopy. There were no significant differences in decay between cedar and ACQ-treated pine over the 18 month period. However, there were significant differences in decay between pine and cedar and between pine and ACQ-

treated pine. The fungal mycelia penetrated the cell walls of pine and were continually observed over 18 months, but were not observed in cedar or ACQ-treated pine. Basidiomycetes containing decay genes lignin peroxidase (Lip), manganese peroxidase (Mnp), and laccase (Lcc) were detected on pine and ACQ-treated pine which also a greater diversity of fungi had compared to cedar. *Phlebia radiata* specific-lignin peroxidase and manganese peroxidase genes were expressed approximately equally on pine and ACQ-treated pine at most sampling times. The expression of *P. radiata* specific Lcc was higher on ACQ-treated pine than untreated pine. No basidiomycete genes were expressed and only a few basidiomycetes were identified on cedar, which also showed little decay. ACQ-treated pine also showed little decay, however basidiomycetes were present and active. Proteins were first detected on pine and ACQ-treated pine at 6 months and continued to increase through 18 months, but were not detected on cedar until 14 months exposure. There were greater numbers of total proteins on pine than on cedar and ACQ-treated pine at each time period. Decay genes were only found on pine but not on cedar and ACQ-treated pine. Additionally, the types of proteins and their score were different among the three wood types. From these results, the natural durability of cedar reduced the wood decay community and its activities. It appears that ACQ-treated wood did not stop the production of the decay enzymes but the chemical treatment did inhibit the effectiveness of the wood decay genes.

# DEDICATION

The author would like to dedicate this research to Kyoungok No (Spouse), Serjin Kang (Daughter), and Eugene Kang (Son), Yongun Kang (Father), Gyeongja Choi (Mother) as well as Prof. Myung-Suk Choi (Mentor) in Republic of Korea.

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# CHAPTER I

# OVERVIEW OF RESEARCH

## INTRODUCTION

Wood decay results in a loss of billions of dollars (US) annually in the United States (Preston 2000). The main causal agents of wood decay are fungi belonging to the Basidiomycota and Ascomycota (Basu and Ghose 1962). Traditional methods of protecting wood from decay involve the application of broad spectrum chemical preservatives without knowing often the specific identity of the microorganisms involved in the decay process. The identification of the microorganisms and their genes causing decay is important in order to deign to develop a complete understanding of the roles that different microorganisms play in wood decay. The time required for wood decay is long and variable depending on the wood and environmental conditions. Naturally durable woods require decades for complete deterioration compared to non-resistant woods which may deteriorate in several years. Use of a soil bed decay test reduces the amount of time required for microorganisms to colonize and decay wood, thus allowing research on wood decay and preservatives to proceed in a reasonable time frame (Li *et al.* 2007).

Wood degradation is initiated by the action of specific microbial enzymes produced mainly by fungi in order to obtain food for their survival (Cease *et al.* 1989). Ascomycota fungi generally cause surface soft rot decay, while Basidiomycota cause a

more destructive brown rot or white rot decay (Nilsson *et al.* 1989). Lignin is three dimensional complex polymer effectively degraded only by white-rot fungi. Thus, the lignification of wood cell walls is an obstacle for wood biodegradation. White rot fungi are capable of extensive degradation of lignin, hemicellulose and cellulose components of wood including the middle lamella (Leonowicz *et al.* 1999). Although brown rot fungi do not totally degrade lignin, they are able to overcome the lignin barrier by non-enzymatic mechanisms and degrade cellulose and hemicellulose.

During the process of wood decay, fungi express different genes involved in both wood decay and fungal metabolic processes. Gene expression results in abundant mRNA and includes all transcripts in the cell. The transcriptome represents the genes that are being actively expressed at a given time, but does not necessarily mean that the corresponding enzyme is also abundant or active in the cell (Prabakaran *et al.* 2001). The proteome is the complete set of proteins produced from the genome (Grigoriev 2001). Proteomics is much more complex than either genomics or the transcriptomics because each protein can be chemically modified in different ways after biosynthesis. Expressional proteomics can provide a snap shot of all proteins expressed by an organism at the time of extraction (Prabakaran *et al.* 2001). These profiles can be compared among different wood types and over time to determine what proteins are being uniquely expressed during the decay process.

**Objectives**

The purpose of this study was to determine how the suite of decay genes and proteins are expressed during biodeterioration of three different wood types in forest soil

over time. The working hypothesis is that the expression of wood decay genes and proteins will be influenced by the decay resistance of the wood being degraded.

Specific objectives were:

1. To characterize structural and physical changes occurring on decay-resistant, non-resistant, and chemically treated woods during microbial colonization over 18 months.
2. To determine how the expression level of genes coding for selected decay enzymes vary during microbial colonization of decay-resistant, non-resistant, and chemically treated woods over 18 months.
3. To determine how the pattern of protein expression varies during microbial colonization of decay-resistant, non-resistant, and chemically treated woods over 18 months.

As an experimental approach, macro and microscopic observation and dynamic modulus-of-elasticity (MOE) were used to characterize structural and mechanical changes occurring during the decay of three wood types. Real-time quantitative PCR was used to quantify selected wood decay genes expressed by a specific decay fungus during this study. A standard proteomics procedure using two-dimensional gel electrophoresis (2-DE) followed by the peptide mass fingerprints obtained by matrix assisted laser desorption/ionization time of flight-mass spectrometry (MALDI/TOF-MS) and matched against sequence databases was used for protein identification. The expected outcomes of this study were to identity and quantify wood decay genes and proteins that are expressed during degradation of different wood types.

There are many unanswered questions concerning wood decay and wood protection research, and no single research study or method will ever answer all of these

questions. The process of wood decay is very complex. There are a variety of molecular techniques currently being used that can allow researchers to probe these wood decay organisms and their activities.

This study was conducted by adopting molecular approaches to characterize biodeterioration genes expressed by microorganisms while degrading untreated pine, cedar, and ACQ treated pine. The chapters in this dissertation are:

Chapter 1: Overview of research

Chapter 2: The structural and physical property changes of wood cell walls during biodeterioration of three wood types

Chapter 3: Gene expression of selected decay enzymes during biodeterioration of three different wood types

Chapter 4: Comparative protein profiling during biodeterioration of three different wood types

The studies of the genome, transcriptome, and proteome of microbial communities present during wood decay will provide some insight into how these communities degrade three wood types.

BIBLIOGRAPHY

Basu, S.N. and R. Ghose. 1962. A microscopical study on the degradation of jute fiber by microorganisms. Textile Research Journal 32: 677–694.

Cease, K.R., R.A. Blanchette, and T.L. Highley. 1989. Interactions between *Scytalidium* species and brown- or white-rot basidiomycetes in birch wood decayed in the laboratory. Wood Science and Technology 23: 151-161.

Grigoriev, A. 2001. A relationship between gene expression and protein interactions on the proteome scale: analysis of the bacteriophage T7 and the yeast Saccharomyces cerevisiae. Nucleic Acids Research 29(17):3513-3519.

Leonowicz, A., A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtaś-Wasilewska, N.-S. Cho, M. Hofrichter, and J. Rogalski. 1999. Biodegradation of lignin by white rot fungi. Fungal Genetics and Biology 27: 175-185.

Li, G., D.D. Nicholas, and T.P. Schultz. 2007. Development of an accelerated soil-contact decay test. Holzforschung 61: 214-218.

Nilsson, T., G. Daniel, T. Kirk, and J.R. Obst. 1989. Chemistry and microscopy of wood decay by some higher ascomycetes. Holzforschung 43:11-18.

Prabakaran, P., J. An, M. Gromiha, S. Selvaraj, H. Uedaira, H. Kono, and A. Sarai. 2001. Thermodynamic database for protein-nucleic acid interactions (ProNIT). Bioinformatics 17:1027-1034.

Preston, F. 2000. Wood preservation: trends of today will influence the industry tomorrow. Forest Products Journal 50: 12–19.

# CHAPTER II

# THE STUCTURAL AND PHYSICAL PROPERTY CHANGES OF CELL WALLS DURING BIODETERIORATION OF THREE WOOD TYPES

## INTRODUCTION

Wood is an important renewable and natural resource with a multitude of uses. The microbial decay of wood is a critical process in carbon recycling and is essential for maintaining forests. Although wood decomposition is a necessary process in forests, it also results in damaged wood products contributing to billions in economic loss annually (Preston 2000). The primary biotic decomposers of wood belong to the basidiomycetes such as white rot and brown rot fungi. Celluloses and hemicelluloses are broken down by reactions catalyzed by enzymes produced by white, brown, and soft rot fungi. However, lignin is effectively degraded only by white-rot fungi (Cease *et al.* 1989). Wood cell wall degrading enzymes produced by these fungi are too large to penetrate into the woody cell wall, thus small mass molecules are needed initially to open up the structure (Eriksson *et al.* 1990).

Traditional methods for wood protection from decay involve treatment with a wide range of chemical preservatives without knowing the specific identity of the microorganisms causing decay. Identification of these microorganisms is important in order to develop a complete understanding of the roles different microorganisms play in

decay. Macrostructural studies of the wood cell wall undergoing degradation by fungi and bacteria over the last 100 years have been invaluable in confirming decay patterns. Light microscopy (LM, ~500X) is the most popular technique to observe the macro-structure of wood. The study of wood micro- structure is possible with scanning electron microscopy (SEM, ~500,000X) and transmission electron microscopy (TEM, ~1,000,000X) and can provide details of the ultrastructure of wood cell wall layers and the microorganisms. Environmental SEM (ESEM, ~1,000,000X) permits the same level of examination without the need to desiccate the samples (Collins *et al.* 1993; Egerton-Warburton *et al.* 1993).

LM, SEM, and TEM was used to determine the extent of decay and to measure dynamic MOE loss measured in woods with different durability over 18 months. The results from this study will provide a better understanding about the different structural changes and microbial decay patterns in untreated pine (non- decay resistant wood), cedar (naturally decay resistant), and alkaline copper quaternary (ACQ, 0.15 pcf) treated pine (chemically treated to be decay resistant).

## LITERATURE REVIEW

### Microbial wood decay process in forest ecosystem

Wood is a natural material that undergoes biodegradation by a variety of microorganisms. The wood degrading organisms are diverse groups of bacteria and fungi. Fungi are the most important organisms with respect to biodeterioration of wood. However, bacteria may co-work fungi in the degradation process. Wood degradation also

constitutes an important part of carbon cycling, a process that is required for a balanced ecosystem and sustenance of life on earth. In forest soils, various microorganisms play an important part in the break down of the rigid structural parts of the trees (Daniel 2003). As fungal mycelia remain in the soil for indefinite periods, they can initiate their physiological functions by imbibing water when available. It is often possible to observe a dense network of fungal mycelia spreading throughout the forest soil litter while hyphae of different fungi, especially Basidiomycetes, appear on the surface.

**Factors that determine occurrence of fungi on wood**

Degradation of wood is a natural process resulting in breakdown of its components into constituent elements. Wood acts as a source of food and energy for many microbes (Beguin and Aubert 1994). Most decay fungi are saprophytes that obtain their nutrients from dead or dying biomass. Some fungi can form aggregates of hyphae which makes it possible for them to reach substrates over long distances because the hyphal system can translocate essential elements (Highley and Micales 1990). Various environmental factors can affect fungi that degrade wood.

The water content of wood is critical for decay to occur. Water is present in wood as bound water in the cell walls and as free water in the cell lumens. At the fiber saturation point, approximately 30%, the cell walls are saturated with water, however the wood decay process occurs at moisture levels above this point. Wood decay basidiomycetes are most active at wood moisture contents between 35-80% (Cunningham *et al.* 2007).

Temperature is another important factor that influences the growth of decay fungi. Most basidiomycetes grow at temperatures between 20-40°C (Cunningham *et al.* 2007). Oxygen is also an important factor for the biodegradation of lignin in wood. Cellulose and hemicellulose may be degraded under anoxic conditions, but there are no reports of degradation of lignin under these conditions. In fact, the reduction of oxygen is known to protect wood. Cronyn (1990) found that timbers enclosed in sealed plastic envelopes absorbed oxygen and produced carbon dioxide by the parenchyma cells providing protection to the wood because of the anoxic condition. Essential nutrients such as nitrogen, carbon, iron, manganese, and sulphur are important factors for the growth of wood degrading fungi. However, the wood decay activities of basidiomycetes require low concentrations of these elements. The decay activity of soft rot fungi is greatly stimulated by the addition of nitrogen. This also explains why soft rot attack is more severe in fertile soil conditions (Terziev and Nilsson 1999).

**Wood anatomy**

Trees are generally classified as softwoods (gymnosperms) and hardwoods (angiosperms). Gymnosperms are trees such as fir, spruce, and pine which are evergreen with world wide distribution. Angiosperms are trees such as oak, poplar, and birch which have broad-leaves (Wardrop 1964). In softwoods, the axial wood cell types are tracheids which align in chains. In hardwoods, the axial wood cells are composed of vessels, fibers, and parenchyma cells. The alignment of these cell types is much more complex and distinct alignment is not apparent because of a variety of different cell types.

Wood is composed of cells containing several wall layers which are categorized as primary (P) and secondary (S) cell walls (Siau 1971). The P cell wall is comprised of a single layer with randomly orientated cellulose microfibrils. The orientation of the microfibrils contributes to the physical strength properties of wood. The S cell walls exist as three layers called S1, S2, and S3. The S1 and S3 layers are comparatively thin with the S2 layer comprising the majority of the S cell wall. The S1 and S3 layers have a horizontal orientation of the microfibrils while the S2 layer orientation is vertical. Vertical orientation of the S2 layer is important because it provides mechanical strength to wood products (Howard and Manwiller 1969). The middle lamella is located between the cells and consists primarily of lignin. Individual cells are connected to each other by the middle lamella (ML).

Wood is composed of ordered axial and radial cell systems (Wardrop and Harada 1965). The axial system is composed of elongated cells orientated in the longitudinal direction of the trunk which provides mechanical support, transport and the storage of nutrients. The radial system is comprised of rays that form horizontal groups of cells extending from the bark to the pith with their main function being the storage and redistribution of starch. Wood cells are produced in the vascular cambium from two special meristem cell types termed fusiform and ray initials (Wardrop 1964). Fusiform cells give rise to all cell types of the axial cell systems and the ray initials provide radial cell systems in both softwoods and hardwoods.

Wood formed during a single year can be divided into earlywood and latewood (Stamm 1964). Earlywood forms during the spring and is recognized by the relatively large tracheids and thin cell walls. Latewood forms during the summer and is recognized

by smaller radial dimensions and thicker cell walls. The function of the tracheids is conduction of fluids and support (Boddy and Rayner. 1983). Earlywood tracheids usually have a greater number of larger bordered pits than latewood tracheids. The function of pits is the movement of water and gas. All tracheids are in contact with one or more rays. Softwoods have two types of parenchyma cells, longitudinal and epithelial. Softwoods have a small number of longitudinal parenchyma and the cells are easily recognized by the presence of simple pits. Epithelial cells produce resin and are only associated with softwood species that contain resin canals (Siau 1971).

**Influence of wood properties on decay**

The importance of durable wood species in Europe and USA has been increasing in the past decades due to increased environmental awareness (Daniel 2003). The natural durability classification of wood is related to the performance and service life while in contact with the ground (Raberg *et al.* 2005). Western Red Cedar (WRC, *Thuja plicata*) is an attractive and important wood because it contains natural extractives with antifungal activity (Edlin 1969). WRC is a conifer and a mainstay of the timber industry in the Western part of USA. It is exceptionally light in weight and remarkably strong. Also, it is naturally durable and resistant to decay outdoors, under most climates, without any preservative treatment (Panshin and Zeeuw 1970). Needs for the development of wood preservatives has continually increased. Many chemical wood preservatives has used for various purposes to protect wood. ACQ is an alkaline, copper, and quaternary chemical preservative that offers resistance to fungal decay. Copper in ACQ produces antifungal

activity which contributes to its decay resistance in wood (Groot and Woodward 1996; Watanabe *et al.* 1998; Goodell 2003).

**Wood structural changes caused by microorganisms**

White rot is caused by a large number of basidiomycetes. The two main morphological decay types described as simultaneous and selective attack are the principle ways in which wood cell walls are degraded by white rot fungi (Nicole *et al.* 1995). Fungal hyphae are typically found in the wood cell lumen during the decay process. In simultaneous white rot decay of wood, all the wood components (cellulose, hemicellulose, and lignin) are degraded from the cell lumen outwards (White and Boody 1992). This type of attack is best observed in the latewood cells of softwoods where the zones of decay are easily recognized as distinct rings progressing across the wood cell walls. TEM observations of decay zones have often shown a marked orientation of the lamella in wood cell walls during selective decay by white rot fungi (Rayner and Boddy 1998). Selective decay is the decay of lignin and hemicellulose first but not cellulose (Perez *et al.* 2002). Wood cell wall degradation is usually localized to cells colonized by fungal hyphae of white rot and substantial amounts of undecayed wood remain even after advanced decay has occurred (Kleman-Leyer *et al.* 1992).

Metals such as Mn, Fe and Cu have been implicated in the oxidative mechanisms of both white rot and brown rot fungi. Using TEM X-ray, the presence of Mn has been shown to be associated with fungal hyphae (*Phlebia radiata),* extracellular slime and to penetrate into the characteristic decay zones during wood decay (Daniel *et al.* 2004). Mn

within cell walls has also been shown to be associated with lignin removal and delamination in both the S2 and middle lamella layers.

Brown rot fungi are characterized by their rapid depolymerization of cellulose leading to a rapid loss in wood strength at early stages of the decay process (Goodell 2003). Brown rot attack is normally characterized by an excessive removal of cellulose and hemicelluloses. Hyphae grow in the cell lumen degrading carbohydrates. Hemicelluloses are degraded in the initial stages followed by extensive degradation of the cellulose, finally leaving a lignin skeleton. The initial attack has been observed to start in the S2 layer; later S1 and S3 are also degraded (Zabel and Morell 1992). Attack on the middle lamella is only occasionally observed. Early attack by brown rot is very difficult to detect using light microscopy. It is only when a substantial amount of the cellulose has been lost, that brown rot decay can be detected.

Bacterial degradation of pit membranes in water-logged or water-sprinkled wood has been observed and bacteria were confirmed to degrade even highly lignified wood cell walls (Blanchette *et al.* 1989; Collins *et al.* 1993). This evidence comes from electron microscopy studies, where bacteria involved in active degradation of cell walls can be seen. The distinction made between different types of bacterial attack is based on the micro-morphology and not on chemical changes occurring in wood. Detailed TEM studies have shown a number of characteristic features of bacteria decaying wood including direct cell wall penetration from the cell lumen and of the highly lignified middle lamella (Collins *et al.* 1993; Fromm *et al.* 2003).

There are two main types of bacteria that degrade wood: tunneling bacteria and erosion bacteria (Rayner and Boddy 1998). Tunneling bacteria are quite uncharacteristic

in their mode of attack. The attack is initiated by single bacteria that bore into the wood cell walls. At later stages the bacteria can be seen at the front of tunnels within the cell walls. The bacteria divide and the new individuals bore new tunnels (Helms *et al.* 2004). Erosion bacteria are rod shaped and gram negative (Rayner and Boddy 1998). They erode the cell walls starting at the S3 layer and progress towards the middle lamella. During degradation erosion bacteria have been observed to align themselves along the cellulose fibrils (Daniel 2003).

Other fungi such as stains and molds belong to the Ascomycetes and Deuteromycetes (Eriksson *et al.* 1990). Stain fungi thrive on extracts and sugar present in wood. Pit membranes are also degraded, increasing the porosity of wood. Stain fungi are also capable of direct penetration of the wood fibers, but none can degrade lignified wood cells. Like stain fungi, molds live on simple sugars and extracts, but do not generally penetrate very deep into the wood. Molds are able to grow through wood fibers and some species are cellulolytic. Wood discoloration and visible decay by stains and molds develops at the later stages of fungal attack on the wood (Daniel 2003).

## MATERIALS AND METHODS

### Preparation of wood stakes

Pine (*Pinus* sp.) and cedar (*Thuja plicata*) boards (5.1cm×10.2cm×180cm, 2.5cm×10.2cm×180cm respectively) used for this study were purchased from Lowes's Home Improvement Center, Starkville, Mississippi. Each board was cut into strips and numbered sequentially. The strips were cut into stakes measuring 14mm×14mm×115mm

(T×R×L). One set of pine stakes were treated with ACQ to a retention of 0.15 pcf by the full cell method using the following schedule: 73.7cm/Hg vacuum for 15 min followed by 150 psi pressure for 15 min (T1-08, 2008 AWPA standard). After treatment, all samples were wrapped in plastic and equilibrated for 7 days. The samples were then air dried for one week and equilibrated to approximately 12% moisture content. After drying ACQ-treated pine stakes as well as pine and cedar stakes were leached in deionized water. This process was repeated for a total of 7 days changing the water daily. The stakes were then air-dried for several days until they reached 60% moisture content (MC), weighed and dynamic modulus of elasticity (MOE) was measured using a Grindo Sonic, MK3 instrument (J.W. Lemmens Co).

**Soil bed decay test**

The decay test was carried out in small plastic containers (250mm×365mm×220mm) containing soil (silty clay soil) that was collected from the test site at Dorman Lake, Oktibbeha County, Mississippi. The soil used in this study was obtained from the top three inches of a forested area and then sieved through a screen to remove any large roots, rocks, and other debris. Eight circular holes (5mm diameter) were placed in the outer perimeter around the bottom of each plastic container. A piece of screen (250mm × 365mm) was placed on the bottom of each container followed by gravel (20mm deep) and the Dorman soil (100mm deep). The container was filled with soil up to 120mm from the bottom of the container. The MC of soil in each container was adjusted to 90% of its water holding capacity. Prepared stakes of pine, cedar, and ACQ treated pine were placed vertically in the plastic containers (Figure 2.1). The containers

(18 total, 6 for each wood type) were placed in a greenhouse at 25°C with a relative humidity 30-50% from November to March and outside from April to October. Two stakes per container were covered with nylon stocking material and used to monitor the moisture content weekly (Figure 2.2). The study was conducted over an 18 month period and described in Figure 2.3.

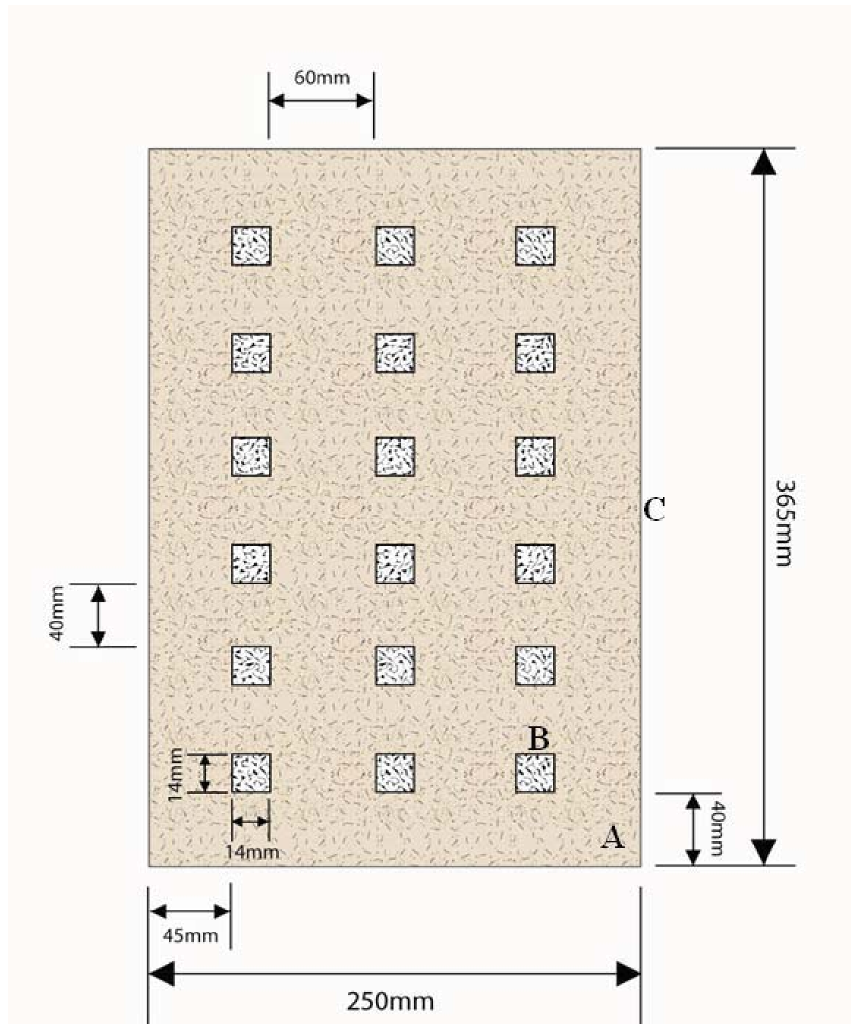



Figure 2.1. The soil bed decay test design. (A) Soil, (B) Wood stake, and (C) Body of container. Wood stakes are untreated pine, untreated cedar, and alkaline copper quaternary (ACQ, 0.15 pcf) treated pine. The container was 250mm×365mm×220mm (width×length×height).

Figure 2.2. Monitoring of control wood stakes during soil bed decay test. Temperature, humidity, and moisture content were monitored during decay test. The sensor detects temperature and humidity in the container. Wood stakes with nylon stocking material were used to monitor the moisture content of wood stakes.

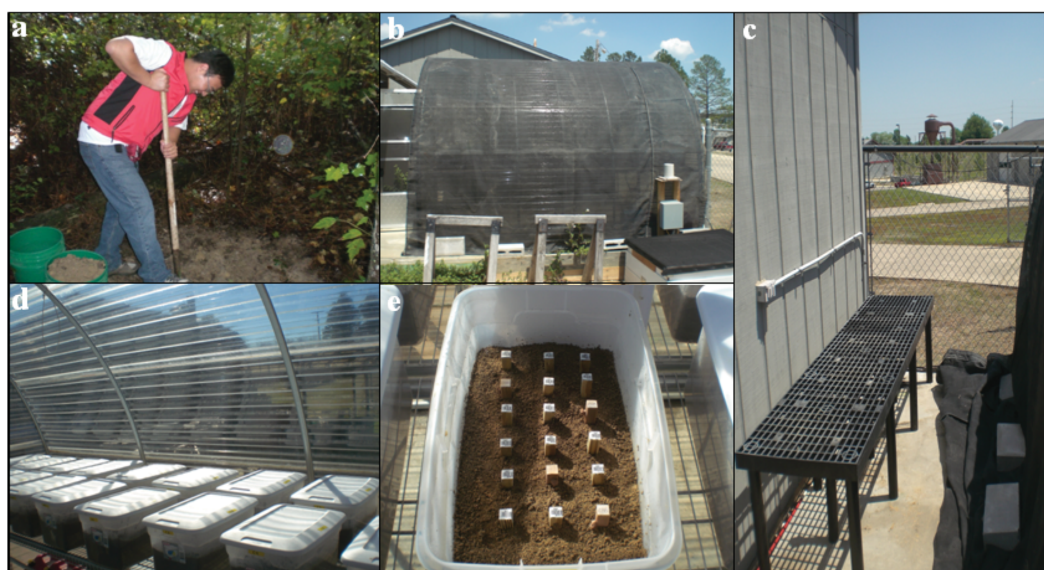



Figure 2.3. Organization of the soil bed decay test. (a) Author collects the soil from Dorman Lake, MS, (b) A view of greenhouse located at Forest Products Department at MSU, (c) A table placed beside greenhouse which held the test containers from April to October, (d) The soil bed decay test containers placed in greenhouse from November to March, and (e) The wood stakes in the soil bed decay test containers during this study.

**Decay rating, dynamic MOE test, and moisture content**

The visual decay rating was determined using method E7-01-2001 of the American Wood Protection Association (AWPA 2001) where 10 denotes no decay and 0 denotes failure (Table 2.1).

Table 2.1. Description of decay rating by American Wood Protection Association (AWPA) standard-E7. Tyhe range from 10 to 0 indicates the decay condition of wood stake during decay.

| Rating | Condition | Description |
|---|---|---|
| 10 | Sound | No sign or evidence of decay, wood softening or discoloration caused by microorganism attack. |
| 9.5 | Trace-suspect | Some areas of discoloration and/or softening associated with superficial microorganism attack. |
| 9 | Slight Attack | Decay and wood softening present. Up to 3% of the cross sectional area is affected. |
| 8 | Moderate Attack | Similar to "9", but more extensive attack with 3-10% of cross sectional area affected. |
| 7 | Moderate/ Severe Attack | Sample has between 10-30% of cross sectional area decayed. |
| 6 | Severe Attack | Sample has between 30-50% of cross sectional area decayed. |
| 4 | Very Severe Attack | Sample has between 50-75% of cross sectional area decayed. |
| 0 | Failure | Sample has functionally failed. It can either be broken by hand due to decay, or the evaluation probe can penetrate through the sample. |

The dynamic modulus of elasticity (MOE) for each of the stakes was measured approximately bimonthly. MOE was measured using a Grindo Sonic, MK3 (J.W. Lemmens Co). The average percentage of MOE change was calculated using the formula: [(initial MOE – current MOE) / initial MOE] ×100%. The moisture content (MC) was determined using the formula: [(current weight – oven dried weight) / oven

dried weight] ×100%. The MC of the control wood stakes from each container was calculated weekly and water was added to each container as needed to maintain the wood MC in the range of 40 to 80%. The statistical analysis of MOE, decay rating, and gene expression was performed by two-way analysis of variance (ANOVA) and Tukey's test (*alpha*=0.05) for randomized complete block design (RCBD) using SAS program (SAS 9.1, SAS Institute Inc., Cary, NC).

**Sample preparation and observation for ESEM**

Environmental scanning electron microscopy (ESEM) was used to visually determine the extent of decay and whether or not fungal tissues were present in selected samples of pine, cedar, and ACQ treated pine. For ESEM observation, small specimens of wood samples were collected at day 0 and 4 months and fixed with 3% glutaraldehyde in 0.1M phosphate buffer overnight (Fromm *et al.* 2003). Samples were washed three times for 10 minutes each (total 30 minutes) with distilled water. Post-fixation was carried out with 2% osmium tetraoxide in 0.1 M phosphate buffer for 4 hours. The fixed specimens were washed with distilled water then broken or cut into small fragments (<20×20 mm) mounted onto aluminum stubs and examined with a Zeiss EVO 50, variable pressure scanning electron microscope (Peabody MA). A variable pressure ESEM was used so that samples could be observed ‘wet’ to eliminate possible cell damage from alcohol dehydration.

**Sample preparation and observation for TEM**

Transmission electron microscopy (TEM) was used to determine the extent of internal decay and whether or not fungal tissues were present on selected samples (pine, cedar, and ACQ treated pine). Small wood sections were removed bimonthly over 18 months from selected wood stakes using a razor blade and were fixed in ½ strength Karnovsky's fixative in 0.1 M phosphate buffer (pH 7.2) (Karnovsky 1965). Samples were then washed four times in the buffer (for 15 min each time) and postfixed in 2% osmium tetroxide in 0.1 M buffer for 2 hours. Samples were rinsed, dehydrated in a graded ethanol series, and infiltrated with Spurr's resin (Spurr 1969). Sections of the embedded material were cut (primarily transverse sections) with a Reichert-Jung Ultracut E ultramicrotome.

Initially, 0.5μm-thick sections from wood samples at 0, 4, 8, 10, 14, and 18 months were made to identify an area of interest for TEM observation. These sections were collected on a microscope slide and were stained for 3 min with 1% Toluidine Blue. They were then examined with a light microscope (Nikon ECL PSE E600, Tokyo, Japan). Once wood sections of interest (14 and 18 months) were identified, ultra thin sections (60-90 nm) were cut, collected on copper grids, and stained with uranyl acetate and lead citrate (Reynolds 1963). Sections were examined with a JEOL JEM-100CX Transmission Electron Microscope (Peabody, MA) at 80 kv.

**Sample preparation and observation for light microscopy after TEM thick section**

Additional sample analysis was done using light microscopy. Samples were fixed in formalin-acetic acid alcohol (FAA), rinsed, dehydrated in a graded ethanol series, and

embedded in Paraplast Plus (Oxford Labware, St. Louis, MO) using CitriSolve (Fisher Scientific, Houston, TX) as transitional fluid. Both cross and transverse sections (8 micron) were cut with an American Optical 820 rotary microtome and were stained with safranin (0.1%)-fastgeen (0.2%) using a modification of a method by Conn (Clark 1981). Stained paraffin sections and toluidine blue stained resin sections were examined with a Nikon ECL PSE E600 light microscope (Tokyo, Japan).

## RESULTS

### Decay ratings, dynamic MOE, and moisture content during 18 months exposure of three wood types

Visual decay ratings of three wood types were significantly different at 4 months through 18 months between pine and cedar and between pine and ACQ-treated pine (Figure 2.4). From this data it is apparent that the decay ratings for the ACQ treated pine and cedar samples show only a minimal decrease over the 18 months exposure period. In contrast, the untreated pine samples show considerably more decay which progressively increased over the 18 months exposure period. There was no significant difference between cedar and ACQ -treated pine over 18 months (*alpha*=0.05).

Decrease in wood stiffness was measured by a decrease in MOE. The average % MOE loss, attributed to decay, was greater in pine than in cedar and ACQ-treated pine over 18 months (Figure 2.5). Initially the average MOE in pine, cedar, and ACQ-treated pine was $9.3\times10^9$, $5.9\times10^9$, and $10.0\times10^9$, respectively. At 2 and 4 months sampling times,

the MOE loss ranged from 5-7% in pine, less than 1% in cedar, and 2-5% in ACQ-treated pine, however this loss was not significantly different.

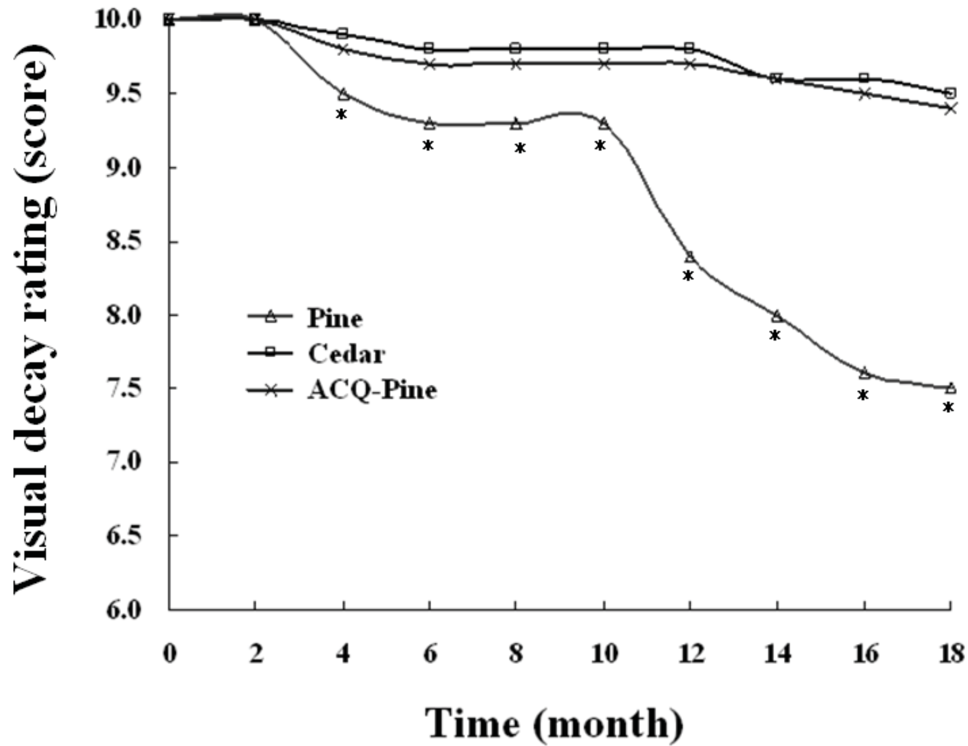



Figure 2.4. Visual decay rating during biodeterioration of three wood types over 18 months. "*" indicates that the decay rating for pine stakes was significantly different (*alpha*=0.05) from cedar and ACQ-pine stakes at the given time. A 10 score denotes no decay and a 6 score denotes severe decay.

At 6 months, a significantly higher % MOE loss (26%) was found in pine compared to 6% in cedar and 7% in ACQ-treated pine. The % MOE loss at 8 months in pine was also significantly higher (29%) compared to cedar (11%) and ACQ-treated pine (14%). The % MOE loss at 10 months in pine was again significantly higher (26%) compared to 9% in cedar and 11% in ACQ-treated pine. The % MOE loss from 12 months to 18 months in pine was significantly higher (32%, 34%, 42%, and 45%,

respectively) compared to cedar (10%, 14%, 16%, and 18%, respectively) and ACQ-treated pine (12%, 15%, 16%, and 19%, respectively). The % MOE loss was much greater for the untreated pine (45%) compared to the visual decay rating at 18 months (7.5 represents 25% loss) indicating the greater sensitivity of MOE for detecting decay. There was no significant difference (*alpha* =0.05) between cedar and ACQ-treated pine at any sampling date. The average moisture contents of all three wood types in each container varied between 40% and 100% over 18 months (Data not shown). Moisture contents were lowest (~38%) at 4 months in all samples.

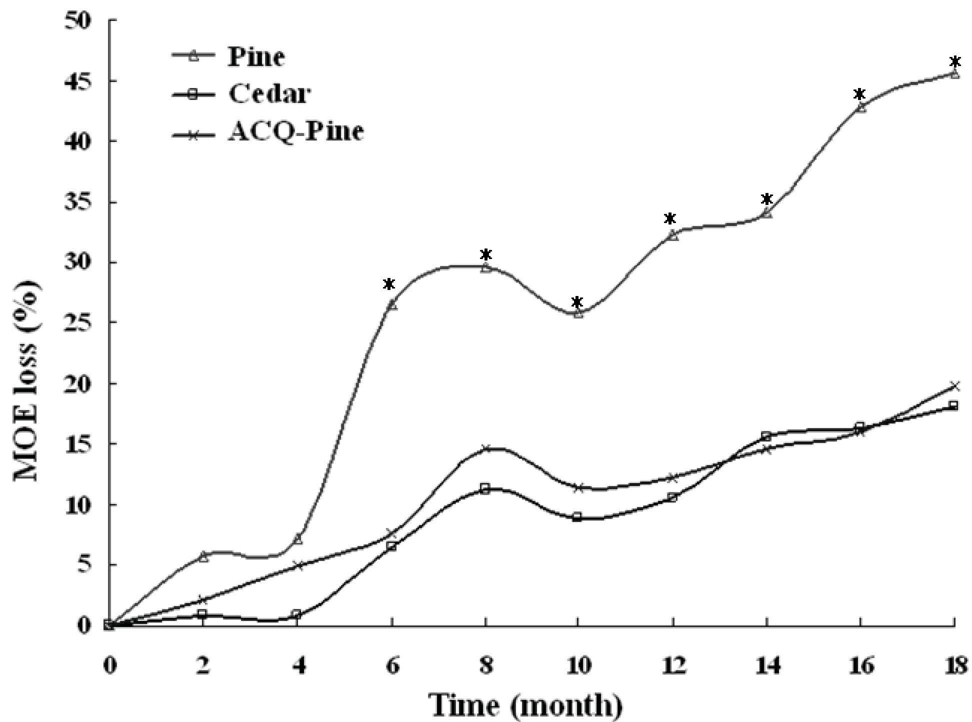



Figure 2.5. The average MOE loss (%) in pine, cedar, and ACQ treated pine over 18 months. "*" indicates that the % MOE loss in pine stakes was significantly greater (*alpha*=0.05) than cedar or ACQ-pine stakes at the given time.

**Macrostructural changes in wood stakes**

Selected stakes from each wood type were visually observed bimonthly over 18 months. Pine stakes developed discoloration at 4 months (Figure 2.6) however cedar (Figure 2.7) and ACQ-treated pine (Figure 2.8) showed only mild discoloration at 4 months. ESEM showed the tracheids for all wood types were clean and well organized at the beginning of the study. However, cell wall degradation in pine at 4 months was observed (Figure 2.9). In comparison to pine, the cell walls of cedar (Figure 2.10) and ACQ-treated pine at 4 months (Figure 2.11) were well organized and their cell structure remained unaffected.LM images of the three wood types were made at 4 months. A few fungal hyphae were observed in the secondary cell wall of pine and cell wall structure showed separation (Figure 2.12). No fungi were observed in cedar or ACQ-treated pine at 4 months and there was no difference between cell wall structure at 0 and 4 months (Figure 2.13) and (Figure 2.14).

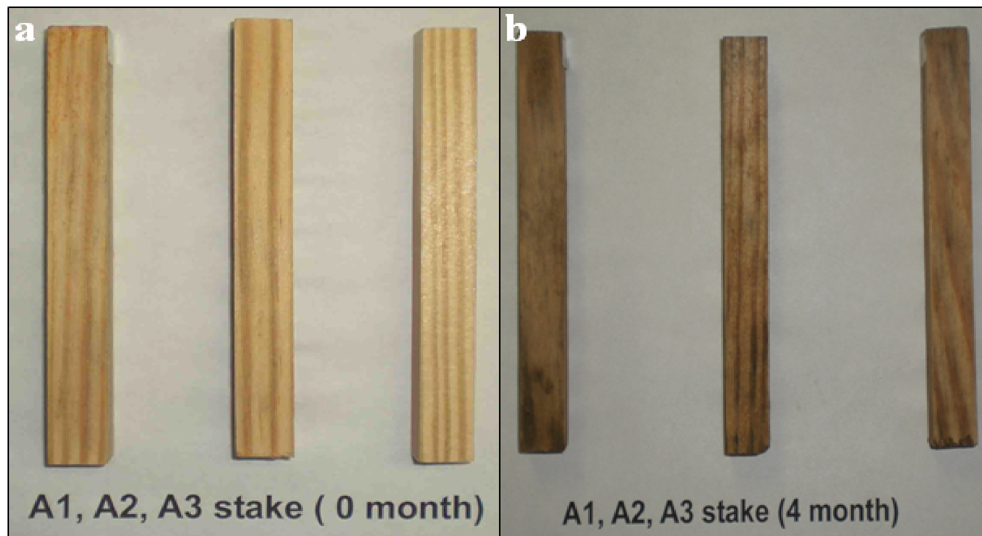



Figure 2.6. Visual observation of pine wood stakes (a) at the beginning of the decay test and (b) at 4 months.

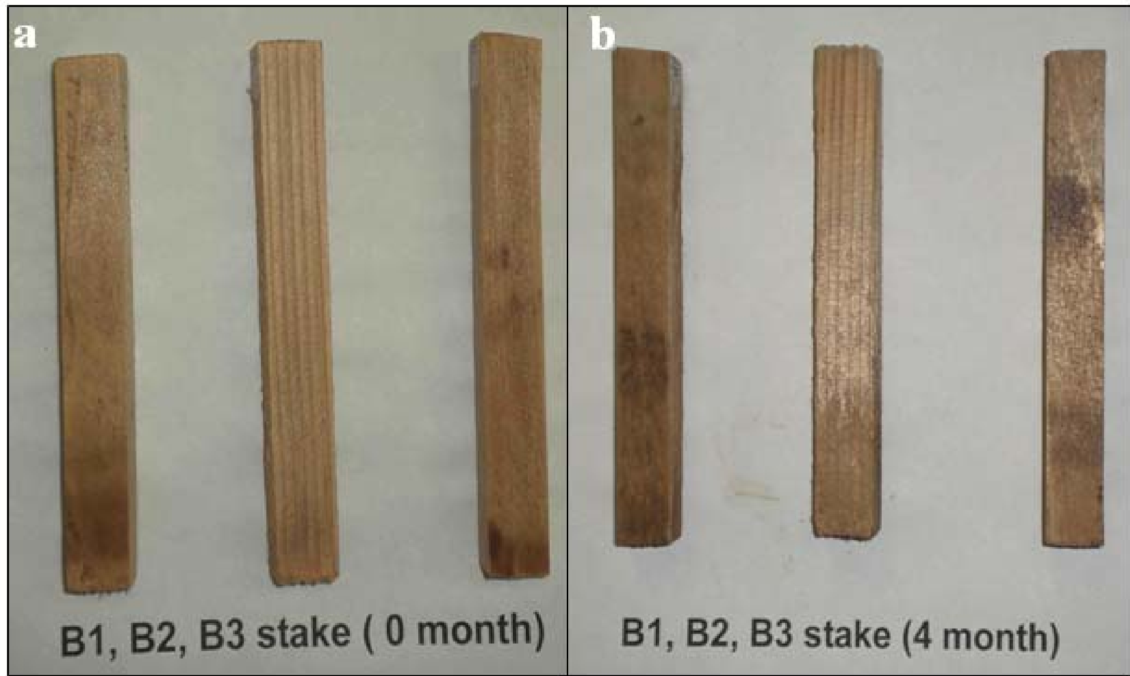



Figure 2.7. Visual observation of cedar wood stakes (a) at the beginning of the decay test and (b) at 4 months.

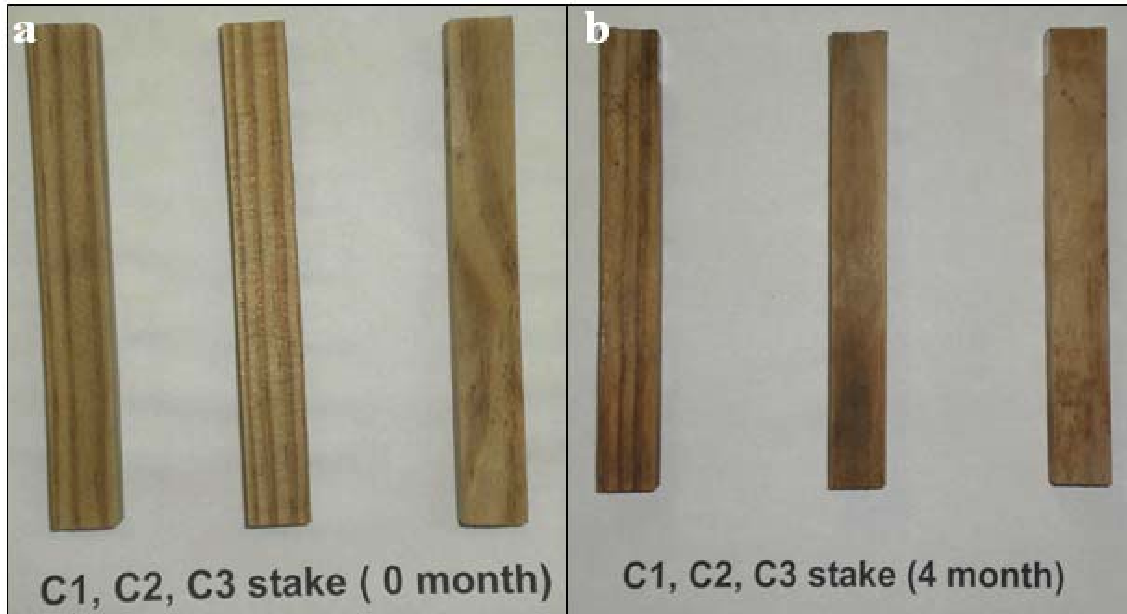



Figure 2.8. Visual observation of ACQ-treated pine wood (a) at the beginning of the decay test and (b) at 4 months.

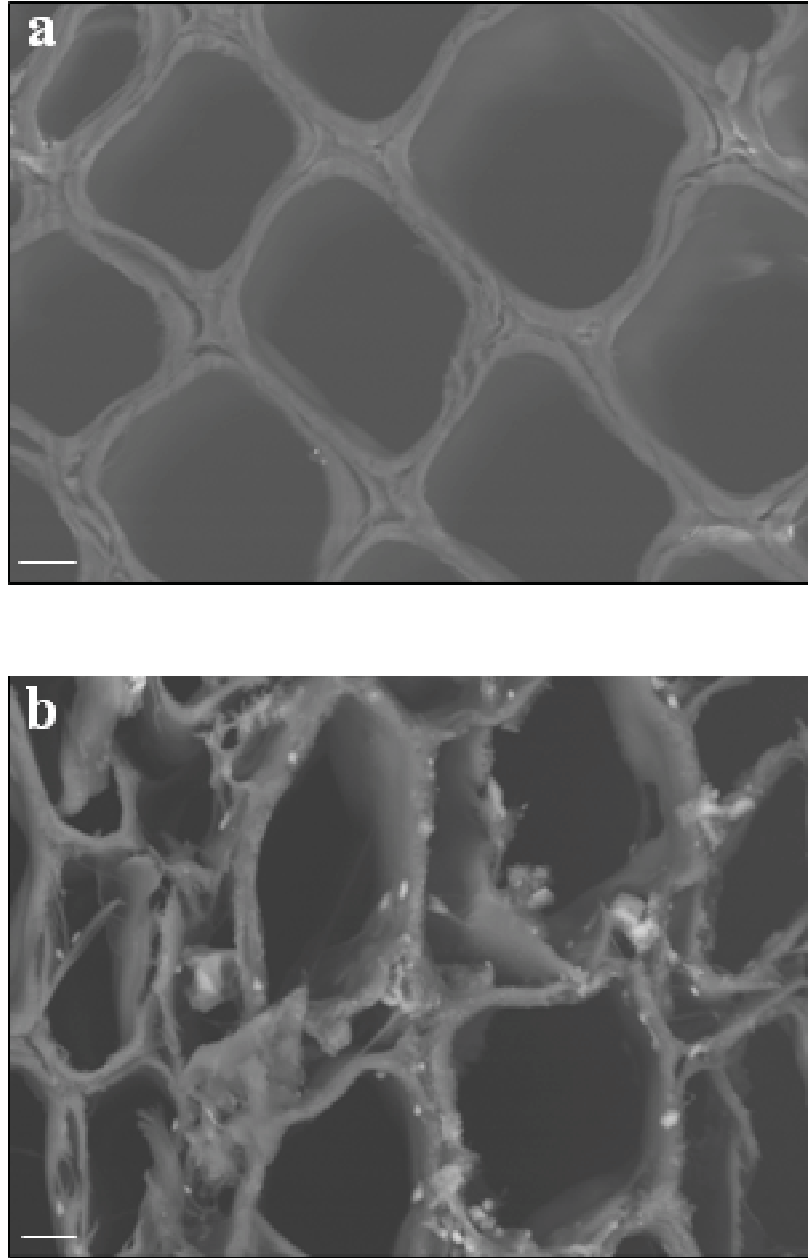
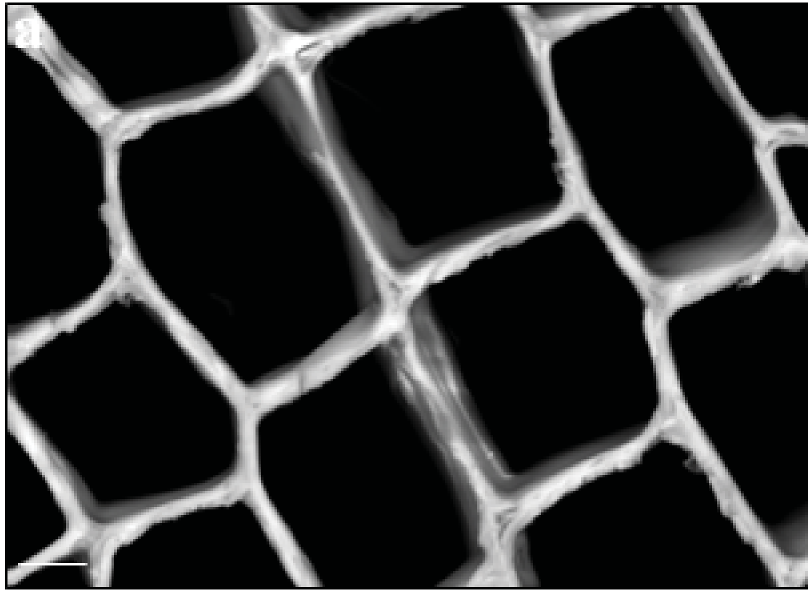



Figure 2.9. Environmental scanning electron micrographs (ESEM) of a cross section of pine stakes (bar =10μm) (a) at the beginning of decay test and (b) at 4 months.



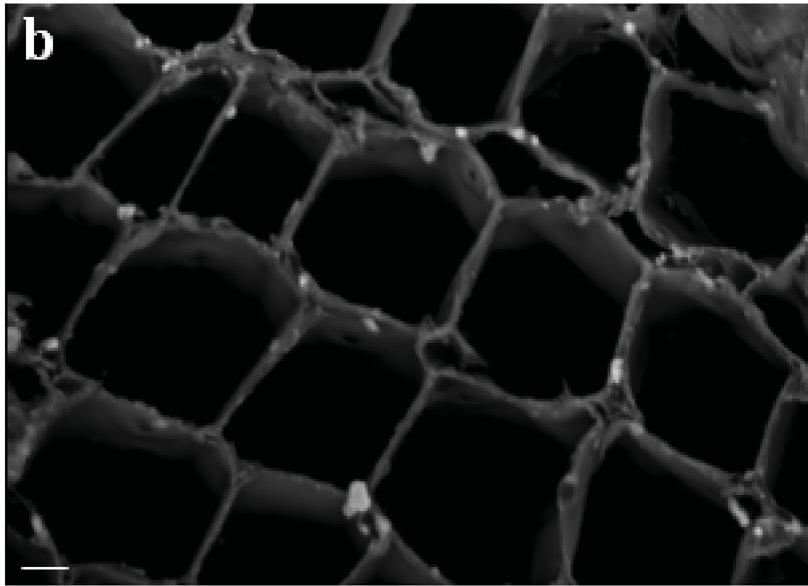
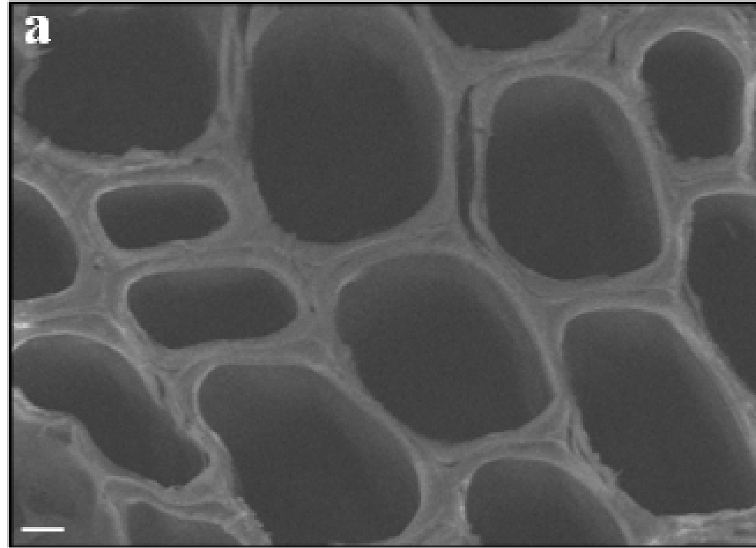




Figure 2.10. Environmental scanning electron micrographs of the cross section of cedar stakes (bar =10μm) (a) at the beginning of decay test and (b) at 4 months.



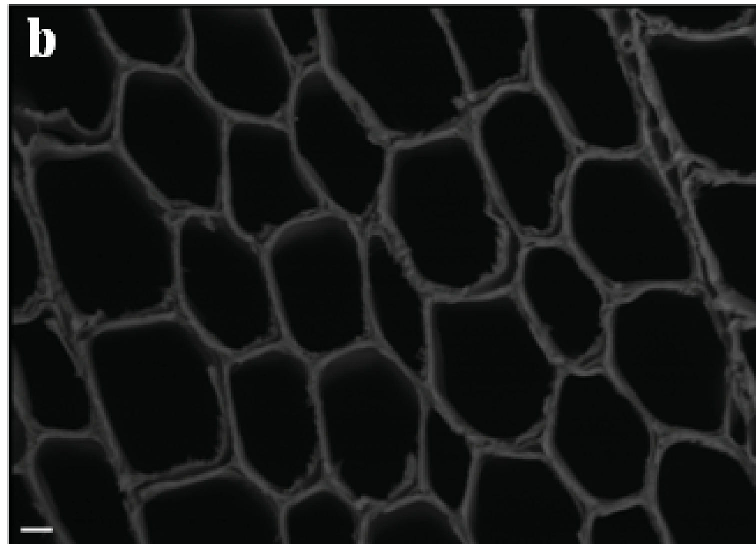
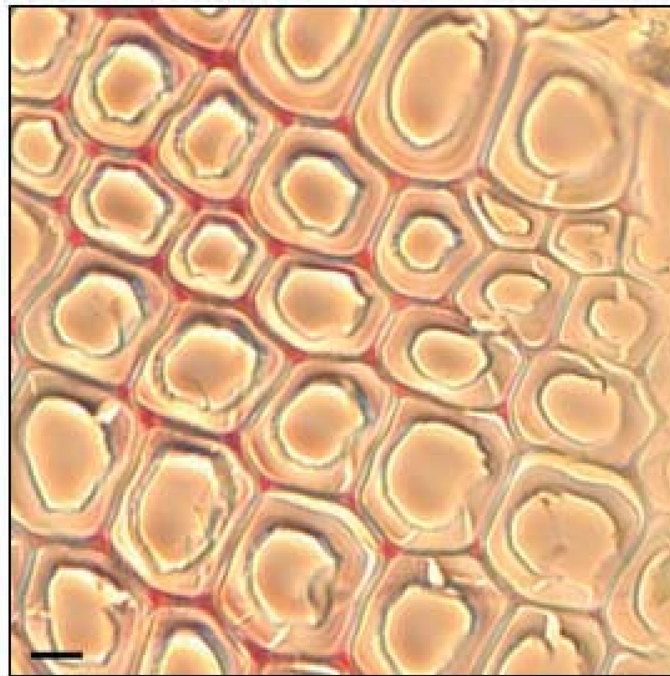




Figure 2.11. Environmental scanning electron micrographs of the cross section of ACQ-treated pine stakes (bar =10μm) (a) at the beginning of decay test and (b) at 4 months.



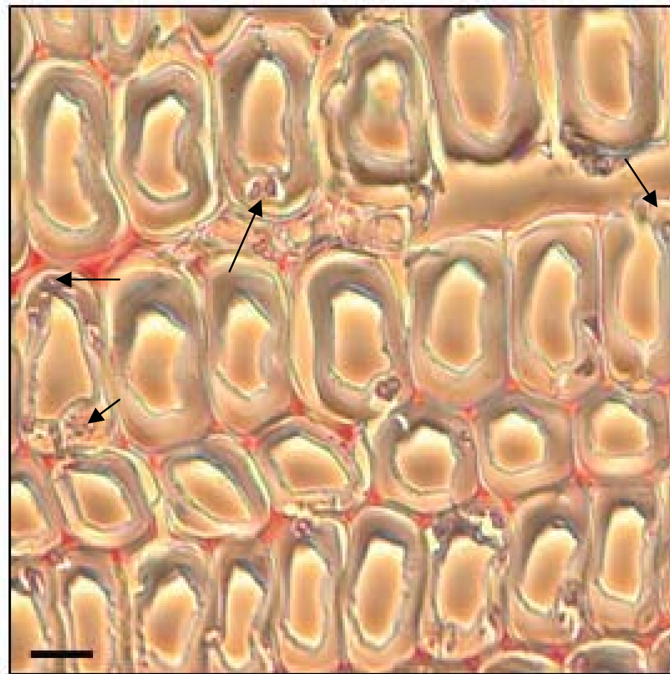
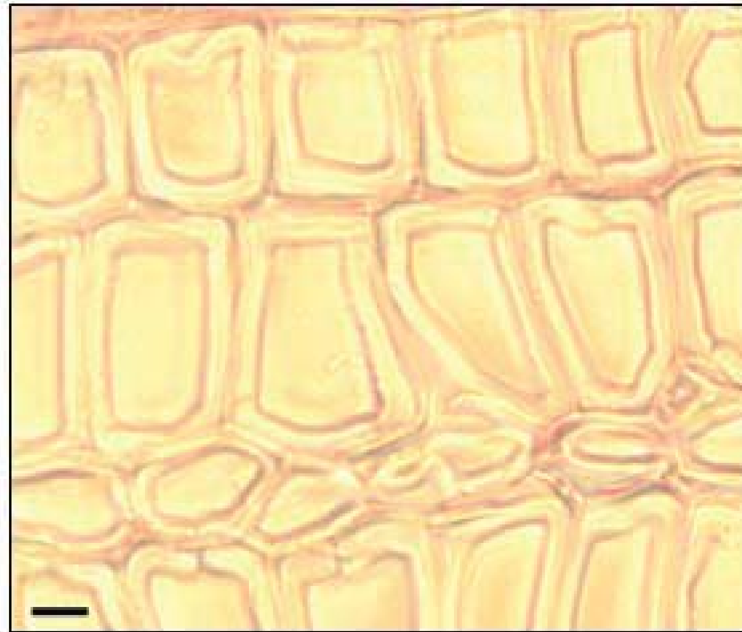


Figure 2.12. Light microscopy images of 0.5μm thick pine stake sections (a) at 0 month and (b) 4 months (bar = 20μm). (a) No fungal hyphae are observed in tracheids cell walls at 0 months and (b) a few fungal hyphae were observed in secondary cell wall at 4 months. The arrows indicate fungal hyphae.



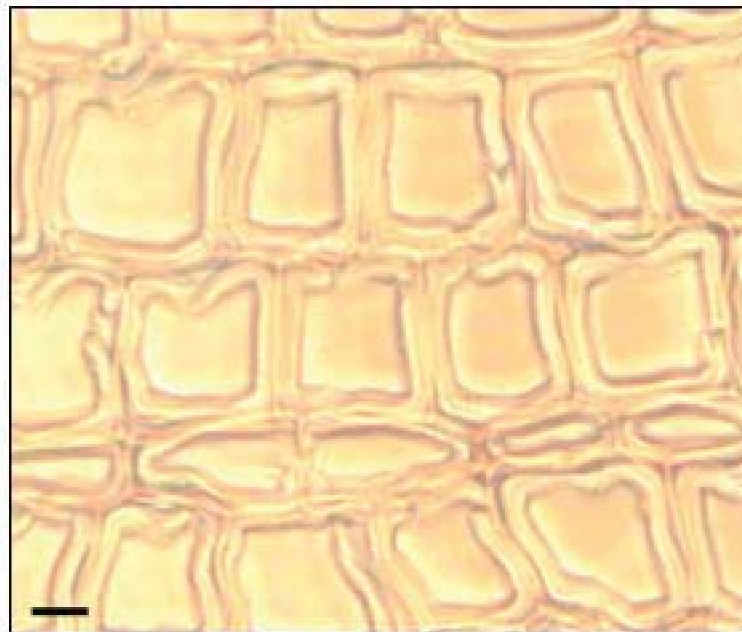
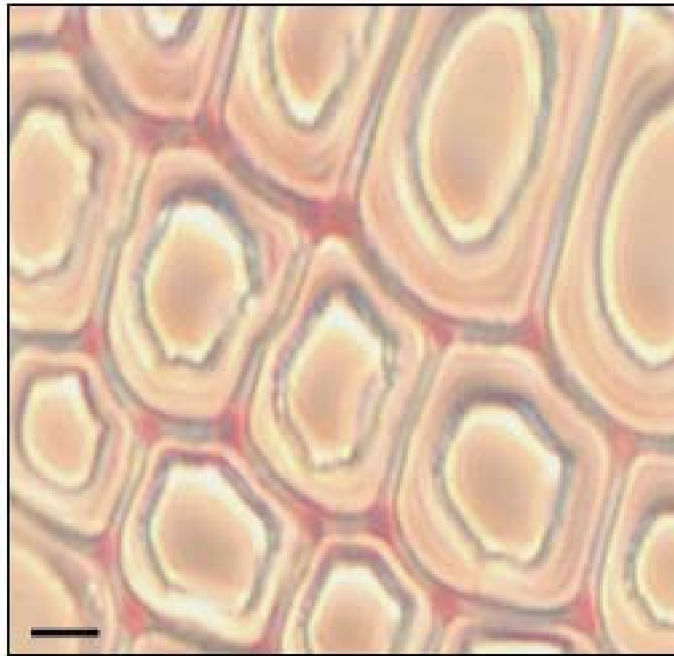


Figure 2.13. Light microscopy images of 0.5μm thick cedar stake sections (a) at 0 month and (b) 4 months (bar =30μm).



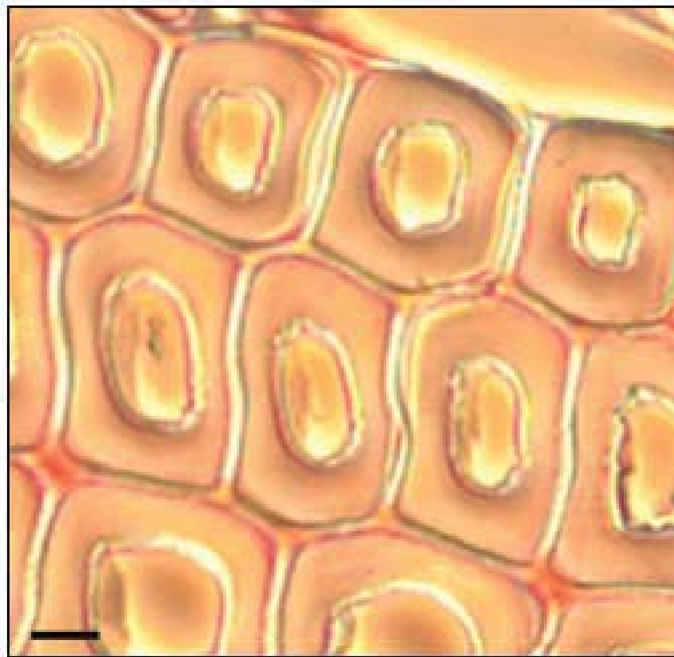


Figure 2.14. Light microscopy images of 0.5µm thick ACQ-treated pine stake sections (a) at 0 month and (b) at 4 months (bar =40µm).

After 6 months exposure fungal mycelia were visually observed on pine stakes and covered the surface of the stakes (Figure 2.15). Fungal mycelia were not observed on either cedar or ACQ-treated pine stakes at 6 months (Data not shown).

Figure 2.15. Mycelia growing on pine stake after 6 months exposure. (M) mycelia and (D) discoloration.

Similar to prior months, visual changes such as discoloration occurred at 8 and 10 months on pine (Figure 2.16), cedar (Figure 2.17), ACQ-treated pine (Figure 2.18).

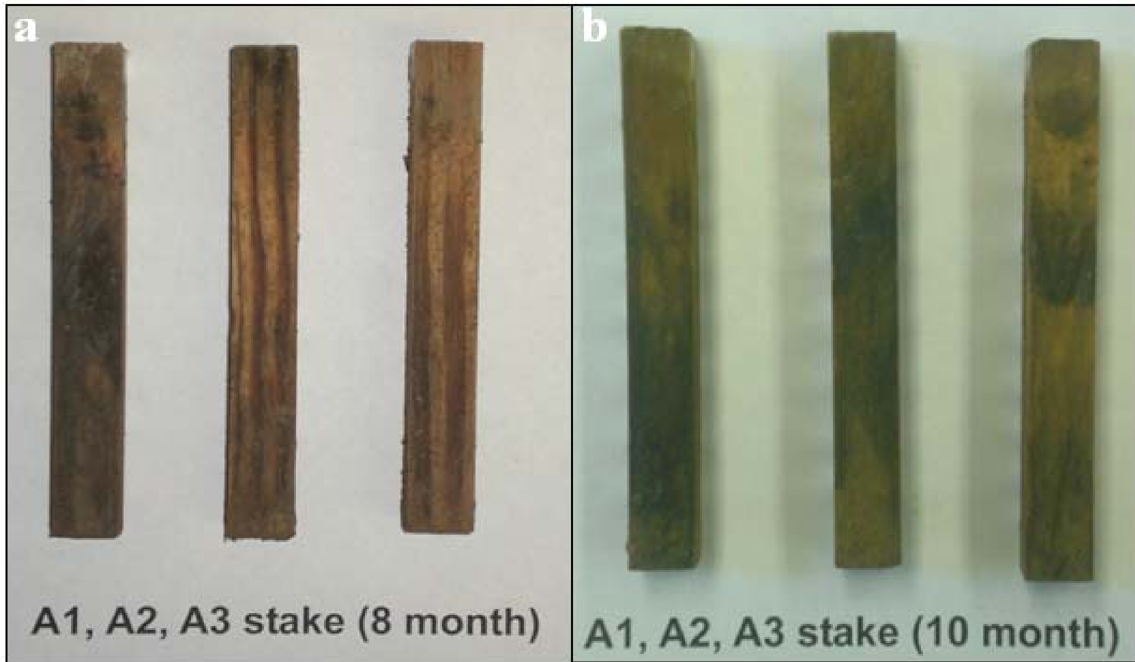



Figure 2.16. Visual observation on pine wood stakes (a) at 8 months and (b) 10 months.

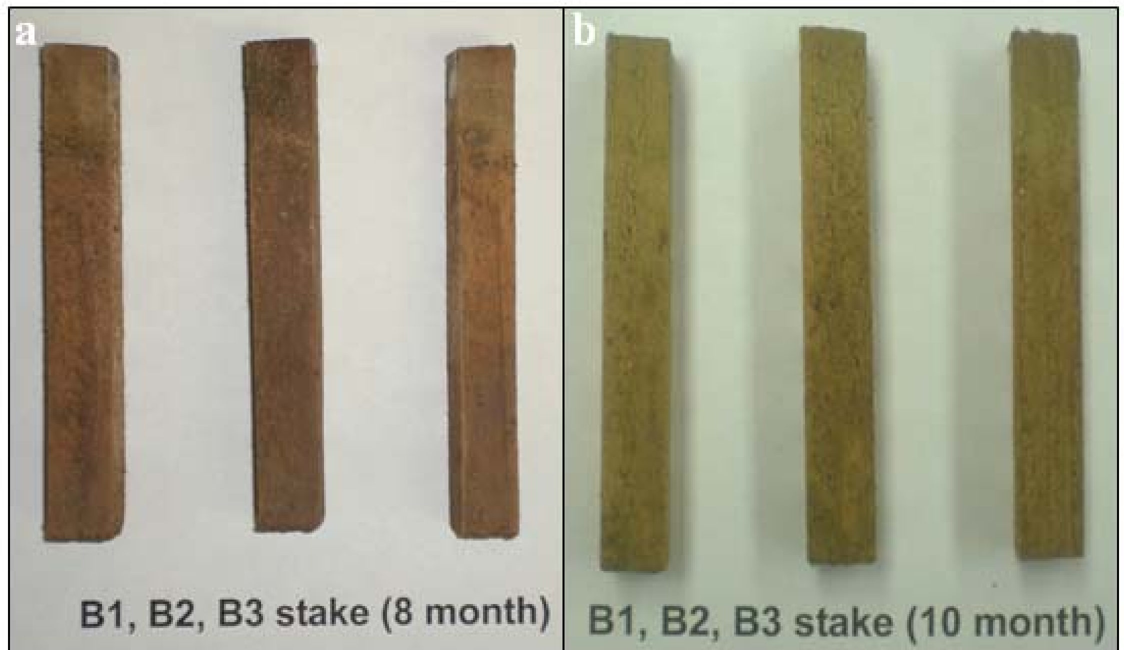



Figure 2.17. Visual observation of cedar wood stakes (a) at 8 months and (b) 10 months.

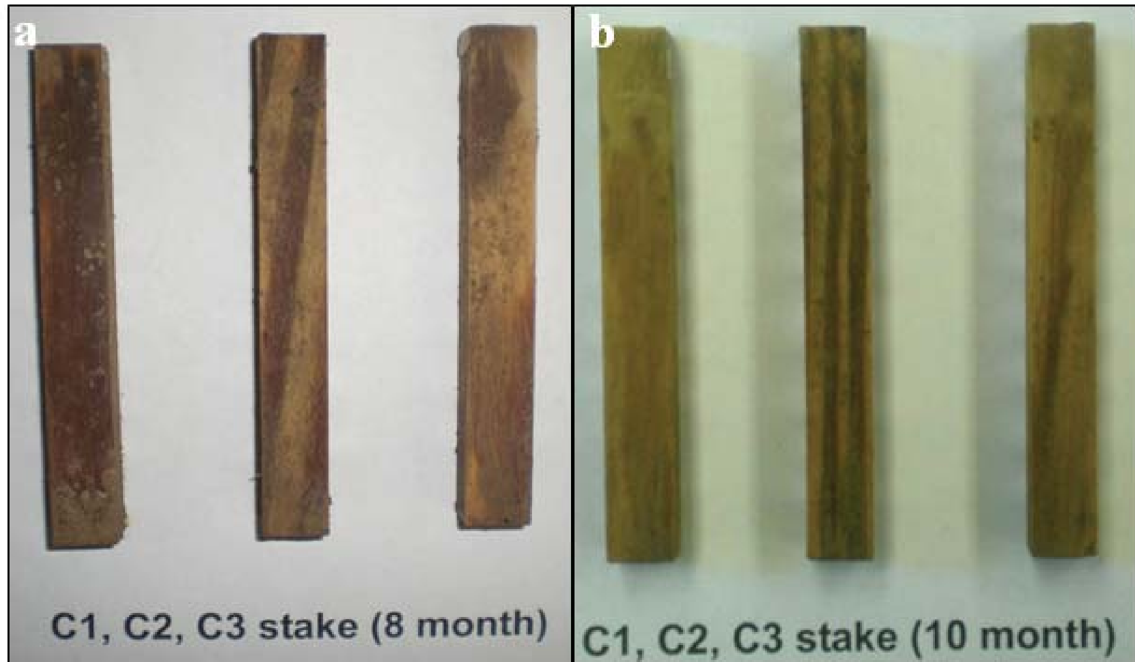
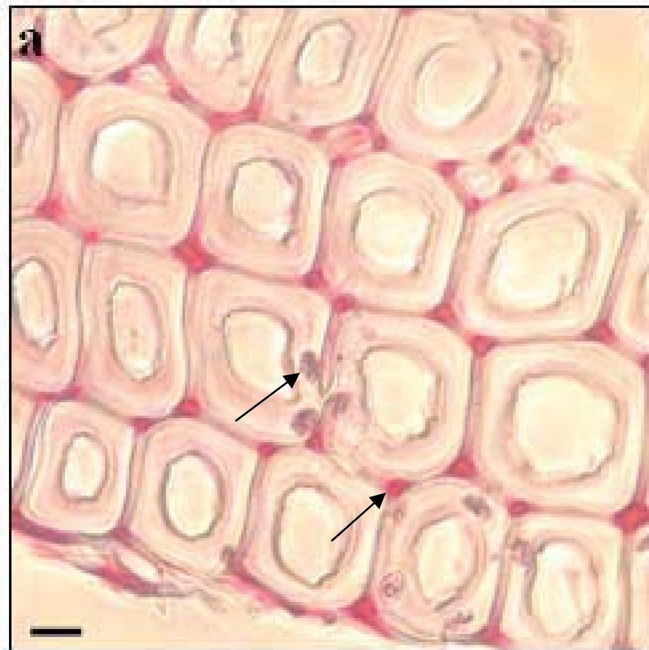



Figure 2.18. Visual observation of ACQ-treated pine wood stakes (a) at 8 months and (b) 10 months.

LM images of the three wood types were made at 8 and 10 months. Fungal hyphae were detected in the secondary cell walls and middle lamella of pine stakes at 8 and 10 months exposure (Figure 2.19). On the other hand, no fungi were observed in cell lumens or in any of the cell walls of the cedar stakes at either 8 and 10 months exposure (Figure 2.20) and tracheids showed good alignment. Similar to cedar, the ACQ-treated pine also did not show any fungal growth in the cell lumens and cell walls at 8 and 10 months (Figure 2.21) but cell wall separation was observed both at 8 and 10 months on ACQ-treated pine.



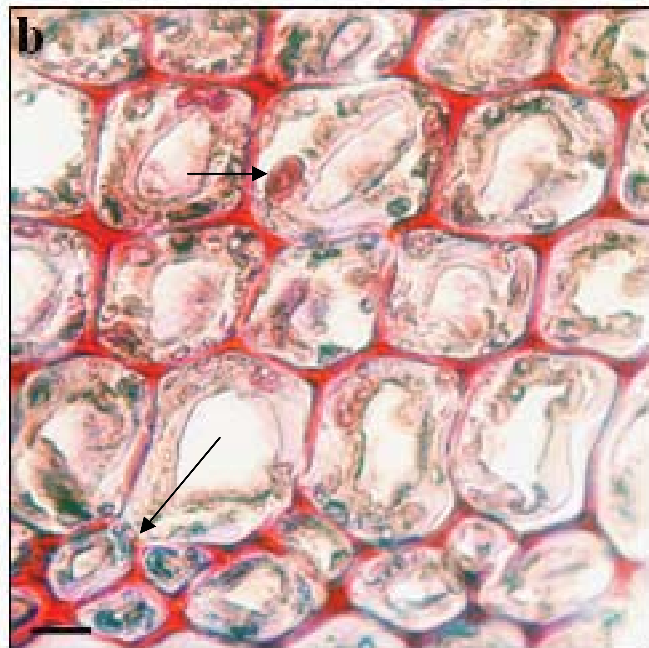
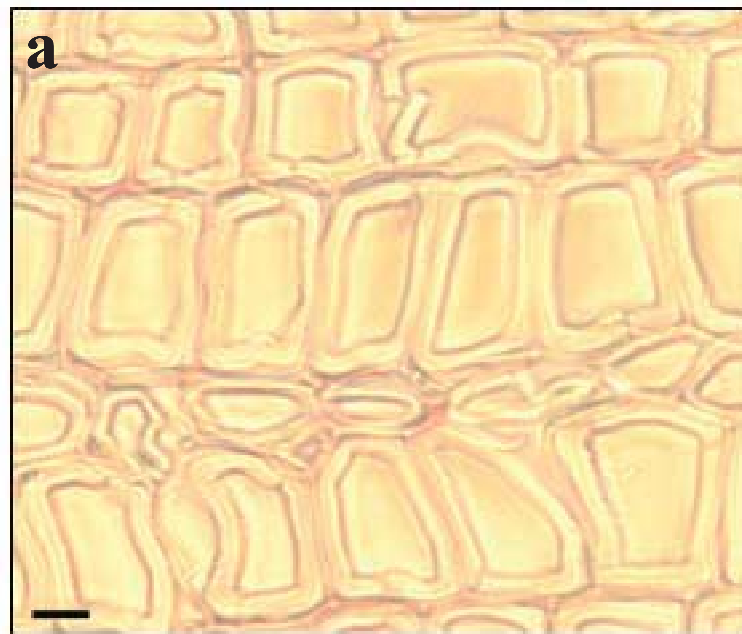




Figure 2.19. Light microscopy images of 0.5μm thick pine stake sections (bar = 30μm). (a) fungal hyphae in secondary cell wall of pine at 8 months (b) fungal hyphae in secondary cell wall and middle lamella at 10 months. The arrows indicate fungal hyphae in secondary cell wall (S) and middle lamella (M).



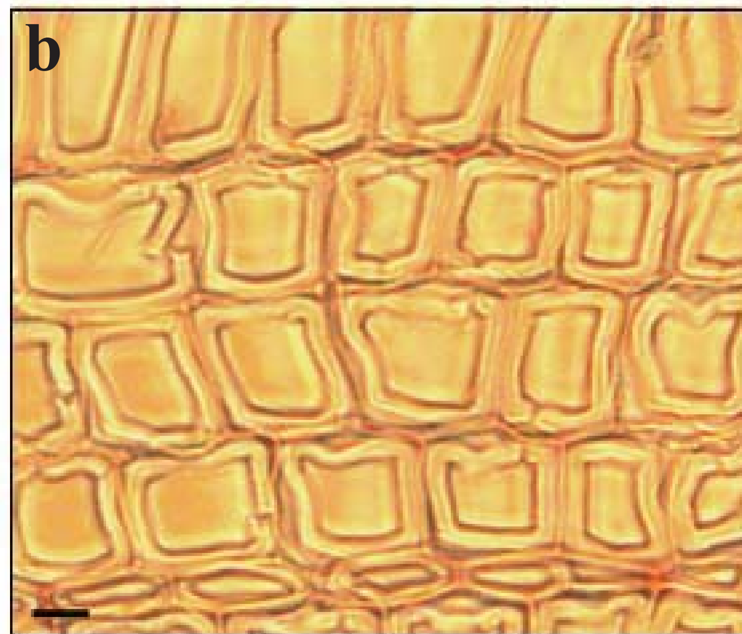
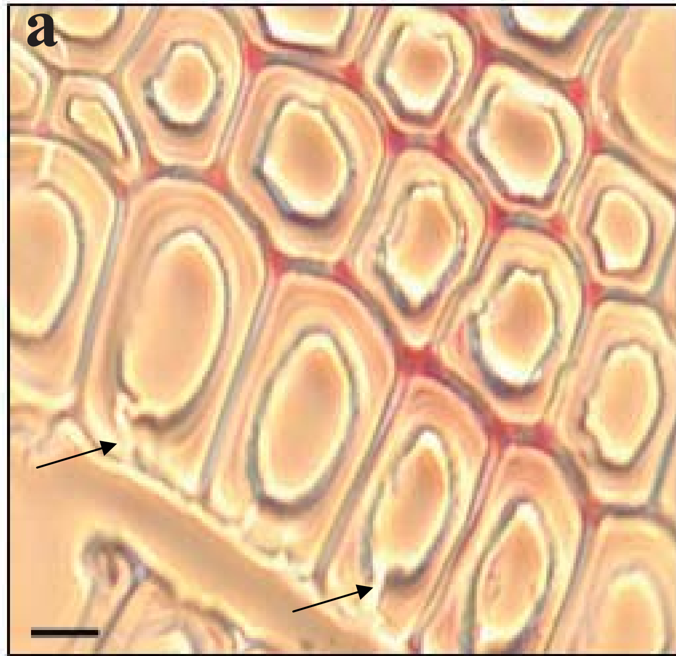




Figure 2.20. Light microscopy images of 0.5μm thick cedar stake sections (bar = 20μm) (a) at 8 months and (b) at 10 months.



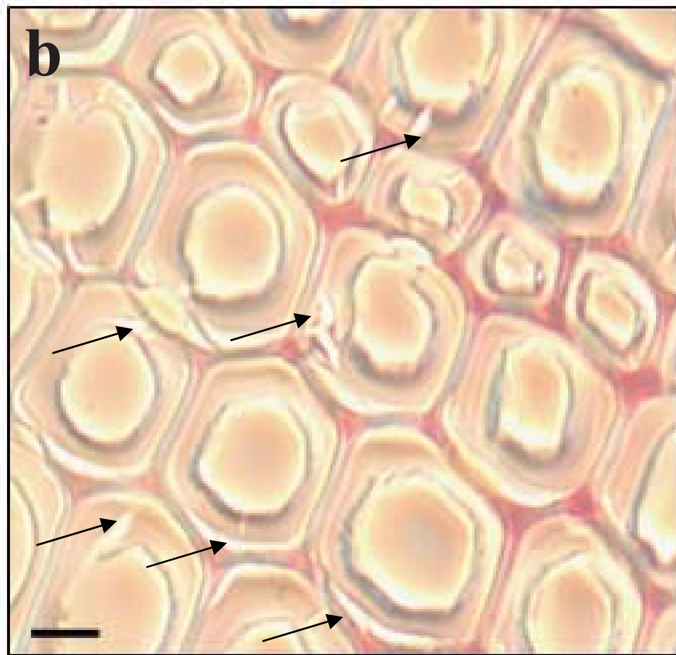




Figure 2.21. Light microscopy images of 0.5µm thick ACQ-treated pine stake sections (bar = 30µm). (a) The separation of wood cell walls are observed in the wood at 8 months (b) More wood cell wall separation was observed at 10 months. The arrows indicate the separation of cell wall.

At 14 months and 18 months, many microorganisms (molds, stains, and decay fungi) colonized the exterior of pine stakes and caused discoloration. The microbial growth was profuse on the surface of pine stakes at the end of 18 months (Figure 2.22). A few microbes colonized the exterior of cedar stakes at 14 and 18 months (Figure 2.23). At 14 and 18 months, some microbes also colonized the exterior surface of ACQ-treated pine stakes causing minor visual discoloration (Figure 2.24).

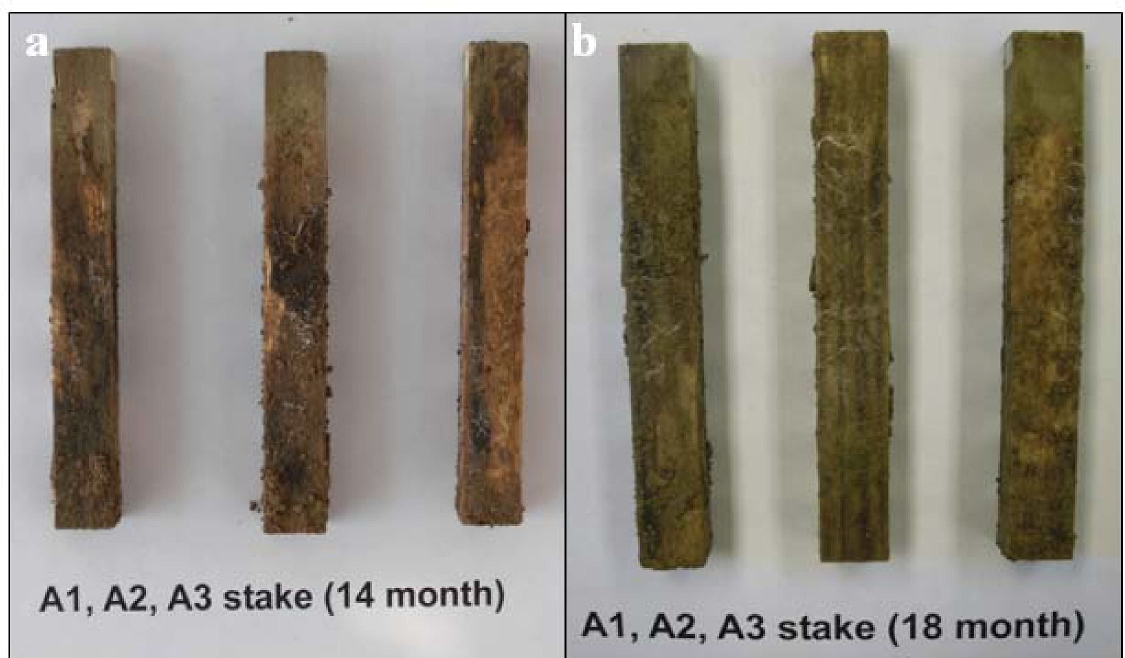



Figure 2.22. Visual observation on pine wood stakes (a) at 14 months and (b) at 18 months.

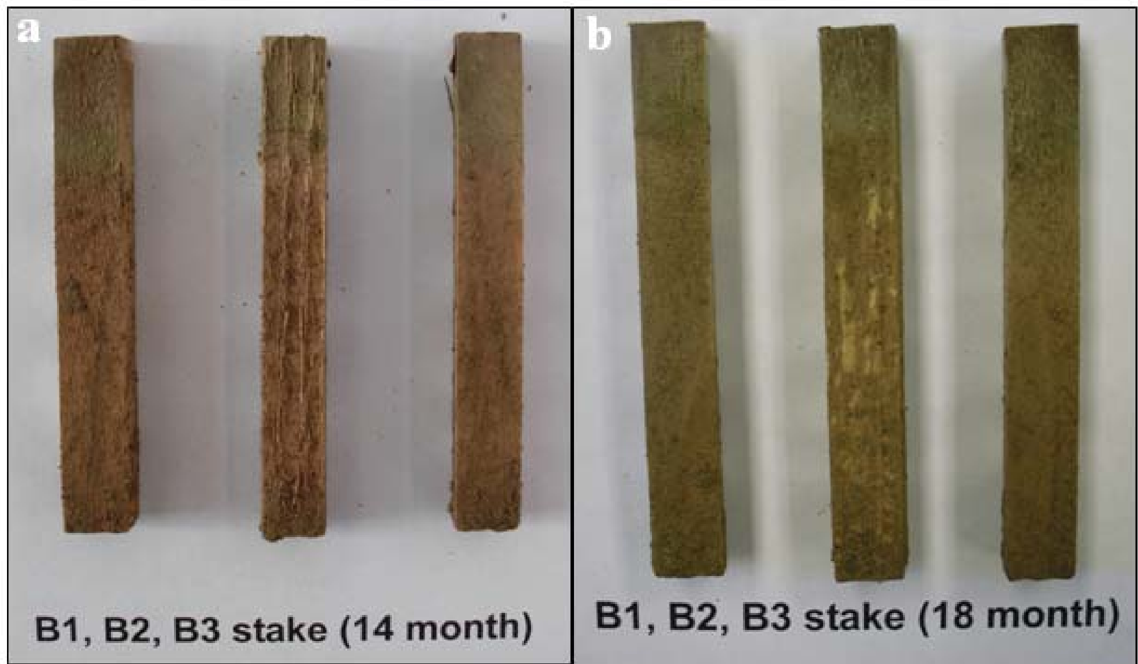



Figure 2.23. Visual observation of cedar wood stakes (a) at 14 months and (b) at 18 months.

Figure 2.24. Visual observation of ACQ-treated pine wood stakes (a) at 14 months and (b) at 18 months.

**Microstructural changes in wood stakes**

The TEM micrographs of ultra thin layers of wood samples were collected at 14 months and 18 months. Cedar and ACQ-treated pine did not show any fungal hyphae inside wood cells (Data not shown). However, TEM images of pine show fungal hyphae in the S2 region at 14 months (Figure 2.25) and showed numerous fungal hyphae attached to secondary cell walls at 18 months (Figure 2.26).

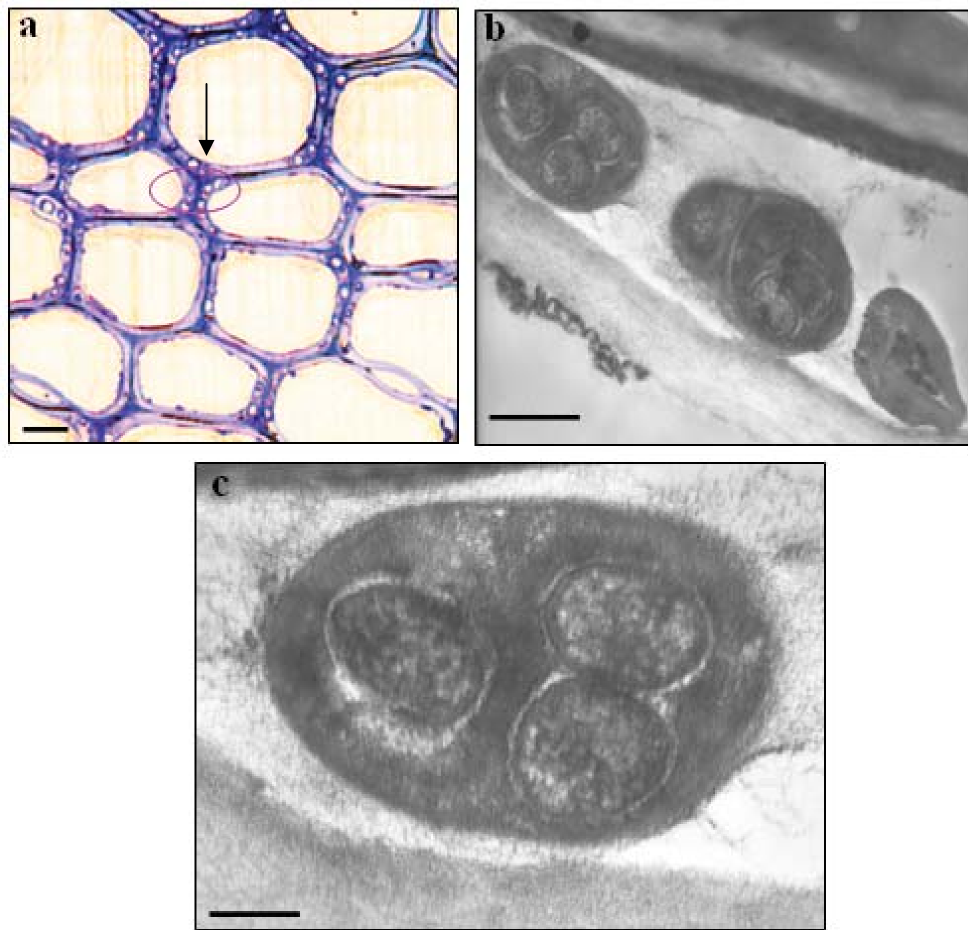



Figure 2.25. LM image of thick pine stake section (a) (0.5  m) and TEM micrographs of ultra thin section (b and c) (85nm) at 14 month. The circle indicates the location of hyphae (a) Fungal hyphae are observed in secondary cell wall at 14 months (bar = 10μm), (b) Fungal hyphae are visible in S2 region (bar =0.5μm), and (c) fungal hyphae in the fiber wall (bar=300nm).

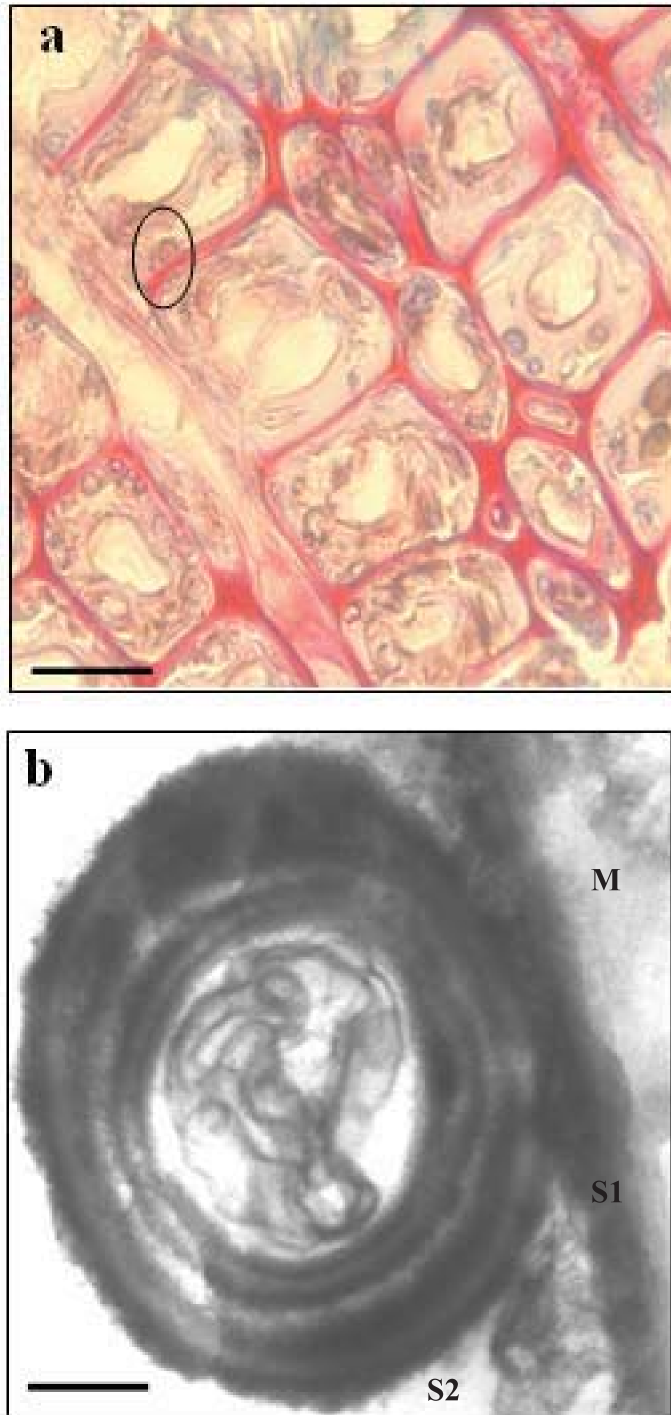



Figure 2.26. LM image of thick pine stake section (0.5µm) and TEM micrographs of ultra thin section (85nm) at 18 months. The circle indicates the location of hyphae. (a) Fungal hyphae observed in secondary cell wall at 18 months (bar = 10µm) and (b) fungal hyphae in S2 regions (bar=300nm).

# DISCUSSION

This study highlighted the different durabilities of three wood types during microbial degradation in forest soil. Pine is composed of different ratios of hemicellulose, cellulose, and lignin compared to cedar (Rayner and Boddy 1998). Pine is considered non-durable although the heartwood has some antifungal activity. However, sapwood of pine does not have enough antifungal properties to protect against fungal attack (Morita *et al.* 2001). Western red cedar is resistant to fungal decay (Edlin 1969). Durability of the cedar is based on natural extractives. The ACQ-treated pine is protected from fungal decay by chemicals. One of the major factors controlling the biodegradation of wood cells is the availability of the cellulose and hemicelluloses within wood cell walls. Visual observations can only evaluate the surface of wood. Dynamic MOE can provide information on the extent of wood decay (loss of stiffness) within the wood cell walls. Nicholas *et al.* (2004) reported a good correlation between the reduction in dynamic MOE and the extent of fungal decay.

In this study, surface fungal colonization was observed on all three wood types after 4 months of exposure. There were no significant differences in % MOE loss between cedar and ACQ-treated pine over the 18 month period. However, there were significant differences between pine and cedar and between pine and ACQ-treated pine, indicating that cedar and ACQ-treated pine were more durable against fungal degradation than pine. Visual decay rating data paralleled MOE loss at each sampling time. The fungal mycelia penetrated the cell walls of pine at 4 months, but not cedar and ACQ-treated pine although microbes were detected on the exterior surfaces at 4 months. Fungal mycelia were continually observed on pine over 18 months.

## BIBLIOGRAPHY

AWPA. 2001. Standard method of evaluating wood preservatives by field tests with stakes. AWPA Method E7-01. Book of Standards. American Wood Protection Association.

AWPA. 2008. Processing and treatment standard. AWPA Method T1-08. Book of Standards. American Wood Protection Association.

Beguin, P. and J.P. Aubert. 1994 The biological degradation of cellulose. FEMS Microbiology Reviews 13: 25–58.

Blanchette, R.A. T. Nilsson, G. Daniel, and A. Abad. 1989. Biological degradation of wood. Chapter 6, pp 141–174. In: Archaeological Wood Advances in Chemistry Vol. 225 American Chemical Society Publication.

Boddy, L. and A. D. M. Rayner. 1983. Origins of Decay in Living Deciduous Trees: The role of moisture content and a re-appraisal of the expanded concept of tree decay. New Phytologist 94(4):623-641.

Cease, K.R., R.A. Blanchette, and T.L. Highley.1989. Interactions between Scytalidium species and brown- or white-rot basidiomycetes in birch wood decayed in the laboratory. Wood Science and Technology, 23: 151-161.

Clark, George (ed.) 1981. Stain Procedures. Williams and Wilkins. Baltimore pg. 325.

Collins, S.P., R.K. Pope, R.W. Scheetz, R.I. Ray, P.A. Wagner, and B.J. Little. 1993. Advantages of environmental scanning electron microscopy in studies of microorganisms. Microscopy Research and Technique 25: 398-405.

Cronyn, J. M. 1990. The Elements of Archaeological Conservation. Routledge Publisher. London and New York.

Cunningham, M., R. Keey, and C. Kerdemelidis. 2007. Isothermal moisture transfer coefficients in Pinus radiata above the fiber-saturation point using the moment method. Wood and Fiber Science 21(2): 112-122.

Daniel, G. J. Volc, and M. Niku-Paavola. 2004. Cryo-FE-SEM & TEM immuno-techniques reveal new details for understanding white-rot decay of lignocellulose. Comptes rendus biologies 327(9):861-871.

Daniel, G.. 2003. Microview of wood under degradation by bacteria and fungi. P. 34-72.

In: Wood Deterioration and Preservation. Goodell, B., D.D. Nicholas, T.P. Schultz (eds), ACS Symposium Series. vol. 845, American Chemical Society, Washington, DC.

Edlin, H. L. 1969. What wood is that: a manual of wood identification. Viking Penguin Inc. Tames and Hudson Limited, London, England.

Egerton-Warburton, L.M., B.J. Griffin, and J. Kuo. 1993. Microanalytical studies of metal localization in biological tissues by environmental SEM. Microscopy Research and Technique 25: 406-411.

Eriksson, K.E.L, R.A. Blanchette, and P. Andr. 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin Heidelberg New York.

Fromm, J., B. Rockel, S. Lautner, E. Windeisen, and G..Wanner. 2003. Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques. Journal of Structural Biology 143 (1): 77 -84.

Goodell, B. 2003. Brown-rot fungal degradation of wood. Our evolving view. In: Goodell, B., D.D. Nicholas, and T.P. Schultz (eds) Wood Deterioration and Preservation. American Chemistry Society, Washington, DC, pp. 97-118.

Groot, R.C.D. and B. Woodward. 1996. A laboratory soil-contact decay test: an accelerated method to determine durability of treated wood shakes. Wood and Fiber Science 28: 214-226.

Helms, A.C. A.C. Martiny, J.Hofman-Bang, B.K. Ahring and M. Kilstrup. 2004. Identification of bacterial cultures from archaeological wood using molecular biological techniques. International Biodeterioration & Biodegradation 53(2):79-88.

Highley, T.L. and J.A. Micales. 1990. Effect of aromatic monomers on production of carbohydrate-degrading enzymes by white-rot and brown-rot fungi. FEMS Microbiology Letter 66:15-22.

Howard, E.T. and F.G. Manwiller. 1969. Anatomical characteristics of southern pine stem wood. Journal of Wood Science 2(2):77-86.

Karnovsky, J.J.1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. Journal of Cell Biology 27: 137-138.

Kleman-Leyer, K., E. Agosin, A. H. Conner, and T. K.Kirk. 1992. Changes in molecular size distribution of cellulose during attack by white rot and brown rot fungi. Applied and Environmental Microbiology 58(4): 1266-1270.

Morita, S. Y. Yazaki, and G. C. Johnson. 2001. Mycelium growth promotion by water extractives from the inner bark of radiata pine (Pinus radiata D. Don). Holzforschung 55(2): 155–158.

Nicholas, D.D., H. Borazjani, and T.P. Schultz. 2004. Accelerated laboratory soil contact decay test using soil amended with composted wood. International Research Group on Wood protection, Document No. IRG/WP 04-20284.

Nicole, M. H. Camberland, D. Rioux, X. Xixuan, R.A. Blanchette, J.P. Geiger, and G.B. Ouellette. 1995. Wood degradation by Phellinus noxius :ultrastructure and cytochemistry. Canadian Journal of Microbiology 41:253-265.

Panshin, A.J. and C.D. Zeeuw. 1970. Textbook of wood technology. Vol.I, 3r ed. McGraw-Hill, New York.

Perez, J., J. Munoz-Dorado, T. Rubia, and J. Martinez. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology 5:53-63.

Preston, F. 2000. Wood Preservation: Trends of today will influence the industry tomorrow. Forest Products Journal 50:12–19.

Raberg, U., M.L. Edlund, N.Terziev, and C. J. Land. 2005. Testing and evaluation of natural durability of wood in above ground conditions in Europe – an overview. Journal of Wood Science 51:429–440.

Rayner, A.D.M. and L. Boddy. 1998. Fungal decomposition of wood: its biology and ecology. John Wiley, Chichester Publisher.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, 17: 208-212.

Siau, J.F. 1971. Flow in Wood. Syracuse wood science series, Syracuse University Press, Syracuse, New York.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26: 31-43.

Stamm, A.J. 1964. Wood and Cellulose Science. Ronald Press Co., New York.

Terziev, N. and T. Nilsson. 1999. Effect of soluble nutrient content in wood on its susceptibility to soft rot and bacterial attack in ground test. Holzforschung 53: 575–579.

Wardrop, A.B. 1964. The structure of formation of cell wall in xylem. In: M.H. Zimmermann (ed.), The Formation of Wood in Forest Trees: 87–134. Academic Press, New York, London.

Watanabe, T., K. Koller, and K. Messner.. 1998. Copper-dependent depolymerization of lignin in the presence of fungal metabolite, pyridine. Journal of Biotechnology 62(3): 221-230.

Wardrop, A.B. and H. Harada. 1965. The formation and structure of the cell wall in fibers and tracheids. Journal of Experimental Botany 16: 356–371.

White, N.A. and L. E. Boddy. 1992. Differential extracellular enzyme production in colonies of Coriolus versicolor, Phlebia radiata and Phlebia rufa: effect of gaseous regime. Journal of General Microbiology138: 2589-2598.

Zabel, R.A. and J.J. Morell. 1992. Wood Microbiology: Decay and Its Prevention, Academic Press, San Diego.

# CHAPTER III

# GENE EXPRESSION OF SELECTED DECAY ENZYMES DURING BIODETERIORATION OF THREE DIFFERENT WOOD TYPES

## INTRODUCTION

The microbial decay of wood is a critical process in carbon recycling and is essential for maintaining forests. However, decay of structural wood products caused by insects and microorganisms results in a loss of billions of dollars annually in the United States (Preston 2000). The primary microbial decomposers of wood are basidiomycete decay fungi which attack and degrade wood cell wall components: cellulose, hemicellulose, and lignin. However, little is known about how different wood species affect the microbial decay community.

The time required for wood decay varies depending on the wood species and environmental conditions. Naturally durable woods require decades for complete deterioration compared to non-resistant woods which may deteriorate in a few years. Traditional methods for protecting wood from decay are treatments with broad spectrum chemical preservatives, usually without knowing the identity of fungi or the kinds of enzymes they produce during decay (Baechler 1959; Baechler *et al*. 1964). Use of an accelerated soil bed decay test reduces the amount of time required for microorganisms

to colonize and decay wood, thus allowing research on wood decay and preservatives to proceed in a reasonable time (Schultz *et al*. 2007).

Wood cell walls are composed of cellulose, hemicelluloses, and lignin. Some bacteria and fungi are capable of partial wood degradation by secreting cellulose and hemicellulose-degrading enzymes. However, lignin is effectively degraded only by white rot fungi. White rot fungi secrete lignin degrading enzymes such as lignin peroxidase (Lip), manganese peroxidase (Mnp), and laccase (Lcc) externally into their surrounding mucilaginous sheath in order to decay wood and provide food for their survival (Cease *et al*. 1989; Pointing *et al*. 2003). Wood cell wall degrading enzymes produced by fungi are too large to penetrate into the woody cell wall, thus some other mechanism is needed to initially open the structure for degradation by enzymes (Eriksson *et al.* 1990). One white rot fungus, *Phanerochaete chrysosporium*, has had its total genome sequenced (Martinez *et al*. 2004). Extracellular enzymes in the lignin-degrading system of *P. chrysosporium* include 10 Lips, 5 Mnps, and 5 Lccs (Paice *et al*. 1993; Janse *et al*. 1998; Pointing *et al*. 2003; Pointing *et al*. 2005). White rot fungi are capable of causing extensive degradation of lignin, hemicellulose and cellulose components (Leonowicz *et al*.1999).

Brown rot fungi primarily degrade cellulose and hemicellulose leaving a modified lignin (Goodell 2003; Cohen *et al.* 2004). In the initial stages of brown rot decay, non-enzymatic oxidative agents have been proposed to be involved in the depolymerization of cellulose (Cowling and Brown 1969). It has been suggested that they have both an oxidative and hydrolytic pathway and hydrogen peroxide was involved in hydroxyl and oxygen radical production during the decay process (Highley 1977; Gilbert *et al*. 1984). The mechanism of wood decay by brown rot fungi include non-

enzymatic low molecular weight compounds such as phenolates and glycopeptides with the ability to reduce $Fe^{3+}$ to $Fe^{2+}$ and generate hydroxyl radicals were found in a brown rot fungus, *G. trabeum* (Koenigs 1974; Jellison et *al.* 1991; Kerem *et al.* 1999).

The amount of decay enzymes produced by fungi has been traditionally determined by enzyme assay methods which are based on substrate utilization as measured by UV/Vis spectroscopy (Gotz and Karst 2007). However, these methods are not specific to any group of organisms and most of these methods have signal interference problems, especially when a fluorescent substrate and product are involved (Orlando *et al*. 1998; Xu and Ewing 2005). Detection of enzymes is also done by polymerase chain reaction (PCR) technology which allows for the amplification of the target gene by in-vitro replication (Mullis *et al*. 1986). The first stage of gene expression is gene transcription followed by translation to proteins (Phillips and Bogyo 2005). Cohen *et al*. (2004), using Real-time PCR for quantification of gene expression in *Gloeophyllum trabeum* liquid- and wood-grown cultures, showed that expression of two quinone reductases were maximal during incipient wood decay which could drive the biodegradative quinone redox cycle. Real-time PCR can provide efficient quantification with the continuous monitoring (Heid *et al*. 1996). However, no studies have measured the expression of decay enzyme genes on different decay resistant woods during biodeterioration.

The objective of this study was to compare the wood decay fungi and their wood decay genes expressed during biodeterioration of three different wood types in a soil bed decay test over time. Specific objectives were to: 1) determine the presence of basidiomycetes, 2) screen for the presence of decay enzymes in Basidiomycetes, and 3)

determine the expression level of selected decay enzymes of *Phlebia radiata* (species specific) on different wood types (pine, cedar, and ACQ-treated pine). Results from this study should increase the basic knowledge of the microbial wood decay process.

## LITERATURE REVIEW

### Wood decay organisms

The wood associated organisms in the fungi kingdom are found in three phyla; Zygomycota, Ascomycota, and Basidiomycota (Zabel *et al.* 1991; Wong *et al.* 1992). Members of *Zygomycota* have limited involvement in wood decay. The majority of mold, stain, and soft rot fungi are in the *Ascomycota* phyla. While most members of *Ascomycota* cannot completely degrade wood, they may produce discolorations at the surface. True wood-decay fungi, termed white and brown rot fungi, are found primarily in *Basidiomycot*a (Highley and Micales 1990; Temp and Eggert 1999).

White rot fungi represent a very large number of basidiomycetes that live on wood and wood products (Temp and Eggert 1999). They often cause a bleaching of normal wood coloration by degrading the brown colored lignin component leaving the whitish cellulose. Their ability to metabolize large amounts of lignin in wood is unique among microorganisms. White rot fungi cause either simultaneous or selective decay (Jaszek *et al.* 2006). Simultaneous decay extensively degrades the lignin, hemicellulose and cellulose components of wood as well as the middle lamella (Leonowicz *et al.* 1999). During this decay, wood components are mostly degraded at the same time and at a similar rate of decay. White rot fungi are the only microorganisms that cause a complete

degradation of the wood structure. Selective decay is the decay of lignin and hemicellulose first but not cellulose (Perez *et al.* 2002). Wood cell wall degradation is usually localized to cells colonized by fungal hyphae of white rot and substantial amounts of undecayed wood remain even after advanced decay has occurred (Kleman-Leyer *et al.* 1992).

Brown rot fungi have the unique function of degrading cellulose and hemicellulose, leaving behind modified lignin (Goodell 2003; Cohen *et al.* 2004). Hemicellulose is degraded during the initial stages followed by extensive degradation of cellulose. The remaining lignin skeleton is also chemically modified (Kerem *et al.* 1999; Jensen *et al.* 2001). The depolymerization of the cellulosic fraction of the $S_2$ cell wall initiated by brown rot fungi dramatically and rapidly reduces the strength of the wood. Because most enzymes are too large to penetrate intact cell wall layers, the $S_2$ layer is first degraded in advance of the hyphae and then later $S_1$ and $S_3$ layers are also degraded. The brown rot fungal hyphae are therefore thought to produce a freely diffusible extracellular degradation system which causes cell wall components to breakdown at some distance from the hyphae (Green and Highley 1997). A low molecular weight compound such as a non-enzymatic oxidative agent which diffuses through cell walls has been proposed to be involved in the depolymerization of cellulose by brown-rot fungi (Cowling and Brown 1969). These non-enzymatic agents participate in Fenton's reactions, which generates hydroxyl radicals (HO•) when a two-valenced transition metal ion such as $Fe^{2+}$ is oxidized by transfer of its electron to hydrogen peroxide. Goodell *et al.* (1997) reported that brown rot fungi have acquired mechanisms to reduce $Fe^{3+}$ to $Fe^{2+}$, and to produce $H_2O_2$.

Soft rot fungi only penetrate a few millimeters into the wood surface and live on parenchyma cells they do attack wood cell wall components (Smith 1975). Soft rot fungi exhibit two decay types; cavity formation (type I) and erosion (type II) (Zabel *et al.* 1991). The type I attack is characterized by the formation of disconnected cavities by hyphae that penetrate into the cell walls and cause erosion (Wong *et al.* 1992).

**Enzymatic decay of wood cell walls**

Wood cell wall components are degraded by either non-enzymatic processes or extracellular enzymes and are further metabolized by intracellular enzymes for energy and fungal biomass production (Cullen 1997). Complete degradation of wood almost always requires multiple enzyme systems (Beguin and Aubert 1994). Cellulases, hemicellulases, and ligninases are the major groups of enzymes catalyze reaction that lead to wood degradation. Each enzyme group has a different function in the overall degradation process.

Enzymes are classified according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, http://www.chem.qmul.ac.uk/iupac/jcbn). In this classification, enzymes are grouped into six classes based on their function: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Each enzyme in this classification scheme is assigned an EC number which indicates the type of reaction it mediates. The majority of decay enzymes involved in fungal degradation belongs to the oxidoreductase and hydrolase groups. Oxidoreductases catalyze oxidation and reduction reactions by transferring hydrogen and/or electrons (Gianfreda *et al.* 1999). Examples of oxidoreductase enzymes that are

involved in lignin degradation are lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.1.1.13), and laccase (EC 1.10.3.2). Hydrolases catalyze the hydrolysis of chemical bonds of glucosides and peptides. Cellulose hydrolases include β-glucosidase (EC 3.2.1.21) and cellulose 1,4-β-cellobiosidase (EC 3.2.1.91). Hemicellulose hydrolases include endo-1,4-β-xylanase (EC 3.2.1.8) and xylan 1,4-β-xylosidase (EC 3.2.1.37).

Cellulose consists of β-1,4-linked glucose units and constitute the major structural component of wood cell walls (Beguin and Aubert 1994). In native cellulose, hydrogen bridges exist between the OH groups of neighboring glucose units and neighboring cellulose molecules forming cellulose chains. The long cellulose polymer chains are attached to each other by networks of hydrogen bonds that form microfibrils that are ordered (crystalline regions) and less ordered (amorphous regions) (Teeri 1997). Fungal degradation of cellulose occurs in various ways based on the types of decay fungi. The common cellulose degradation enzymes produced by fungi are cellulases. Cellulases are divided into two main classes; cellobiohydrolases (EC 3.2.1.91) and endoglucanases (EC 3.2.1.4) (Yoshida *et al.* 2004). Cellobiohydrolases remove cellobiose repeating units of the cellulose polymer from the reducing ends of the chain. Endoglucanases cleave bonds randomly in the middle of the chains.

Hemicelluloses of wood are complex polymers formed mainly from xylose, galactose, arabinose, mannose, and glucose (Puls 1992). Hardwood hemicelluloses are often called xylan because the major polymer is *O*-Acetyl-xylan with β-1-4-linked xylose units (Timell 1976; Uffen 1997). In softwoods, galactoglucomannan is the major hemicellulose polymer and has a backbone of 1,4-β-linked D-glucose and D-mannose

units depending on the species (Eriksson 1990). Hemicellulose components in wood are often degraded first followed by cellulose and/or lignin (Puls 1992). The common hemicellulose degradation enzymes in fungi are endo-1,4,-β-xylanase, xylan-1,4-β-xylosidase, mannan endo-1,4-β-mannosidase, and β-mannosidase (Eriksson *et al.* 1990). The complex hemicellulose polymers are degraded into simple sugars by many different enzymes depending on structure.

Lignin is a phenolic polymer in wood composed of phenylpropane units that polymerize into three-dimensional macromolecules which are highly hydrophobic (Janse *et al.* 1998). The common lignin degradation enzymes in fungi are lignin peroxidase, manganese peroxidase, and laccase (Martinez *et al.* 2005; Gregorio *et al.* 2006). Auxiliary enzymes such as alcohol oxidase are decay associated enzymes that contribute to the decomposition of lignin (Pointing *et al.* 2005).

Lignin peroxidases (Lip) are a group of hydrogen peroxide ($H_2O_2$) dependent enzymes produced by many white rot fungi (Janse *et al.* 1998). Lip directly oxidizes non-phenolic lignin structures to aromatic radicals in the presence of veratryl alcohol (Smith 1975). The common names designated for Lip are ligninase I, diarylpropane peroxidase, and hydrogen-peroxide oxidoreductase. Manganese peroxidases (Mnp) are a group of isozymes that catalyze the oxidation of $Mn^{2+}$ to $Mn^{3+}$ with hydrogen peroxide as an oxidant (Cohen *et al.* 2002; Datta *et al.* 1991). Mnp is independent of hydrogen peroxide producing enzymes and also known as peroxidase-M2 and NADH-oxidizing peroxidase (Cullen and Kersten 1996). The activated Mnp system can oxidize phenolic structures in lignin, but cannot directly attack non-phenolic structures except in the presence of unsaturated fatty acids. Mnp has been shown to oxidize non-phenolic structures in lignin

and also called lipid dependent peroxidase (Bogan *et al*. 1996). Laccases (Lcc) are phenol-oxidases that are produced by many different organisms and perform many different functions (Heinzkill *et al.* 1998). Lcc is often produced in the form of several isozymes, depending on the microbial species. Laccase contains four copper ions that are active in the catalytic cycle. The substrate specificity of Lcc is very broad, but important substrates such as phenols, are oxidized to phenolic radicals (Gianfreda *et al.* 1999).

**General mechanism of gene regulation**

The gene is the basic unit of DNA in living organisms that encodes information that ultimately produces a functional product in the form of a protein (Watson 1968). The regulation of gene expression is the cellular control of the amount of the functional product of a gene (Nelson and Cox 2005). Some genes are continuously expressed and normally referred to as housekeeping genes or as constitutive genes, while other genes expressed only in response to molecular signals are known as inducible or regulated genes.

Protein synthesis starts with the conversion of DNA to RNA (transcription) and RNA into protein (translation). During transcription RNA is produced when RNA polymerase binds to a DNA segment (gene) synthesizing a complimentary RNA strand called messenger RNA (mRNA) (Nelson and Cox 2005). Promoters are generally located in the vicinity of the genes and usually facilitate transcription (Gessner and Raeder 1994). The regulation of transcription is dependent on the extent of promoter interaction with RNA polymerase.

There are two ways of regulating transcription: one involves repressors which inhibit transcription and the other involves activators which enhance transcription (Nelson and Cox 2005). Repressors function by binding to non-coding sequence sites on the DNA, overlapping the promoter region, and inhibiting RNA polymerase movement along DNA. Depending on the type of repressor, the gene can be turned on when the signal ligand is present or it can be turned off when the signal is absent. This avoids wasteful production of unnecessary transcripts (Nelson and Cox 2005). Most genes are on by default and need to be repressed when not require (Gessner and Raeder 1994). In contrast, activators bind to DNA elements in the vicinity of genes and enhance the activity of RNA polymerase at a promoter when gene products are needed (Lamph *et al*. 1990). The interactions of repressors and activators can result in a decrease or an increase of transcriptional levels. Although a functional gene product may be RNA or a protein, the majority of known mechanisms regulate protein coding genes.

The second step in protein production is translation which occurs in the ribosomes (Weaver 2005). Translation is the process whereby genetic instructions are converted into sequences of amino acids called peptides. Translation occurs in three steps: initiation, elongation, and termination. Ribosomes are complex structural subunits of organelles in cells that bind to the mRNA (initiation) and translate mRNA into polypeptides using amino acids attached to transfer RNA. The ribosomes add an amino acid sequentially to the growing polypeptide chain (elongation). Finally, the mRNA and finished polypeptide are released from ribosomes (termination).

**Regulation of lignin modifying enzymes in basidiomycetes**

As previously discussed, wood decay fungi secrete several extracellular enzymes including Lip, Mnp, Lcc, and auxiliary enzymes such as alcohol oxidase in order to break down lignin (Pointing *et al*. 2005). These enzymes are usually referred to as lignin modifying enzymes (LMEs) and are involved in the free radical reactions (Reading *et al*. 2003). Other factors such as hydrogen peroxide, oxalate, small molecule mediators, and the plasma membrane redox potential mediate free-radical generation. These free-radical reactions may involve both direct and indirect oxidations and reductions.

Two or more enzymes that catalyze the same reaction but are encoded from different genes are known as isozymes. Several isozymes perform the same reaction with different effects so optimized physiological and biochemical conditions are important factors for isozymes regulation (Wilkinson 1965). The regulation of metabolic pathways in organisms is often made complex by the presence of isozymes. During gene regulation, a single enzyme or multiple enzymes may undergo reduced metabolic activity when they are not needed. However, enzyme activity may not be completely eliminated due to presence of other active isozymes

Lip is translated as a group of ten closely related isozymes classified as LipA through LipJ (Gaskell *et al*. 1994) and is mostly produced by white rot fungi (Machuca and Ferraz 2001). Lip has a heme iron as cofactor that serves as a source or sink of electrons required for redox reactions (Piontek *et a.l* 2001). The activation of Lip is based on its high reduction potential due to the presence of oxidized iron. Li *et al.* (1994) reported that nitrogen limitation regulates post-translational Lip expression through heme mediation.

Transcription and regulation of Lip isozymes are differentially controlled under carbon and nitrogen environments. Transcript levels of the ten known Lip genes have been characterized in the presence of organic compounds, contaminated soils, cultured media, and in wood (Stewart *et al*. 1992; Bogan *et al*. 1998; Janse *et al*. 1998; Stewart and Cullen 1999). Under carbon limitation, LipD transcripts were upregulated and LipA transcripts were undetectable. On the other hand, under nitrogen limitation LipA was the most abundant transcript and LipD expression was relatively low (Stewart and Cullen 1999). Thus a differential regulation of Lip ensues under different environmental situations.

Mnp is produced by many white and brown rot fungi during biodegradation of wood. The principal function of Mnp is to oxidize $Mn^{2+}$ to $Mn^{3+}$, using $H_2O_2$ as an oxidant (Kuwahara *et al*. 1984). Mnp does not directly oxidize lignin and is dependent on manganese to complete its catalytic cycle. At least five closely related isozymes have been identified and designated as Mnp1 through Mnp5 (Bogan *et al*. 1996). Transcription and regulation of Mnps is mainly dependent on concentration of the metal ion, $Mn^{2+}$ (Brown *et al*. 1990). Metal response elements (MREs) have been identified upstream of *P. chrysosporium* Mnp1 and Mnp2 and their transcript levels increase substantially in response to $Mn^{2+}$ supplementation under nitrogen limitations (Gettemy *et al*. 1998). Quantitative transcript analyses of the five known *P. chrysosporium* Mnp genes generally showed variable regulation in colonized soil and decayed wood (Janse *et al*. 1998). Transcript levels of *P. chrysosporium* genes lacking paired MREs were not influenced by the addition of $Mn^{2+}$ (Gettemy *et al*. 1998). It has been suggested that MREs play an important role in transcriptional regulation of *P. chrysosporium* Mnp genes.

Lcc are multiple copper oxidases that catalyze the one-electron oxidation of phenolic, aromatic amines, and other electron-rich substrates with the concurrent reduction of $O_2$ to $H_2O$ (Pointing *et al*. 2005). Lcc is also found in many microorganisms and is included in the broad category of ligninases. Lcc participates in direct oxidation of the phenolic and non-phenolic structures in lignin. Whenever a redox mediator such as $Cu^{2+}$ is available to react with Lcc, it may attack non-phenolic lignin (Maheshwari *et al*. 2000). Thus, Lcc promotes the oxidative coupling of lignols, a family of natural phenolic compounds obtained from lignin degradation (Solomon *et al*. 1996).

Lcc is also differentially regulated and the patterns of regulation differ substantially between species. Most white rot fungi produce laccase, but some do not. The *P. chrysosporium* genome does not reveal Lcc genes, but several related sequences were found. A multi-copper oxidase (MCO) gene in *P. chrysosporium* has been shown to encode multi-laccase enzymes (Cullen 1997; Cullen and Kersten 2004). Copper can influence the transcriptional induction of laccase (Soden and Dobson 2001; Galhaup *et al*. 2002), where a higher concentration (1.0–2.0 mM) of $Cu^{2+}$ induces increased Lcc expression.

## MATERIALS AND METHODS

### Preparation of wood stakes

Pine (*Pinus* sp.) and cedar (*Thuja plicata*) boards (5.1cm×10.2cm×180cm, 2.5cm×10.2cm×180cm respectively) used for this study were purchased from Lowes's Home Improvement Center, Starkville, Mississippi. Each board was cut into strips and numbered sequentially. The strips were cut into stakes measuring 14mm×14mm×115mm

(T×R×L). ACQ treating solutions of the appropriate concentration to achieve the desired retention of 0.15 pcf were prepared. The samples were treated according to the full cell method using the following schedule: 73.7cm/Hg vacuum for 15 min followed by 150 psi pressure for 15 min (T1-08, 2008 AWPA standard). After treatment, all samples were wrapped in plastic and equilibrated for 7 days. The samples were then air dried for one week and equilibrated to approximately 12% moisture content. After drying, ACQ-treated pine stakes, untreated pine, and cedar stakes were leached in deionized water. This process was repeated for a total of 7 days changing the water daily. The stakes were then air-dried for several days until they reached 60% moisture content (MC), weighed and dynamic modulus of elasticity (MOE) was measured using a Grindo Sonic, MK3 instrument (J.W. Lemmens Co).

**Soil bed decay test**

The decay test was carried out in small plastic containers (250mm×365mm×220mm). The soil (silty clay soil) was collected from the top three inches of a forested area at Dorman Lake, Oktibbeha County, Mississippi and then sieved through a screen to remove any large roots, rocks, and other debris. Eight circular holes (5mm diameter) were placed in the bottom perimeter of each plastic container. Screen (250mm × 365mm) was placed on the bottom of each container followed by gravel (20mm deep) and the Dorman soil (100mm deep). The water content of soil in each container was adjusted to provide a MC of 80% based on the oven dry weight of the soil. Pine, cedar, and ACQ treated pine stakes were placed vertically in the plastic containers (Figure 3.1). The containers (18 total, 6 for each wood type) were placed in a greenhouse

at 25°C with a relative humidity 30-50% from November to March and outside from April to October. Two stakes per container were covered with nylon stocking material and used to monitor the moisture content (Figure 3.2). The study was conducted over an 18 month period and described in Figure 3.3.

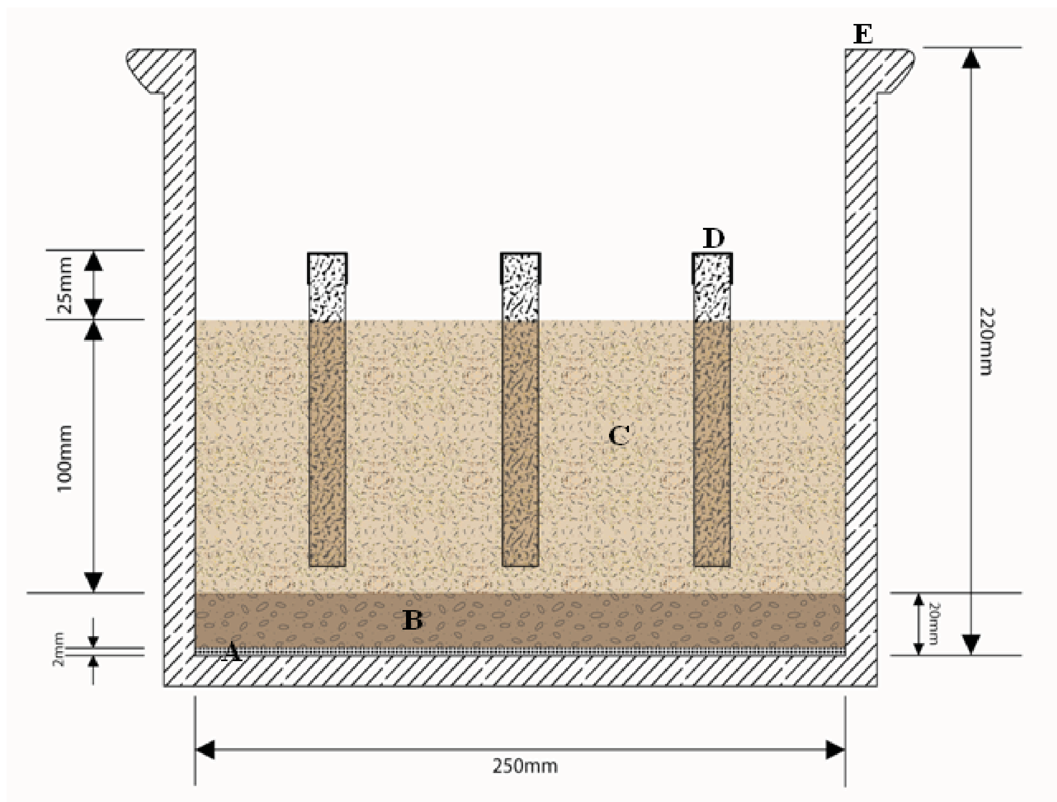



Figure 3.1. The soil bed decay test design. A: screen (2mm), B: gravel, C: soil, D: stake, and E: body of container. Materials are untreated pine (non- decay resistant wood), untreated cedar (naturally decay resistant), and alkaline copper quaternary (ACQ, 0.15 pcf) treated pine (chemically treated to be decay resistant). The container was measured as 250mm×365mm×220mm (width×length×height).

Table 3.1. Description of decay rating by American Wood Protection Association (AWPA) standard-E7. The range from 10 to 0 indicates the decay condition of wood stake during decay.

| Rating | Condition | Description |
| --- | --- | --- |
| 10 | Sound | No sign or evidence of decay, wood softening or discoloration caused by microorganism attack. |
| 9.5 | Trace-suspect | Some areas of discoloration and/or softening associated with superficial microorganism attack. |
| 9 | Slight Attack | Decay and wood softening in present. Up to 3% of the cross sectional area is affected. |
| 8 | Moderate Attack | Similar to "9", but more extensive attack with 3-10% of cross sectional area affected. |
| 7 | Moderate/ Severe Attack | Sample has between 10-30% of cross sectional area decayed. |
| 6 | Severe Attack | Sample has between 30-50% of cross sectional area decayed. |
| 4 | Very Severe Attack | Sample has between 50-75% of cross sectional area decayed. |
| 0 | Failure | Sample has functionally failed. It can either be broken by hand due to decay, or the evaluation probe can penetrate through the sample. |

**Decay rating, MOE test, and moisture content**

Visual decay rating was determined using method E7-01-2001 of the American Wood Protection Association (AWPA 2001) where 10 denotes no decay and 0 denotes failure (Table 3.1). Dynamic modulus of elasticity (MOE) for each of the stakes was measured approximately bimonthly. MOE was measured using a Grindo Sonic, MK3 instrument (J.W. Lemmens Co). The average percentage of MOE change was calculated using the formula: [(initial MOE – current MOE) / initial MOE] ×100%. Moisture content (MC) was determined using the formula: [(current weight – oven dried weight) / oven dried weight] ×100%. The MC of control wood stakes from each container was

calculated weekly and water was added to each container as needed to maintain the wood MC in the range of 40 to 80%. The statistical analysis of MOE, decay rating, and gene expression was performed by two-way analysis of variance (ANOVA) and Tukey's test (*alpha*=0.05) for randomized complete block design (RCBD) using SAS program (SAS 9.1, SAS Institute Inc., Cary, NC).

**Extraction of genomic DNA and total RNA from the wood stakes**

The three stakes showing the largest decrease in MOE from each wood type were selected bimonthly for extraction of genomic DNA and total RNA. Each selected wood stake was cut into 16 equal sections, 4 sections were combined and ground using a rasp for DNA extraction and another 4 sections were combined and ground for RNA extraction. The remaining samples were quick frozen in liquid nitrogen and stored at -70°C. The genomic DNA was extracted from the sawdust (0.05g) in CTAB buffer (1000 μL, 2% (w/v) hexadecyltrimethylammoniumbromide, 100mM Tris, 20mM $Na_2EDTA$, and 1.4M NaCl) by beating in a mini-8 bead mill (Biospec, Bartlesville, OK) for three minutes at maximum speed. The resulting mixture was processed according to the Machery Nagel Nucleospin Plant DNA extraction kit protocol (Easton, PA, USA). Total RNA was extracted from the sawdust (using two tubes each containing 0.05g per sample) according to the protocols of the RNAqueous TM kit (Ambion Co. USA) and genomic DNA was removed from the extracted RNA using TURBO DNA-free™ kit (Ambion Co. USA). The quality and quantity of the extracted genomic DNA and total RNA were determined by UV absorbance at 260 and 280 nm using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.). Extracted genomic DNA

and total RNA were stored at -70 ºC. RNA was converted into cDNA using an iScript cDNA Synthesis kit (Bio-Rad Co. USA).

**Detection of BS 18s rRNA gene and BS degenerative decay enzymes**

The Basidiomycete specific (BS) 18s rRNA gene was amplified using the forward primer: 5´-ACATCCAAGGAAGGCAGCAG–3´and reverse primer: 5´–ATAAGACCCGAAAGAGCCCTATATTG–3´. These primers (Sigma-Aldrich, USA) were designed from an alignment of *Trametes versicolor* (Accession number: AY336751), *Phanerochaete chrysosporium* (Accession number: AF026593), *Gloeophyllum trabeum* (Accession number: AY336761), and *Gloeophyllum sepiarium* (Accession number: AJ540308) using Beacon Designer 4.0 software (Premier Biosoft International, Palo Alto, CA, USA). The amplification of the 18s rRNA gene was carried out using the following parameters: initial denaturation at 94 ºC for 2 min, 35 cycles at 94 ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 30 sec, and a final extension at 72 ºC for 10 min. PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide. Additionally, genomic DNA and cDNA were amplified by degenerative (DG) primers of basidiomycetes and xylariaceous fungi for lignin peroxidase, manganese peroxidase, and laccase, following the published protocol of Pointing et *al.* (2005).

**Cloning and sequencing of PCR products**

Amplified 18s rRNA PCR products from the 4 months and amplified internal transcribed spacer (ITS) products from the 18 months stakes of three wood types were

transformed into *E.coli* plasmids using the TOPO-cloning kit for sequencing (Invitrogen, Co. USA). Amplification was achieved using the forward primer: 5′-CTTGGTCATTTAGAGGAAGTAA–3′ and reverse primer: 5′–TCCTCCGCTTA TTGATATGC–3′ (White *et al.* 1990; Gardes and Bruns 1993). The plasmids of positively transformed *E.coli* were isolated using the PureLink™ Quick Plasmid Miniprep Kit. Plasmids were analyzed for inserts by restriction analysis using digest with EcoRI and then prepared for sequencing using the Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter Co). Automated sequencing was performed using a Beckman CEQ 8000 DNA Analysis System. The sequences were aligned based on NCBI Basidiomycete database using the CLUSTAL W Multiple Sequence Alignment Program, version 1.7 and analyzed data were identified using BLAST search of Basidiomycetes in NCBI (Thompson *et al*. 1994).

**Screening of *Phlebia radiata* using species specific primers**

*Phlebia radiata* (Pr) specific primers (18s rRNA) were designed using the Beacon Designer 4.0 software (Premier Biosoft International, Palo Alto, CA,USA) based on NCBI gene bank database (Table 3.2). The amplification of Pr-18s r RNA gene was carried out using the following procedure: initial denaturation at 94 ºC for 2 min, 35 cycles at 94 ºC for 30 sec, 57 ºC for 30 sec, 72 ºC for 30 sec, and a final extension at 72 ºC for 10 min.

**Gene expression of specific decay enzymes using Real-time Q-PCR**

Real-time-q-PCR (iCycler iQ, Bio-Rad) was performed on day 0 and bimonthly on samples up to 18 months for quantification and expression of Lip, Mnp, and Lcc genes (Table 2.2). Real-time-q-PCR was run as a duplex where one duplex partner was the internal standard gene (18s rRNA) and the other one was either Lip or Mnp or Lcc using iScript Two Step Real-time-PCR kit with SYBR Green (Bio-Rad) programmed for an initial step of 30 min at 50°C and 10 min at 95°C, followed by 45 cycles at 95°C for 15s and 30s at 57°C. The Ct value of each gene was normalized for differences in the amount of total cDNA in the reaction using the Pr 18s rRNA gene as an internal standard control. Relative quantification of gene expression was determined by the standard curve method (Bio-Rad, USA).

Table 3.2. Primers used for *P. radiata* specific gene expression analyses by Real-time PCR. Pr: *Phlebia radiata*, Lip: Lignin peroxidase, Mnp: Manganese peroxidase, and Lcc: Laccase.

| Name | Product Size | Source |
|---|---|---|
| Pr-18s rRNA (F): 5′–ACTCAATTTGATGTGTCGCTCGGC – 3′<br>Pr-18s rRNA (R): 5′–TGGTGCCCTTCCGTCAATTCCTAT – 3′ | 109bp | AY946267 |
| Pr-Lip1 (F): 5′– GCTCATCGCTACGGTTATTC – 3′<br>Pr-Lip1 (R): 5′– GAGGTTCTTGTTCAGGTCATC – 3′ | 126bp | AY743218 |
| Pr-Mnp2 (F): 5′– CACGACGCTATTGCCATC – 3′<br>Pr-Mnp2 (R): 5′– GGAACTGCGAGAGGAAGG – 3′ | 157bp | AJ315701 |
| Pr-Lcc (F): 5′– CGACCGCTTACCATAACC – 3′<br>Pr-Lcc (R): 5′– TGCTTGGAGACTGGAGAG – 3′ | 211bp | X52134 |

# RESULTS

## Changes in decay rating, MOE, and moisture content

Visual decay ratings of the three wood types were significantly different at 4 months through 18 months between pine and cedar and between pine and ACQ-treated pine (Figure 3.2). Pine showed considerably more decay than cedar or ACQ-treated pine which increased over time. ACQ treated pine and cedar samples showed only a minimal decrease in visual decay rating with no significant difference between cedar and ACQ - treated pine over 18 months (*alpha*=0.05).

Decrease in wood stiffness was measured by a decrease in MOE. The average % MOE loss attributed to decay was greater in pine than in cedar and ACQ-treated pine over 18 months (Figure 3.3). At 2 and 4 months sampling times, the % MOE loss ranged from 5-7% in pine, less than 1% in cedar, and 2-5% in ACQ-treated pine, however these losses were not significantly different. From 6 months to 18 months, a significantly higher % loss in MOE was found in pine (26%-45%) compared to cedar (6%-18%) and ACQ-treated pine (7%-19%). The greatest change in % MOE loss was found at 6 months between pine and cedar and pine and ACQ-treated pine. At 18 months the % MOE loss was much greater for the untreated pine (45%) than the visual rating of 25% (7.5). There was no significant difference (alpha = 0.05) in % MOE loss and visual decay rating between cedar and ACQ-treated pine at any sampling date. The average moisture contents of all three wood types in each container generally varied between 40% and 100% over 18 months (Data not shown). Moisture contents were lowest (37%) at 4 months in all samples.

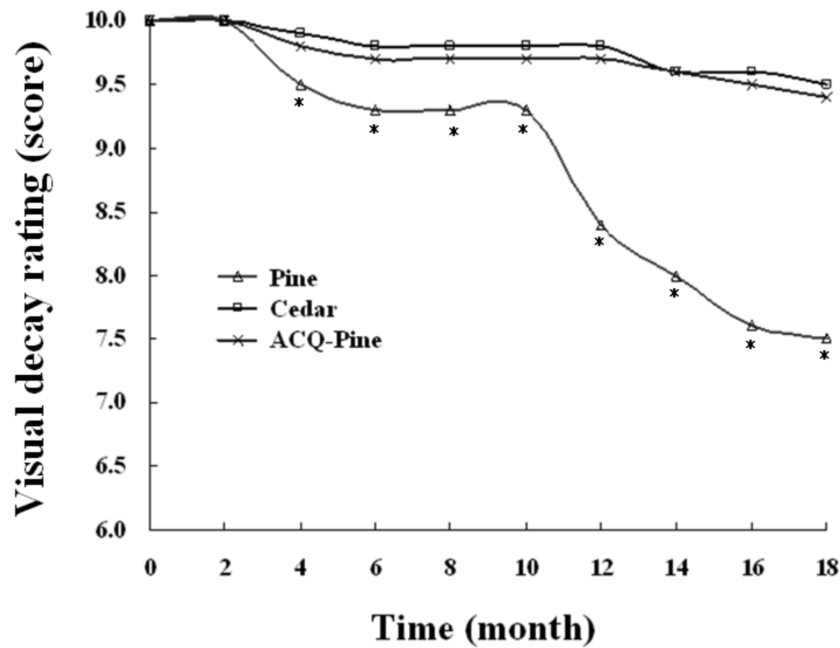



Figure 3.2. Visual decay rating during biodeterioration of three wood types over 18 months exposure. A score of 10 denotes no decay and 6 denote severe decay. "*" indicates that the decay rating for pine stakes was significantly different (*alpha*=0.05) from the cedar and ACQ-pine stakes at the given time.

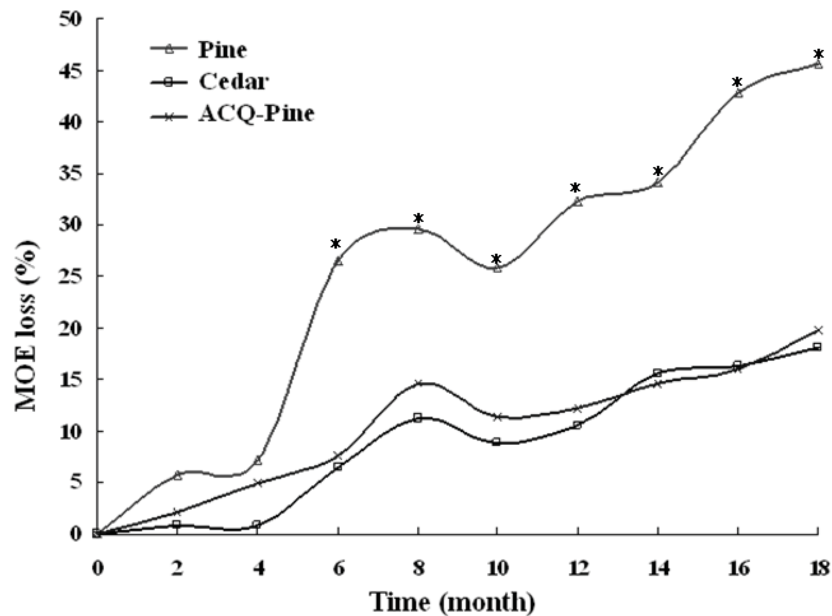



Figure 3.3. The average MOE loss (%) in pine, cedar, and ACQ treated pine over 18 months exposure. "*" denotes the % MOE loss in pine stakes was significantly greater (*alpha*=0.05) than cedar and ACQ-pine stakes at the given exposure time.

**Detection of BS 18s rRNA gene and selected BS specific degenerative enzymes on three wood types**

Basidiomycetes (BS) were present on all samples beginning at 4 months as determined by PCR amplification of BS specific 18s rRNA gene (Figure 3.4). No detection of the BS 18s rRNA gene on pine was found at 0 months and only two pine samples contained BS at 2 months (Figure 3.4). No BS was found on cedar at 0 month and 2 months. One ACQ-treated pine sample contained BS at 2 months, but none were found at 0 month. After 4 months, BS were found on all three types of wood stakes and continued through 18 months (Table 3.3).

Figure 3.4. The Basidiomycete specific 18s rRNA gene at 2 and 4 months on three wood types. M: DNA ladder 1kb, lane 1-3: pine stakes at 2 months, lane 4-6: cedar stakes at 2 months, lane 7-9: ACQ treated pine stakes at 2 months, N: negative control, P: *P. chrysosporium* as positive control, lane 10-12: pine stakes at 4 months, lane 13-15: cedar stakes at 4 months, and lane 16-18: ACQ treated pine stakes at 4 months.

Table 3.3. Presence of basidiomycete specific 18s rRNA gene from genomic DNA in three wood types over 18 months. M: month, "-" means absence of basidiomycetes specific 18s rRNA gene and "+" means presence of basidiomycetes specific 18s rRNA gene.

| | Pine | Cedar | ACQ-Pine |
|---|---|---|---|
| 0M (December) | - | - | - |
| 2M (February) | + | - | + |
| 4M (April) | + | + | + |
| 6M (June) | + | + | + |
| 8M (August) | + | + | + |
| 10M (October) | + | + | + |
| 14M (February) | + | + | + |
| 16M (April) | + | + | + |
| 18M (June) | + | + | + |

Wood samples were screened for selected decay genes using BS degenerative primers to determine the presence/absence of decay enzymes from genomic DNA (Table 3.4) and from cDNA (Table 3.5). Lip gene from genomic DNA was detected on pine at 2, 4, 6, 14, 16, 18 months and ACQ-treated pine at 2, 4, 6, 14, 18 months but was detected on cedar only at 4 months. Mnp gene from genomic DNA was detected on pine at 2, 4, 6, 14, 18 months and ACQ-treated pine at 2, 4, 6, 10, 14, 18 months but was detected on cedar only at 4 months. Lcc gene from genomic DNA was detected on pine and ACQ-treated pine at 2, 4, 6, 8, 10, 14, 16, 18 months and on cedar at 4, 8, 14, 16, 18 months.

Additionally, Lip gene from cDNA was not detected on pine until 14, 16, 18 months and on ACQ-treated pine at 14 and 18 months but was not detected on cedar. Mnp gene from cDNA was detected on pine at 4, 6, 14, and 18 months and on ACQ-treated pine at 4, 14, and 18 months, but was not detected on cedar. Lcc gene from cDNA

was detected on pine at 6, 10, 14, 16, 18 months and ACQ-treated pine at 6, 8, 14, 18 months, but was not detected on cedar.

Table 3.4. The amplification of genomic DNA using Basidiomycete degenerative decay gene primers for three different wood stakes. M: month, “-” means absence of basidiomycete degenerative gene and “+” means presence of basidiomycete degenerative gene. Lip: Lignin peroxidase, Mnp: Manganese peroxidase, and Lcc: Laccase.

| | Pine | | | Cedar | | | ACQ-Pine | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lip | Mnp | Lcc | Lip | Mnp | Lcc | Lip | Mnp | Lcc |
| 0M (December) | - | - | - | - | - | - | - | - | - |
| 2M (February) | + | + | + | - | - | - | + | + | + |
| 4M (April) | + | + | + | + | + | + | + | + | + |
| 6M (June) | + | + | + | - | - | - | + | + | + |
| 8M (August) | - | - | + | - | - | + | - | - | + |
| 10M (October) | - | - | + | - | - | - | - | + | + |
| 14M (February) | + | + | + | - | - | + | + | + | + |
| 16M (April) | + | - | + | - | - | + | - | - | + |
| 18M (June) | + | + | + | - | - | + | + | + | + |

Table 3.5. The amplification of cDNA using Basidiomycete degenerative decay genes primers for three different wood stakes. M: month, "-" means absence of basidiomycete degenerative gene and "+" means presence of basidiomycete degenerative gene. Lip: Lignin peroxidase, Mnp: Manganese peroxidase, and Lcc: Laccase.

| | Pine | | | Cedar | | | ACQ-Pine | | |
|---|---|---|---|---|---|---|---|---|---|
| | Lip | Mnp | Lcc | Lip | Mnp | Lcc | Lip | Mnp | Lcc |
| 0M (December) | - | - | - | - | - | - | - | - | - |
| 2M (February) | - | - | - | - | - | - | - | - | - |
| 4M (April) | - | + | - | - | - | - | - | + | - |
| 6M (June) | - | + | + | - | - | - | - | - | + |
| 8M (August) | - | - | - | - | - | - | - | - | + |
| 10M (October) | - | - | + | - | - | - | - | - | - |
| 14M (February) | + | + | + | - | - | - | + | + | + |
| 16M (April) | + | - | + | - | - | - | - | - | - |
| 18M (June) | + | + | + | - | - | - | + | + | + |

**Identification of basidiomycetes on three wood types after cloning and sequencing**

To identify the microorganisms from each wood type, PCR products of the basidiomycete 18s rRNA gene for 4 month wood samples and general ITS region at 18 month wood samples were cloned and sequenced. At 4 months, four species of basidiomycetes were detected on cedar, six on ACQ treated pine, and twelve on pine (Table 3.6). *Blastosporella zonata*, *Boletaceae sp.*, and *Phlebia radiata* were identified on all three types of wood species, but *P. radiata* was the only wood decay fungus present on all wood types at 4 months. At 18 months, six species of basidiomycetes were detected on pine, three on cedar, and five on ACQ treated pine. *Trametes elegans* and *Trametes lactinea* were found all three wood types. *Gloeophyllum sepiarium* was only found on

pine. *Gloeophyllum trabeum* was found on both cedar and ACQ treated pine at 18 months. *Phlebia radiata* was found on pine and ACQ-treated pine however not on cedar. *P. radiata* was used for species specific gene expression of the decay enzymes.

Table 3.6. Identification of basidiomycetes at 4 months and 18 months after cloning and sequencing from three wood types.

4 months

| Pine | Cedar | ACQ treated pine |
|---|---|---|
| *Auriculariaceae sp.* | | |
| *Arthromyces matolae* | | |
| *Blastosporella zonata* | *Blastosporella zonata* | *Blastosporella zonata* |
| *Boletaceae sp.* | *Boletaceae sp.* | *Boletaceae sp.* |
| *Cryptococcus aerius* | | |
| *Filobasidiella neoformans* | | *Filobasidiella neoformans* |
| *Phlebia radiata* | *Phlebia radiata* | *Phlebia radiata* |
| *Polyporus umbellatus* | *Polyporus umbellatus* | |
| *Serpula himantioides* | | |
| *Trametes sp.* | | |
| *Truncocolumella citrima* | | |
| *Tulashella asymmetrica* | | |
| | | *Gloeophyllum trabeum* |
| | | *Podoscypha petalodes* |

18months

| Pine | Cedar | ACQ treated pine |
|---|---|---|
| *Gloeophyllum sepiarium* | | |
| | *Gloeophyllum trabeum* | *Gloeophyllum trabeum* |
| *Phlebia sp.* | | *Phlebia sp.* |
| *Phlebia radiata* | | *Phlebia radiata* |
| *Pycnoporus sanguineus* | | |
| *Trametes elegans* | *Trametes elegans* | *Trametes elegans* |
| *Trametes lactinea* | *Trametes lactinea* | *Trametes lactinea* |

**Detection of *P. radiata* specific 18s rRNA gene on three wood types**

To confirm the presence of the *P. radiata* on the wood stakes, the *P. radiata* specific 18s rRNA gene was amplified from genomic DNA on all three woods at each sampling period during the 18 month study. *P. radiata* specific 18s rRNA gene was first detected on pine at 4 months (Figure 3.5) and continued to be detected through 18 months (Table 3.7). *P. radiata* specific 18s rRNA gene was detected on cedar only at 4 months. The *P. radiata* specific 18s rRNA gene was detected on ACQ-treated pine at 4 months and continued to be detected through 18 months except at10 months and 16 months.

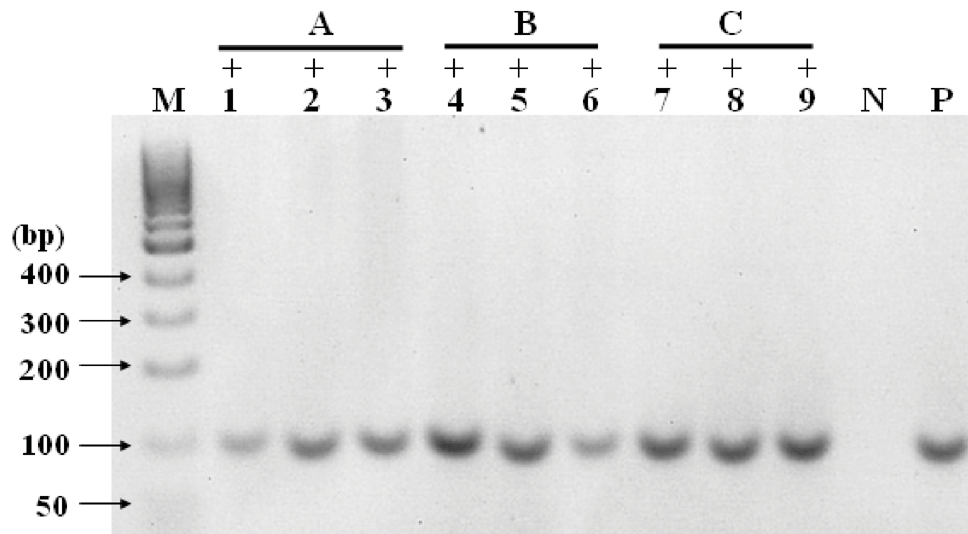



Figure 3.5. The presence of *Phlebia radiata* 18s rRNA gene from genomic DNA at 4 months. M: DNA ladder 1kb, lane 1-3: pine stakes at 4 months, lane 4-6: cedar stakes at 4 months, lane 7-9: ACQ treated pine stakes at 4 months, N: negative control, P: *P. chrysosporium* as positive control.

Table 3.7. Detection of *Phlebia radiata* specific 18s rRNA gene from genomic DNA of three wood types. M: month, "-" means absence of *Phlebia radiata* specific 18s rRNA gene and "+" means presence of *Phlebia radiata* specific 18s rRNA gene.

| | Pine | Cedar | ACQ-Pine |
|---|---|---|---|
| 0M (December) | - | - | - |
| 2M (February) | - | - | - |
| 4M (April) | + | + | + |
| 6M (June) | + | - | + |
| 8M (August) | + | - | + |
| 10M (October) | + | - | - |
| 14M (February) | + | - | + |
| 16M (April) | + | - | - |
| 18M (June) | + | - | + |

**Gene expression of selected decay enzymes from three wood types**

The cDNA from total RNA of each wood stake was screened for *Phlebia radiata* specific Lip, Mnp, and Lcc genes over 18 months (Data not shown). The expression of Lip gene was detected on pine and ACQ-treated pine starting at 14 months (Figure 3.6). The Lip gene expression level on pine at 14 months through 18 months was not significantly different from the expression level on ACQ-pine with the exception of 16 month. The Lip expression on pine at 16 months was significantly higher than on ACQ-treated pine. The Mnp expression level on pine at 4 months through 18 months was very similar to the expression level on ACQ-pine with the exception of 6 months that showed the Mnp expression on pine to be significantly higher than on the other two wood types which was undetectable (Figure 3.7). The Lcc gene was detected at 6 months (Figure 3.8).

The Lcc expression level on pine at 6, 8, 16, 18 months was significantly less than the expression level on ACQ-pine. However at 10 months on pine, the Lcc expression level was significantly higher than on ACQ-treated pine. At 14 months, there was no significant difference in the Lcc expression level on pine and ACQ-treated pine. No expression of Lip, Mnp, and Lcc was detected on cedar at any sampling period.

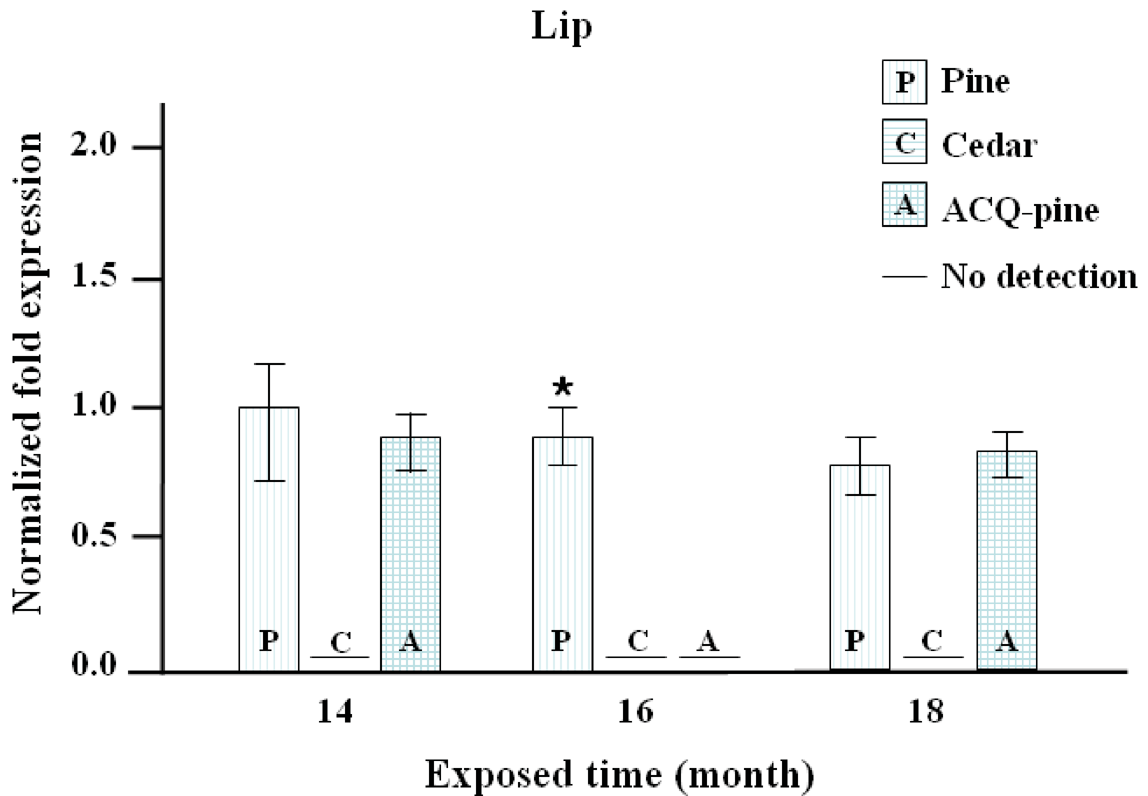



Figure 3.6. Gene expression of *Phlebia radiata* specific Lip on three wood types over 18 months. *Phlebia radiata* specific Lip gene was quantified by Real-time PCR for all three wood types. P: pine, C: cedar, and A: ACQ-treated pine. “*” means that there was significant difference at given month (*alpha*=0.05).

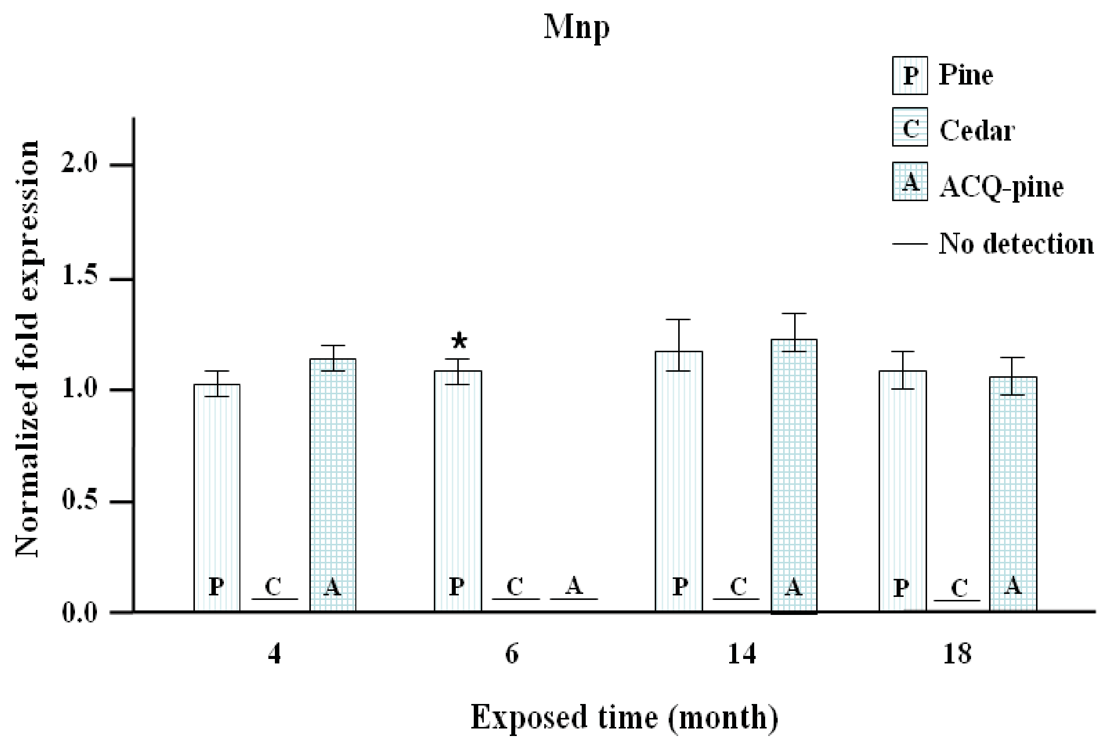



Figure 3.7. Gene expression of *Phlebia radiata* specific Mnp on three wood types over 18 months. *Phlebia radiata* specific Mnp gene was quantified by Real-time PCR for all three wood types. P: pine, C: cedar, and A: ACQ-treated pine. “*” means that there was a significant difference at the given month (*alpha*=0.05).

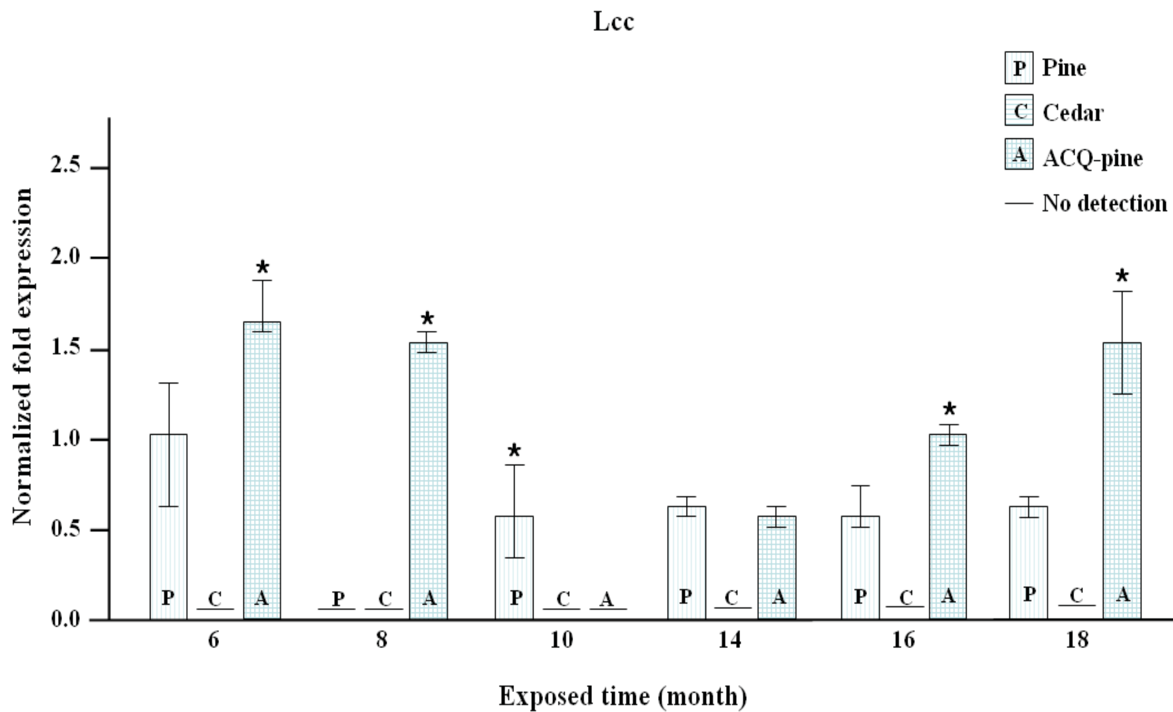



Figure 3.8. Gene expression of *Phlebia radiata* specific Lcc on three wood types over 18 months. *Phlebia radiata* specific Lcc gene was quantified by Real-time PCR for all three wood types. P: pine, C: cedar, and A: ACQ-treated pine. "*" means that there was significant difference at given month (*alpha*=0.05).

## DISCUSSION

Visually evaluating decay only estimates the surface of wood decay, whereas dynamic MOE measures both surface and internal stiffness. A direct relationship between visual decay rating and dynamic MOE loss found a similar relationship (Li et *al.* 2007). Basidiomycetes appeared on pine at 2 months and were present on all wood samples from 4 months until the end of study. After 6 months, a significant decrease in MOE loss was found between pine (26-45%) and cedar (6-18%) and between pine and ACQ-pine (7-19%). The visual decay rating data showed a similar trend. There were no significant

differences in the % MOE loss between cedar and ACQ-treated pine, although at 18 months both cedar and ACQ-treated pine showed a 19% decrease in MOE. The MOE loss (45%) measurement was much greater for untreated pine at 18 months versus the visual decay rating (7.5 score= 25%) illustrating the greater sensitivity of MOE for detecting stiffness loss.

The genomic DNA of untreated pine at 2 months showed the presence of basidiomycetes and basidiomycetes containing the decay genes Lip, Mnp, and Lcc. However, based on the cDNA data, no Lip, Mnp, or Lcc was expressed. Twelve species of basidiomycetes were identified at 4 months and six species at 18 months on pine. Not surprisingly, untreated pine had a greater richness of fungi compared to cedar and ACQ-treated pine most likely because untreated pine possesses no natural durability. The Lip gene was not detected on pine and ACQ-treated pine until 14 months. The expression of Pr-Lip on pine was similar to the expression level on ACQ-pine except at 16 months when no Pr-Lip was detected on ACQ-treated pine. Additionally, Pr-Mnp expression levels on pine from 4 months to 18 months were very similar to the expression level on ACQ-pine except at 6 months when no Mnp was detected on ACQ-treated pine. However, the expression of Pr-Lcc on pine at 6, 8, 16, and 18 months was significantly lower than ACQ-treated pine. The expression levels of Lip, Mnp, and Lcc were only measured for *P. radiata,* but other basidiomyetes likely produced decay enzymes that would also affect wood decay. Loblolly pine (*Pinus taeda*) has sources of both hydrophilic and lipophilic extracts such as resin acids and fatty acids (Morita et *al.* 2001; Porter 2006; Kim et *al.* 2009) although pine wood is considered non-resistant to fungal decay. Pine lumber is

made of sapwood and this sapwood does not contain enough extracts to protect against fungal attack.

Basidiomycetes were present on cedar beginning at 4 months however decay genes such as Lip, Mnp, or Lcc were not expressed. *P. radiata* was identified on cedar at 4 months but not at 18 months. *P. radiata* decay genes were not expressed on cedar during the entire decay exposure period. Three species of basidiomycetes, a brown rot fungus *Gloeophyllum trabeum,* and two white rot fungi, *Trametes elegans* and *Trametes lactinea* were detected on cedar at 18 months. During the entire 18 month study, no basidiomycete decay genes were expressed on cedar. Decay fungi can be present on wood but may or may not express decay genes. Basidiomycetes containing the potential to produce Lcc were observed however no Lcc decay genes were expressed. Thus cedar appears to have the ability to inhibit expression of decay enzymes. Western red cedar, a naturally durable wood, contains toxic chemicals such as thujaplicin and thujic acid that have been reported to possess important antimicrobial properties against decay fungi (Van der Kamp 1986; Nault 1988; Debell *et al.*1997). Cedar strongly reduced the wood decay community and its activities in comparison to untreated pine and ACQ-treated pine. The natural chemicals in cedar most likely reduced the fungal growth and prevented the production of the decay enzymes.

Basidiomycetes were present on ACQ-treated pine beginning at 2 months. The Pr-Lip gene was not detected on ACQ-treated pine until 14 months. At 14 and 18 months, the Pr-Lip gene expression level on ACQ-treated pine was not significantly different from the expression level on pine. The Pr-Mnp expression level on ACQ-treated pine from 4 months to 18 months was very similar to the expression level on pine with the exception

of 10 months. On ACQ-treated pine at 6, 8, 16, and 18 months, there was significantly more Pr-Lcc gene expression than on pine stakes. *P. radiata* colonized ACQ-treated pine and decay genes were expressed but no decay was observed. Interestingly, *Gloeophyllum trabeum* was found at both 4 and 18 months on ACQ-treated pine. *G. trabeum* is a copper tolerant wood decay fungus and may be able to occupy an open niche on ACQ-treated wood that is not available on untreated wood (Goodell 2003). It appears ACQ-treated wood does not stop the growth of fungi or the production of the decay enzymes but does inhibit the effectiveness of the enzyme in decaying the wood. Other studies reported that ACQ-treated pine was colonized with microbes that damaged the surface of wood but did not significantly affect the interior wood structure (Przewloka *et al.* 2008). ACQ is an alkaline, copper, and quaternary chemical preservative that offers resistance to fungal decay. Copper in ACQ produces antifungal activity which contributes to its decay resistance in wood (Groot and Woodward 1996; Watanabe *et al.* 1998; Goodell 2003). Copper can influence the transcriptional induction of laccase where a higher concentration (1.0–2.0 mM) of $Cu^{2+}$ induces increased Lcc expression (Soden and Dobson 2001; Galhaup *et al*. 2002).

## CONCLUSION

This study compared microbial colonization and gene expression on woods with natural and chemical treatment durabilities. These results indicate that different resistant woods have different effects on the microbial communities and their enzymatic activities during decay. In forest soil, there are many fungal species that degrade wood. Although

only *P. radiata* quantitive gene expression was measured, this study also screened for the presence (genomic DNA) and expression (cDNA) of LMEs in basidiomycetes from three wood types. The results showed that the naturally durable cedar reduced the wood decay community and its activities in comparison to untreated pine and ACQ-treated pine. ACQ-treated wood did not stop the expression of the laccase decay gene, but did inhibit the effectiveness of the enzymes. To our knowledge, this is the first report that describes gene expressions of decay enzymes on different wood types in forest soils. The study also provides a decay comparison of natural durable wood versus chemical protected wood.

## BIBLIOGRAPHY

AWPA. 2001. Standard method of evaluating wood preservatives by field tests with stakes. AWPA Method E7-01. Book of standards. American Wood Protection Association.

AWPA. 2008. Processing and treatment standard. AWPA Method T1-08. Book of standards. American Wood Protection Association.

Baechler, R.H. 1959. Improving wood's durability through chemical modification. Forest Products Journal 9: 166-171.

Baechler, R.H., J.O. Blew, and H.G. Roth. 1964. The double-diffusion method of treating wood: a review of studies. Forest Products Journal 14(4):171-178.

Beguin, P. and J.P. Aubert. 1994. The biological degradation of cellulose. FEMS Microbiology Letters. 13:25-58.

Bogan, B., B. Schoenike, R. Lamar, and D. Cullen. 1998. Expression of Lip genes during growth in soil and oxidation of anthracene by Phanerochaete chrysosporium. Applied and Environmental Microbiology 62: 3697-3703.

Bogan, B.W., R.T. Lamar, and K.E. Hammel. 1996. Fluorene oxidation in vivo by Phanerochaete chrysosporium and in vitro during manganese peroxidase-dependent lipid peroxidation. Applied and Environmental Microbiology 62:1788–1792.

Bollag, J.M. and A. Leonowiz 1984. Comparative studies of extracellular fungal laccases. Applied and Environmental Microbiology 48:849-854.

Brown, J., J.K. Glenn, and M.H. Gold. 1990. Manganese regulates expression of manganese peroxidase by Phanerochaete chrysosporium. Journal of Bacteriology 172:3125-3130.

Cease, K.R., R.A. Blanchette, and T.L. Highley.1989. Interactions between Scytalidium species and brown- or white-rot basidiomycetes in birch wood decayed in the laboratory. Wood Science and Technology 23: 151-161.

Cohen, R., M.R. Suzuki, and K.E. Hammel. 2004. Differential stress-induced regulation of two quinone reductases in the brown rot Basidiomycete Gloephyllum trabeum. Applied and Environmental Microbiology 70: 324-331.

Cohen, R., O. Yarden and Y. Hadar. 2002. Lignocellulose affects $Mn^{2+}$ regulation of peroxidase transcript levels in solid-state cultures of Pleurotus ostreatus. Applied and Environmental Microbiology 68: 3156-3158.

Cowling, E.B. and W. Brown. 1969. Structural features of cellulosic materials in relation to enzymatic hydrolysis. In cellulases and their application, eds G.J. Hajny and E.T. Reese. Advance Chemical Series 95:152-187.

Crawford, D.M. 1994. Detection of incipient decay by soft-rot fungi by changes in wood modulus of elasticity. Maters's thesis, Mississppi State University.

Cullen, D. 1997. Recent advances on the molecular genetics of ligninolytic fungi. Journal of Biotechnology 53: 273-289.

Cullen, D. and P.J. Kersten. 1996. Enzymology and molecular biology of lignin degradation. In: Bramble R, Marzluf G (eds) The Mycota III. Springer, Berlin Heidelberg New York, pp 297-314.

Cullen, D. and P.J. Kersten. 2004. Enzymology and molecular biology of lignin degradation. The Mycota III: Biochemistry and Molecular Biology. Berlin; Heidelberg : Springer-Verlag, Pages 249-273.

Datta, A., A. Bettermann, and T.K. Kirk. 1991. Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by Phanerochaete chrysosporium during wood decay. Applied and Environmental Microbiology 57: 1453–1460.

Debell, J.D., J.J. Morrell, and B.L. Gartner. 1997. Tropolone content of increment cores as an indicator of decay resistance in western red cedar. Wood and Fiber Science 29(4):364-369.

Eriksson, K.E.L, R.A. Blanchette, and P. Andr. 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin Heidelberg New York.

Eriksson, K.E.L. 1990. Biotechnology in the pulp and paper industry. Wood Science Technology 24:79-101.

Freeman, M.H. and C.R. McIntyre. A comprehensive review of copper-based wood preservatives: with a focus on new micronized or dispersed copper systems. Forest Products Journal 58(11): 6-27.

Galhaup, C.H., B.Wagner, and D.H. Hinterstoisser. 2002. Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens. Enzyme and Microbiological Technology 30: 529–536.

Gardes, M., and T.D. Bruns. 1993. ITS primers with enhanced specificity basidiomycetes- application to the identification of mycorrhizae and rusts. Molecular Ecology. 2: 113-118.

Gaskell, J.P., P.J. Sterwart, S.F. Kersten, F. Covert, J. Reiser, and D. Cullen. 1994. Establishment of genetic linkage by allele-specific PCR: application to the lignin peroxidase gene family of Phanerochate chrysosporium. BioTechnology 12:1372-1375.

Gessner, M. and U. Raeder. 1994. A histone H4 promoter for expression of a phleomycin-resistance gene in Phanerochaete chrysosporium. Gene 142:237–241.

Gettemy, J.M., B. Ma, M. Alic, and M.H. Gold. 1998. Reverse transcription-PCR analysis of the regulation of the manganese peroxidase gene family. Applied Environmental Microbiology 64:569-574.

Gianfreda, L., F. Xu, and J.M. Bollag. 1999. Laccases: A useful group of oxidoreductive enzymes. Bioremediation Journal 3: 1-25.

Gilbert, B.C., D.M. King, and C.B. Thomas. 1984. The oxidation of some polysaccharides by the hydroxyl radical. Carbohydrate Research 125(2):217-235.

Goodell, B. 2003. Brown-rot fungal degradation of wood. Our evolving view. In: Goodell B, Nicholas DB, Schultz TP(eds) Wood deterioration and preservation. ACS Symp Ser 845, American Chemistry Society, Washington, DC, pp. 97-118.

Goodell, B., J. Jellison, J. Liu, G. Daniel, A. Paszczynski, F. Fekete, S. Krishnamurthy, L. Jun, and G. Xu. 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. Journal of Biotechnology 53:133–162

Gotz, S. and U. Karst. 2007. Wavelength-resolved fluorescence detector for microchip capillary electrophoresis separations. Sensors and Actuators 123(1):622-627.

Green, F. and T.L. Highley. 1997. Mechanism of brown-rot decay: Paradigm or Pardox. International Biodeterioration and Biodegradation 39(2):113-124.

Gregorio, A.P.F., I.R. Silva, M.R. Sedarati, J.N. Hedger. 2006. Changes in production of

lignin degrading enzymes during interactions between mycelia of the tropical decomposer basidiomycetes Marasmiellus troyanus and Marasmius pallescens. Mycological Research 110: 161-168.

Groot, R.C.D. and B. Woodward. 1996. A laboratory soil-contact decay test: an accelerated method to determine durability of treated wood shakes. Wood and Fiber Science 28: 214-226.

Heid, C.A., J. Stevens, and K.J. Livak. 1996. Real time quantitative PCR. Genome Research 6:986-994.

Heinzkill, M., L. Bech, T. Halkier, P. Schneider, and T. Anke. 1998. Characterization of laccases and peroxidases from wood- rotting fungi (Family Coprinaceae). Applied and Environmental Microbiology 64:1601-1606.

Highley, T. L. 1977. Requirements for cellulose degradation by a brown-rot fungus. Material und Organismen 25–36.

Highley, T.L. and J.A. Micales. 1990. Effect of aromatic monomers on production of carbohydrate-degrading enzymes by white-rot and brown-rot fungi. FEMS Microbiology Letters 66: 15-22.

Janse, B.J.H., J. Gaskell, M. Akhtar, and D. Cullen. 1998. Expression of Phanerochaete chrysosporium genes encoding lignin peroxidases, manganese peroxidases, and glyoxal oxidase in wood. Applied and Environmental Microbiology 64(9):3536-3538.

Jaszek, M., J. Żuchowski, E. Dajczak, K. Cimek, M. Grąz, and K. Grzynowicz. 2006. Ligninolytic enzymes can participate in a multiple response system to oxidative stress in white-rot basidiomycetes: Fomes fomentarius and Tyromyces pubescens. International Biodeterioration & Biodegradation 58: 168-175.

Jellison, J., V. Chandhoke, B.Goodell, and F.A. Fekete. 1991. The isolation and immunolocalization of iron-binding compounds produced by Gloeophyllum trabeum. Applied Microbiology and Biotechnology 35:805-809.

Jensen, K.A., JR., C.J. Houtman, Z.C. Ryan, and K.E. Hammel. 2001. Pathways for extracellular Fenton chemistry in the brown rot Basidiomycete Gloeophyllum trabeum. Applied and Environmental Microbiology 67: 2705-2711.

Kerem, Z., K.A. Jensen, and K.E. Hammel. 1999. Biodegradative mechanism of the brown rot basidiomycete Gloephyllum trabeum: evidence of and extracellular

hydroquinone-driven Fenton reaction. FEBS Letters 446:49-54.

Kim, J.W, D. Harper, and A.M. Taylor. 2009. Effect of extractives on water sorption and durability of wood-plastic composites. Wood and Fiber Science 41(3): 279-290.

Kirker, G.T. 2008. Effects of chlorothalonil (CTN) and butylated hydroxyl-toluene (BHT) on microbial communities involved in the deterioration of wood using terminal restriction fragment length polymorphism (T-RFLP) analyses. Ph.D- Dissertation. Mississippi State University.

Kleman-Leyer, K., E. Agosin, A. H. Conner, and T. K.Kirk. 1992. Changes in molecular size distribution of cellulose during attack by white rot and brown rot fungi. Applied and Environmental Microbiology. 58(4): 1266-1270.

Koenigs, J. W. 1974. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood and Fiber Science 6: 66-80.

Kuwahara, M., J.K. Glenn, M.A. Morgan, and M.H. Gold. 1984. Separation and characterization of two extracellular H2O2-dependent oxidases from ligninolytic cultures of Phanerochaete chrysosporium. FEBS Letters 169: 247-250.

Lamph, W.W., V. J. Dwarki, R. Ofir, M. Montminy, and I.M. Verma. 1990. Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation. Proceedings of the National Academy of Sciences 87:4320-4324.

Leonowicz, A., A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtaś-Wasilewska, N.-S. Cho, M. Hofrichter, and J. Rogalski. 1999. Biodegradation of lignin by white rot fungi. Fungal Genetics and Biology 27: 175-185.

Li, D., M. Alic, and M.H. Gold. 1994. Nitrogen regulation of lignin peroxidase gene transcription. Applied Environmental Microbiology 60:3447–3449.

Li, G., D.D. Nicholas, and T.P. Schultz. 2007. Development of an accelerated soil-contact decay test. Holzforschung 61: 214-218.

Machuca, A. and A. Ferraz. 2001. Hydrolytic and oxidative enzymes produced by white- and brown-rot fungi during Eucalyptus grandis decay in solid medium. Enzyme and Microbial Technology 29:386-391.

Maheshwari, R., G. Bharadwaj, and M.K. Bhat. 2000. Thermophilic fungi: their physiology and enzymes. Microbiology and Molecular Biology Reviews 64:461-

488.

Martinez, A., M. Speranza, F.J. Ruiz-Dueñas, P. Feffeira, S. Camarero, F. Guillén, M.J. Martínez, A. Gutiérrez, and J.C. del Río. 2005. Biodegradation of lignocellulosic microbial, chemical, and enzymatic aspects of the fungal attack of lignin. International Microbiology 8:195-204.

Martinez, D., L.F. Larrondo, N. Putnam, M.D.S. Gelpke, K. Huang, J. Chapman, K.G. Helfenbein, P. Ramaiya, J.C. Detter, F. Larimer, P.M. Coutinho, B. Henrissat, R. Berka, D. Cullen, and D. Rokhsar. 2004. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nature Biotechnology 22(6): 695-700.

Morita, S., Y. Yazaki, and G. C. Johnson. 2001. Mycelium growth promotion by water extractives from the inner bark of radiata pine (Pinus radiata D. Don). Holzforschung 55(2): 155–158.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symposium on Quantitative Biology 51:263-273.

Nault, J. 1988. Radial distribution of thudaplicins in old growth and second growth western red cedar (Thuja plicata Donn). Wood Science and Technolgy 22:73-80.

Nelson, D. and M. Cox. 2005. Lehninger Principles of Biochemistry. Fourth edition. W.H. Freeman publisher.

Orlando, C., P. Pinzani, and M. Pazzagli. 1998. Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicine 36: 255– 269.

Paice, M, I. Reid, R. Bourbonnais, F. Archibald, and L. Jurasek. 1993. Manganese peroxidase produced by Trametes versicolor during pulp bleaching demethylates and delignifies. Applied and Environmental Microbiology. 59(1):260-265.

Perez, J., J. Munoz-Dorado, T. Rubia, and J. Martinez. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. International Microbiology 5:53-63.

Phillips, C. and M. Bogyo. 2005. Proteomics meets microbiology: technical advances in the global mapping of protein expression and function. Cellular Microbiology 7(8): 1061–1076.

Piontek, K., A.T. Smith, and W. Blodig. 2001. Lignin peroxidase structure and function. Biochemical Society Transactions 29:111-115.

Pointing, S. A.L. Pelling, G.J.D. Smith, K.D. Hyde, and C.A. Reddy. 2005. Screening of basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific sequences. Mycological Research 109: 115-124.

Pointing, S., M.M. Parungao, and K.D. Hyde. 2003. Production of wood-decay enzymes, mass loss and lignin solubilization in wood by tropical Xylariaceae. Mycological Research, 107, 231-235.

Porter, T. 2006. Wood identification and Use. Guild of master craftsman publications Ltd., Singapore.

Preston, A. 2000. Wood Preservation: Trends of today will influence the industry tomorrow. Forest Products Journal 50:12–19.

Przewloka, S.R., D.M. Crawford, D.R. Rammer, D.L. Buckner, B.M. Woodward, G. Li and D.D. Nicholas. 2008. Assessment of biodeterioration for the screening of new wood preservatives: Calculation of stiffness loss in rapid decay testing. Holzforschung 62(3):270–276.

Puls, J. 1992. Alfa-glucuronidase in the hydrolysis of wood xylan. Progress in Biotechnology 7: 213-224.

Rayner, A.D.M. and L. Boddy. 1998. Fungal decomposition of wood: its biology and ecology. John Wiley, Chichester Publisher.

Reading, N.S., K.D. Welch, and S.D. Aust. 2003. Free radical reactions of wood-degradating fungi. In: Goodell B, Nicholas DB, Schultz TP(eds) Wood deterioration and preservation. ACS Symp Ser 845, American Chemistry Sociey, Washington, DC, pp. 16-31.

Schultz, T.P., D.D. Nicholas, and A.F. Preston. 2007. A brief review of the past, present and future of wood preservation. Pest Management Science 63(8):784-788.

Smith, R.S. 1975. Deterioration of pulpwood by fungi and its control. Canadian Pulp and Paper Association 2:33-37.

Soden, D.M. and A.D. Dobson. 2001. Differential regulation of laccase gene expression in Pleurotus sajor-caju. Microbiology 147: 1755- 1763.

Solomon, E. I., U.M. Sundaram, and T.E. Machonkin. 1996. Multicopper oxidases and oxygenases. Chemical Reviews 96: 2563-2606.

Stewart, P. and D. Cullen. 1999. Organization and differential regulation of a cluster of lignin peroxidase genes of Phanerochaete chrysosporium. Journal of Bacteriology 181:3427–3432.

Stewart, P., P. Kersten, A.V. Wymelenberg, J. Gaskell, and D. Cullen. 1992. The lignin peroxidase gene family of Phanerochaete chrysosporium: complex regulation by carbon and nitrogen limitation, and the identification of a second dimorphic chromosome. Journal of Bacteriology 174: 5036-5042.

Teeri, T.T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolase. Trends in Biotechnology. 15:160-167.

Temp, U. and C. Eggert. 1999. Novel interaction between laccase and cellobiose dehydrogenase during pigment synthesis in the white rot fungus Pycnoporus cinnabarinus. Applied and Environmental Microbiology 65:389-395.

Thompson, T.D., D.G. Higgins, and T.J. Gibson. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 11(22):4673-4646.

Timell, T.E. 1976. Recent progress in the chemistry of wood hemicelluloses. Wood Science and Technology. 1:45-70.

Uffen, R.L. 1997. Xylan degradation: a glimpse at microbial diversity. Journal of Industrial Microbiology & Biotechnology 19: 1-6.

Van der Kamp, B.J. 1986. Effects of heartwood inhabiting fungi on thujaplicin content and decay resistance of western red cedar (Thuja plicata Donn). Wood and Fiber Science 18(3):421-427.

Waston, J.D. 1968. The double helix: a personal account of the discovery of the structure of DNA. Atheneum, New York; p.282-279.

Watanabe, T., K. Koller, and K. Messner.. 1998. Copper-dependent depolymerization of lignin in the presence of fungal metabolite, pyridine. Journal of Biotechnology 62(3): 221-230.

Weaver, R.F. 2005. Molecular biology. Third edition, McGraw Hill higher education.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, Pp. 315-322. In M.A. Innis, D.H., Gelfand, J.J. Sninsky, and T.J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

Wilkinson, J. H. 1965. Isoenzymes. J. B.Lippincott Co. Philadelphia. pp. 43-81.

Wong, A.H.H., R.B. Pearce, and S.C. Watkinson. 1992. Fungi associated with ground line soft rot decay in copper-chrome-arsenic treated hardwood utility poles of Malaysian hardwood. IRG/WP/1567.

Xu, H. and A. Ewing. 2005. High-throughput enzyme assay on a multichannel microchip using optically gated sample introduction. Electrophoresis 26(24):4711-4717.

Yoshida, M., K. Igarashi, R. Kawai, K. Aida, and M. Samejima. 2004. Differential transcription of β-glucosidase and cellobiose dehydrogenase genes in cellulose degradation by the basidiomycete Phanerochaete chrysosporium. FEMS Microbiology Letters 235:177-182.

Zabel, R.A., C.J.K.Wang, and S.E. Anagnost. 1991. Soft-rot capabilities of the major micro fungi, isolated from Douglas-fir poles in the North-East. Wood Fiber Science 23:220-237.

Zabel, R.A and J.J. Morrell. 1992. Wood Microbiology: Decay and Its Prevention. Academic Press, Harcourt Brace Jovanovich. New York.

# CHAPTER IV

# COMPARATIVE PROTEIN PROFILING DURING BIODETERIORATION OF THREE DIFFERENT WOOD TYPES

## INTRODUCTION

During biodeterioration of wood, many different microorganisms colonize and survive on wood at the same time. The ability of wood decay fungi to consume renewable lignocellulosic resources can lead to a dominance of a particular group. The competition among fungi for resources can lead to successions of microorganism diversity (Hackl *et al.* 2005). Recognizing the interactions between wood decay fungi and other microorganisms (such as molds, stains, bacteria, and algae) is important in understanding the complex decay processes (Rayner and Boddy 1988).

Wood degradation is initiated by the action of specific microbial enzymes produced by microorganisms in order to obtain food for their survival. Metagenomics is the study of genomes of all organisms in an environment (Markowitz 2007). The genome is the complete set of an organism's DNA. When organisms such as fungi produce decay enzymes, the first step is transcription of decay genes located on the DNA which is then translated to messenger RNA (mRNA). These mRNAs are called transcripts. This is followed by translation of mRNA into proteins (Phillips and Bogyo 2005). The proteome is the complete set of proteins expressed by the genome and is constantly changing in

response to internal and external conditions including environment and food sources (Phillips and Bogyo 2005). Thus, when fungi degrade wood, proteins (which include decay enzymes) are produced that are unique to the decay process. Metaproteomics explores the proteins expressed by a microbial complex and can provide a better understanding of the microbial network in environmental communities (Gromov and Celis 2000).

The presence of a particular microorganism does not mean it is metabolically active in the decay process. Identification of proteins produced by microorganisms can help develop a better understanding of the complex decay process. Protein extracts obtained from wood degraded by the action of microorganisms will be comprised of many different types of enzymes from a variety of organisms. As the microbial community changes over time or with the environment, changes in the activities of the microorganisms will be reflected in their proteins. This information can help to identify the proteins that are being distinctively expressed during biodeterioration of wood.

The sequencing of the genomes of the white rot fungus *Phanerochaete chrysosporium* (Martinez *et al.* 2004) and the brown rot fungus *Postia placenta* (Martinez *et al.* 2009) can potentially lead to the discovery of new enzymes involved in wood decay and also provide information on the succession of enzymes involved in this process. A proteomic study of oak by *Phanerochaete chrysosporium* detected lignin peroxidase, manganese peroxidase and laccase being expressed during the decay of wood (Abbas *et al.* 2005). A study of the secretome, products secreted by a cell or an organism, of *P. chrysosporium* growing on cellulose medium instead of wood substrate found 268 proteins using mass spectrometry peptide identifications (Vanden Wymelenberg *et al.*

2005). The decay related enzymes identified included endoglucanases, exocellobiohydrolases, endoxylanases, cellobiose dehydrogenase, and glucose oxidase. Protein profiles from Southern yellow pine sapwood inoculated with the brown rot fungus, *Gloeophyllum trabeum* were compared to a pure culture of the fungus (Kang *et al.* 2009). The fungus on wood produced decay related proteins including alcohol oxidase, lipoxygenase, and catalase, while the pure culture of the fungus produced mostly metabolic proteins. Many hypothetical proteins were also detected highlighting a limitation of proteomic analysis for wood decay research which is the low number of protein sequence information annotated on the public databases. None of the hypothetical proteins, which were a majority of the proteins, were found in both the pure culture of fungus and the fungus inoculated on wood. This indicated there was a very different mechanism of growth between the pure cultures and the fungus inoculated on wood.

The objective of this study is to determine the variations in protein expression when microorganisms colonize non-resistant, naturally durable, and chemically treated woods. To our knowledge, this study is the first to compare metaproteomics from microbial communities decaying different wood types over time and therefore will likely provide baseline information for future studies.

## LITERATURE REVIEW

### Microorganism communities in wood decay

Wood is a major source of nutrients in forest ecosystems and its decomposition occurs by microbial communities (Zhang *et al.* 2008). The process of decomposition of

wood varies greatly from one site to another, depending on temperature, moisture, and wood species (Eriksson *et al.* 1990). Multiple microorganism species naturally inhabit wood and interactions during wood decay exist among species. Microorganisms display an impressive diversity in their metabolic activities and in their interactions with other microbes. Microorganisms may play roles in wood deterioration individually, simultaneously, or gradiently (Rayner and Boddy 1998). Gradient decomposition is where some microorganisms inhabit wood first and then other microorganisms inhabit later.

Although microbial communities interact to assist in wood decay, true decay of wood occurs primarily by wood decay fungi. White rot decay shows extensive and rapid decay of all wood components due to enzymatic degradation and is characterized by wood bleaching due to lignin removal. Brown rot decay shows a very rapid cellulose and hemicellulose degradation. The changes in wood during brown rot are attributed to non-enzymic degradation with relatively little lignin modification leaving behind a brown crumbly cubical wood. The white rot and brown rot fungi are classified as basidiomycetes (Beguin and Aubert 1994). Soft rot decay is an enzymatic decay of cellulose and hemicellulose accompanied by little or no lignin degradation. This type of decay occurs in the surface layers of wood and is caused by fungal species belonging to Ascomycetes (Zabel *et al.* 1991).

**Biochemical approaches to understanding microbial communities in wood decay**

Many microorganisms are difficult to culture in an artificial medium. Molecular methods target DNA extracted directly from environmental samples and have been

successfully applied to microbial communities in a variety of environments (Millsa *et al.* 2007; Zwolinski 2007). The majority of molecular methods currently being used for community analysis involve DNA or RNA. This can provide a more complete picture of microbial communities and microbial species. Identification of the microorganisms involved in decay is necessary in order to develop a complete understanding of the roles the different microorganisms play in the decay process. However, the presence of a particular microorganism does not mean it is metabolically active in the decay process. Wood decay is caused by enzymes and other metabolites secreted by fungi in their efforts to obtain a carbon source for their survival (Pointing *et al.* 2003). Thus, in order to develop a better understanding of the decay process, identification of the enzymes produced by microorganisms is needed.

All enzymes are proteins with roles in the organism's metabolism. Protein extracts obtained from wood deteriorated by the action of microorganisms will be comprised of many different types of enzymes. Protein extracts do not identify microorganisms, but could serve as a potential source for the identification of microbial enzymes produced during wood decay. Enzymes are protein molecules synthesized from linear heterogeneous polymers consisting of 20 different amino acids (Mathewson 1998). The protein expressed by a genome (the complete set of an organism's DNA) is called a proteome (Liebler 2002; Phillips and Bogyo 2005). The proteome of a given organism is constantly changing in response to internal and external conditions including its environment and food sources. Thus, when fungi degrade wood, unique proteins (enzymes and some mediators) are produced depending upon the decay process. Proteomics has become a vital tool in the field of functional genomics, because only

proteins are involved in metabolic function. While traditional biochemistry or molecular real-time methods can measure the activity of individual enzymes, proteomics can show all proteins or enzymes expressed at a given time.

The most extensively used method in the field of proteomics is Two-Dimensional PolyAcrylamide Gel Electrophoresis (2D-PAGE) (O'Farrell 1975). One of the largest applications is protein profiling which compares the protein expression levels under different biological conditions. However, the combination of 2D-PAGE and mass spectrometry (MS) detects only the most abundant proteins, indicating that the 2D-PAGE and MS approach does not provide complete coverage of the entire proteome but does provide information on the expressed proteins at a given time. The technique of matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS) was developed in the 1980s (Karas et *al.* 1985). In MALDI-TOF/MS, signal protein ion fragments in the mass range 200-20,000 Da is obtained. The spectral data gathered offers diagnostic and comparative advantages in cases of unknown fungal protein samples. The peptide mass fingerprints typically obtained by MALDI/TOF or LC/MS/MS are matched against sequences found among databases using dedicated bioinformatics tools (Zhou *et al.* 2004).

**Application of proteomics in the study of biodeterioration of wood**

Many studies have shown that there is low correlation between mRNA and protein expression levels (Koker and Kersten 2002; Pevsner 2003). Gene expression results in mRNA which should include all transcripts in the cell. The transcriptome reflects the genes that are being actively expressed at any given time, but it does not

necessarily mean that the corresponding protein is produced in the cell (Zhou *et al.* 2004). Proteomics is much more complex than either genomics or transcriptomics because each protein can be chemically modified in different ways after synthesis. Therefore characterization of both the genes and their proteins will provide a more complete picture.

Expressional proteomics can provide a snap shot of all proteins expressed by an organism at the time of extraction (Prabakaran *et al.* 2001). These profiles can be compared to individual microbes to determine what proteins are being uniquely expressed during the wood decay process. A proteomic study of inoculated *Phanerochaete chrysosporium* detected lignin peroxidase, manganese peroxidase and laccase expressed while growing on oak substrates (Abbas *et al.* 2005). Also cellulose and hemicellulose degrading enzymes were identified from proteomic analysis. A study of the secretome of the white rot fungus *P. chrysosporium* growing on cellulose containing medium instead of wood substrate found 268 proteins using mass spectrometry peptide identifications (Vanden Wymelenberg *et al.* 2005). The multiple endoglucanases, exocellobiohydrolases, endoxylanases, cellobiose dehydrogenase, glucose oxidase were identified. Proteins from the brown rot fungus, *Gloeophyllum trabeum* growing on Southern yellow pine sapwood and from pure cultures were compared using 2D-PAGE and MALDI-TOF/MS (Kang *et al.* 2009). The fungus decaying wood produced 76 proteins, including the Fenton-chemistry related enzymes, alcohol oxidase, lipoxygenase and catalase. The pure culture of the fungus produced 111 mostly metabolic proteins. There was very little overlap of proteins between both sets of environmental conditions, indicating a very different mechanism of action when the fungus is growing on a cellulose-based nutrient (wood) versus glucose media. This study

also highlighted a current limitation of this approach which is the limited protein and genomic sequence information found annotated in the public databases.

## MATERIALS AND METHODS

### Preparation of wood stakes

Pine (*Pinus* sp.) and cedar (*Thuja plicata*) boards (5.1cm×10.2cm×180cm, 2.5cm×10.2cm×180cm respectively) used for this study were purchased from Lowes's Home Improvement Center, Starkville, Mississippi. Each board was cut into strips and numbered sequentially. The strips were cut into stakes measuring 14mm×14mm×115mm (T×R×L). ACQ treating solutions of approximately 38% was used to treat pine stakes to a desired retention of 0.15 pcf. The samples were treated using the full cell method according to the following schedule: 73.7cm/Hg vacuum for 15 min followed by 150 psi pressure for 15 min (T1-08, 2008 AWPA standard). After treatment, all samples were wrapped in plastic and equilibrated for 7 days. The samples were then air dried for one week and equilibrated to approximately 12% moisture content. After drying the treated pine stakes untreated pine and cedar stakes were then leached in deionized water for 7 days, with water changes daily. The stakes were then air-dried for several days until they reached 60% moisture content (MC), weighed and the dynamic modulus of elasticity (MOE) was measured using a Grindo Sonic, MK3 instrument (J.W. Lemmens Co).

### Soil bed decay test

The decay test was carried out in small plastic containers (250mm×365mm×220mm). The soil (silty clay soil) was collected from the top three inches of a forested area in Dorman Lake, Oktibbeha County, Mississippi and then sieved through a screen to remove any large roots, rocks, and other debris. Eight circular holes (5mm diameter) were placed in the bottom perimeter of each plastic container followed by screen (250mm × 365mm), gravel (20mm deep), and the Dorman soil (100mm deep). The water content of soil in each container was adjusted to provide a MC of 80% based on the oven dry weight of the soil. Pine, cedar, and ACQ treated pine stakes were placed vertically in the plastic containers (Figure 4.1).

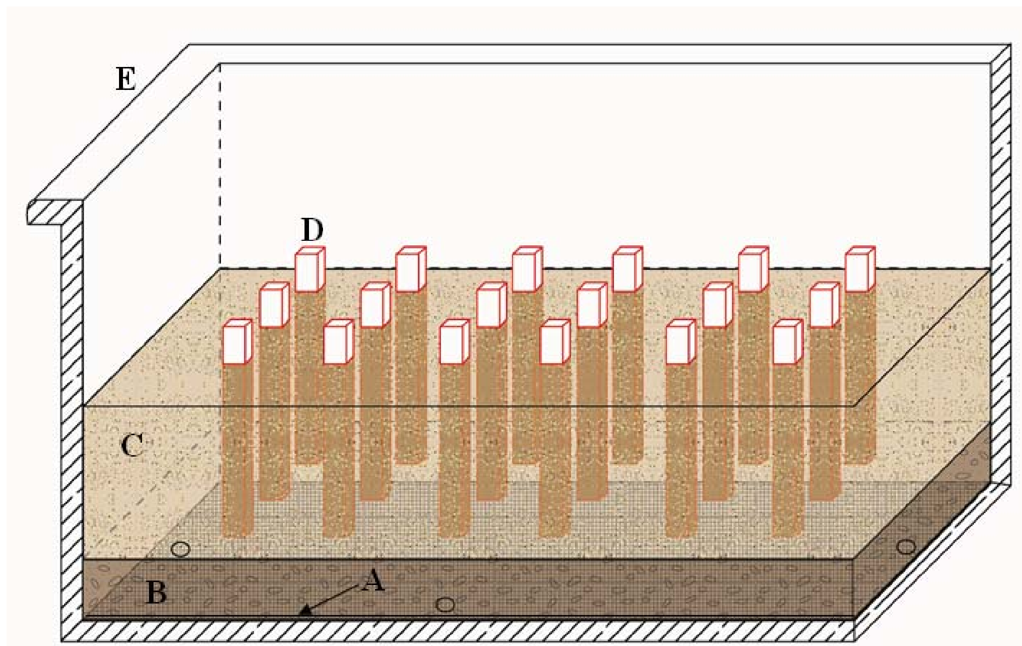



Figure 4.1. The soil bed decay test design. (A) Screen, (B) Gravel, (C) Soil, (D) Wood stake, and (E) Body of container. Wood stakes were untreated pine, untreated cedar, and alkaline copper quaternary (ACQ, 0.15 pcf) treated pine.

The containers (18 total, 6 for each wood type) were placed in a greenhouse at 25°C with a relative humidity 30-50% from November to March and outside from April to October. Two pine stakes per container were covered with nylon stocking material and used to monitor moisture content of the wood. The study was conducted over an 18 month period.

**Decay rating, dynamic MOE test, and moisture content**

The visual decay rating was determined using method E7-01-2001 of the American Wood Protection Association (AWPA 2001) where 10 denotes no decay and 0 denotes failure (Table 4.1).

Table 4.1. Description of decay rating by American Wood Protection Association (AWPA) standard-E7. The range from 10 to 0 indicates the decay condition of wood stake during decay.

| Rating | Condition | Description |
|---|---|---|
| 10 | Sound | No sign or evidence of decay, wood softening or discoloration caused by microorganism attack. |
| 9.5 | Trace-suspect | Some areas of discoloration and/or softening associated with superficial microorganism attack. |
| 9 | Slight Attack | Decay and wood softening in present. Up to 3% of the cross sectional area is affected. |
| 8 | Moderate Attack | Similar to "9", but more extensive attack with 3-10% of cross sectional area affected. |
| 7 | Moderate/ Severe Attack | Sample has between 10-30% of cross sectional area decayed. |
| 6 | Severe Attack | Sample has between 30-50% of cross sectional area decayed. |
| 4 | Very Severe Attack | Sample has between 50-75% of cross sectional area decayed. |
| 0 | Failure | Sample has functionally failed. It can either be broken by hand due to decay, or the evaluation probe can penetrate through the sample. |

The dynamic modulus of elasticity (MOE) for each of the stakes was measured approximately bimonthly. MOE was measured using a Grindo Sonic, MK3 instrument (J.W. Lemmens Co). The average percentage of MOE change was calculated using the formula: [(initial MOE – current MOE) / initial MOE] ×100%. The moisture content (MC) was determined using the formula: [(current weight – oven dried weight) / oven dried weight] ×100%. The MC of control wood stakes from each container was calculated weekly and water was added to each container as needed to maintain the wood MC in the range of 40 to 80%. The statistical analysis of % MOE change and decay rating was performed by two-way analysis of variance (ANOVA) and Tukey's test (*alpha*=0.05) for randomized complete block design (RCBD) using SAS program (SAS 9.1, SAS Institute Inc., Cary, NC).

**Extraction of microbial proteins from three wood types**

Stakes representing each of three wood types were collected bimonthly and selected for extraction of microbial proteins. The most decayed stake from each wood type was cut into 16 equal sections. The 4 sections showing the most decay were combined and ground using a rasp. The proteins were extracted and quantified from all three samples according to published methods (Bradford 1976; Kang *et al.* 2009). In general, 1.0 g of each sample was homogenized using a clean pestle and protein extraction buffer (5.0 ml for 1.0 g wet weight of biomass). The protein extraction buffer was composed of 0.7 M sucrose, 0.5 M Tris-HCl (pH 8.5), 0.05 M $Na_2EDTA$, 0.1 M KCl, and 2% (v/v) 2-mercaptoethanol. Water-saturated phenol was then used to extract total proteins from the buffer. Proteins in the phenol phase were precipitated with five volumes of ammonium acetate in methanol (0.1 M $NH_4OAc$ and 1% (v/v) 2-mercaptoethanol in

methanol) at -70 ºC for 2 hr. The resultant pellets were sequentially washed with ammonium acetate in methanol and 80% acetone. The protein samples were then air-dried and stored at -70 ºC until needed. The frozen protein pellet was dissolved with protein rehydration buffer (9.5M Urea, 4% CHAPS, 1% DTT, 0.2% Ampholites). The concentration of the proteins from the wood samples was quantified by the 2D-Quant Kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as the standard.

**Analysis of the protein patterns using Two Dimensional PAGE (2D-PAGE)**

Each soluble protein (300 µg) with the rehydration buffer was transferred to a tray for the isoelectric focusing (IEF) system (Bio-Rad, Hercules, CA). IEF was carried out in Bio-Rad PROTEAN IEF CELL on immobilized pH Gradient (IPG) strips (Dry Strips, 7 cm, pH 3–10 non-linear, Bio-Rad, Hercules, CA). The IEF of sample proteins was accomplished using the following step gradient: 500 volts for 1 hr, 1000 volts for 1 hr, and 8000 volts until a total of 67,500 V-hr had been achieved then passively rehydrated overnight. After IEF, the strips were equilibrated in the buffer containing 7 M urea, 2% SDS, 375 mM Tris (pH 8.8), and 10% glycerol plus either 50 mM DTT for reduction or 100 mM iodoacetamide for alkylation. Equilibrated IPG strips were loaded onto a 12% acrylamide sodium dodecyl sulfate (SDS) – PAGE gel and sealed with 1% agarose.

Second dimensional electrophoresis was carried out using a Protean IIxi system (Bio-Rad, Hercules, CA) until the dye front reached the bottom of gel (Fryksdale *et al.* 2002). Protein molecular markers were purchased from Amersham Biosciences Corp (Piscataway, NJ, USA). IEF and 2D-PAGE were conducted three times from each protein

extraction. The proteins in the gels were stained with Commassie Brilliant Blue R-250, and images were acquired with a digital camera. Protein spots were detected and numbered with PD Quest software (Bio-Rad, Hercules, CA). The proteins on each gel were cut using a robotic digester and spot cutter (Robotic Bio-Rad proteome work stations, Ann Arbor, MI) in the Life Sciences and Biotechnology Institute (LSBI) at Mississippi State University following the procedure of Pechanova *et al.* (2008). After cutting from the gel, all proteins were digested by trypsin following the in-gel digestion method of Pechanova *et al.* (2008) using the ProPrep Robotic Digester (Genomics Solution). The digested peptides were desalted with C18 ZipTips and subjected to MALDI-TOF/TOF/MS (Matrix Assisted Laser Desorption Ionization – Time of Flight/ Mass Spectrometry, ABI 4700, Applied Biosystems, Foster City, CA) analysis.

**Identification of proteins and functional grouping**

From the mass spectral data, protein identification was automatically performed with ABI GPS Explorer software using the Result Dependent Analysis Mode. This system ran a search for each protein against the NCBInr (National Center for Biotechnology non-redundant database) using the MASCOT (version 1.8.0, Matrix Science Ltd., London, UK) search engine (Pappin *et al.* 1993). Identifications via MASCOT were performed by searching mono-isotopic peptide masses against the general bacteria, general fungi, and basidiomycete databases in NCBI. In these searches the peptide mass tolerance was set at 150 ppm, mass tolerance for fragmented ions was set to 0.2 Da allowing one missed cleavage by trypsin. Protein modifications included oxidation of methionine and carbamidomethylation of cysteine, when appropriate.

Proteins with a MASCOT high Cross Confidence Interval (C.I. % > 95) score were considered identified. Proteins that matched with a lower confidence score were considered tentative and proteins with unknown or un-confirmed function were listed as hypothetical proteins. In addition, hypothetical proteins were placed into predicted functional groups if possible by searching the NCBI Conserved Domain Database (CDD) and PDBJ (Protein Data Bank Japan) using the BLAST search engine. Functional categories were based on those listed at PDBJ. Based on protein identification, protein taxonomical grouping was performed among three wood types from each collection time and classified as bacteria, fungi, basidiomycetes or others.

## RESULTS

### Changes of decay rating, MOE, and moisture content over time

Visual decay ratings of the three wood types were significantly different at 4 months through 18 months between pine and cedar or between pine and ACQ-treated pine (Figure 4.2). There was no significant difference between cedar and ACQ -treated pine over 18 months (*alpha*=0.05).

Decrease in wood stiffness was measured by a decrease in MOE. The average % MOE loss, which is attributed to decay, was greater in pine than in cedar or ACQ-treated pine over 18 months (Figure 4.3). From 6 months through 18 months, the % MOE loss in pine was significantly greater than cedar or ACQ-treated pine. Additionally, there was no significant difference (*alpha* = 0.05) between cedar and ACQ-treated pine at any sampling date.

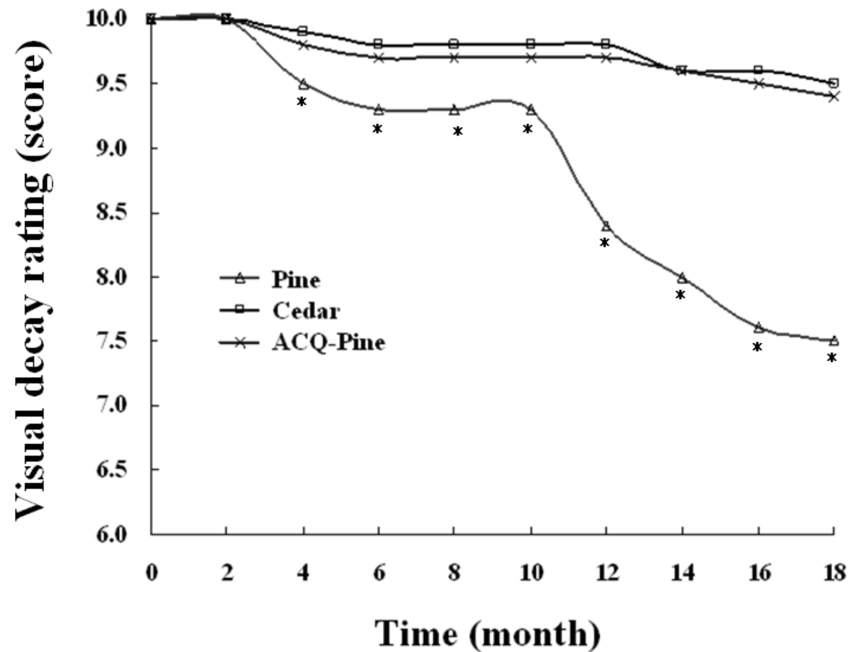



Figure 4.2. Visual decay rating during biodeterioration of three wood types over 18 months. "*" indicates that the decay rating for pine stakes were significantly different (*alpha*=0.05) from cedar and ACQ-pine stakes at the given time.

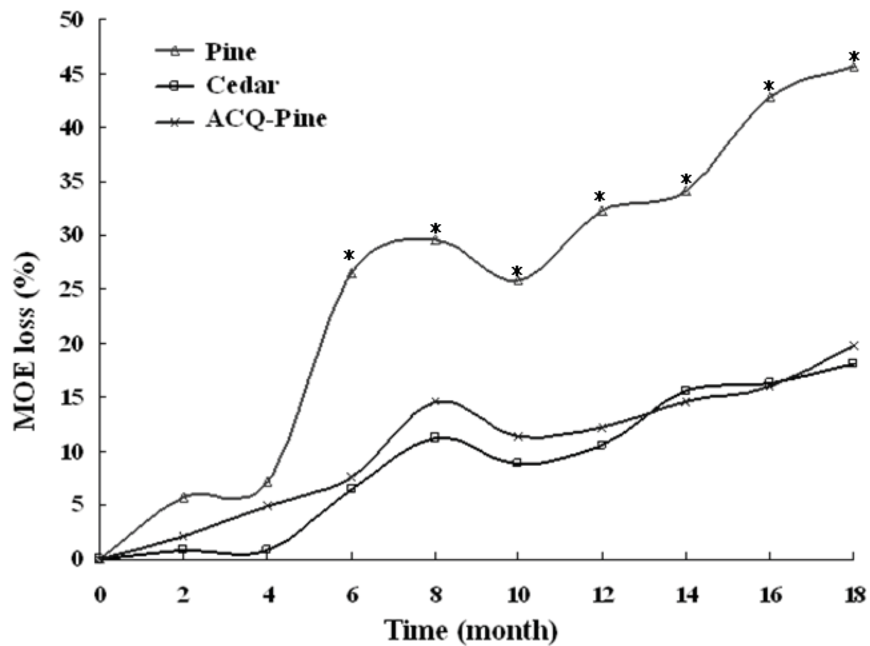



Figure 4.3. The average MOE loss (%) in pine, cedar, and ACQ treated pine over 18 months. "*" indicates that the % MOE loss in pine stakes was significantly higher (*alpha*=0.05) than cedar and ACQ-pine stakes at the given time.

## 2D -PAGE of three wood types at 18 months of decay

Three 2D gels were run for each of the three samples, untreated pine, cedar, and ACQ-treated pine at each collection date. No proteins were detected from any of the three wood types until 6 months. This is not unexpected because a critical level of microbial biomass is needed before proteins can be detected on a gel. Proteins were first detected on pine at 6 months and the numbers continued to increase through 18 months. No 2D-PAGE was performed at the 12 month sampling period for all three wood types. From pine, there were 14 protein spots at 6 months (Figure 4.4), 16 protein spots at 8 months (Figure 4.5), 29 protein spots at 10 months (Figure 4.6), 64 protein spots at 14 months (Figure 4.7), 113 protein spots at 16 months (Figure 4.8), and 124 protein spots at 18 months (Figure 4.9). The proteins on gels from 6, 8, 10, and 18 months equaling 360 proteins were selected for spot cutting and identification.

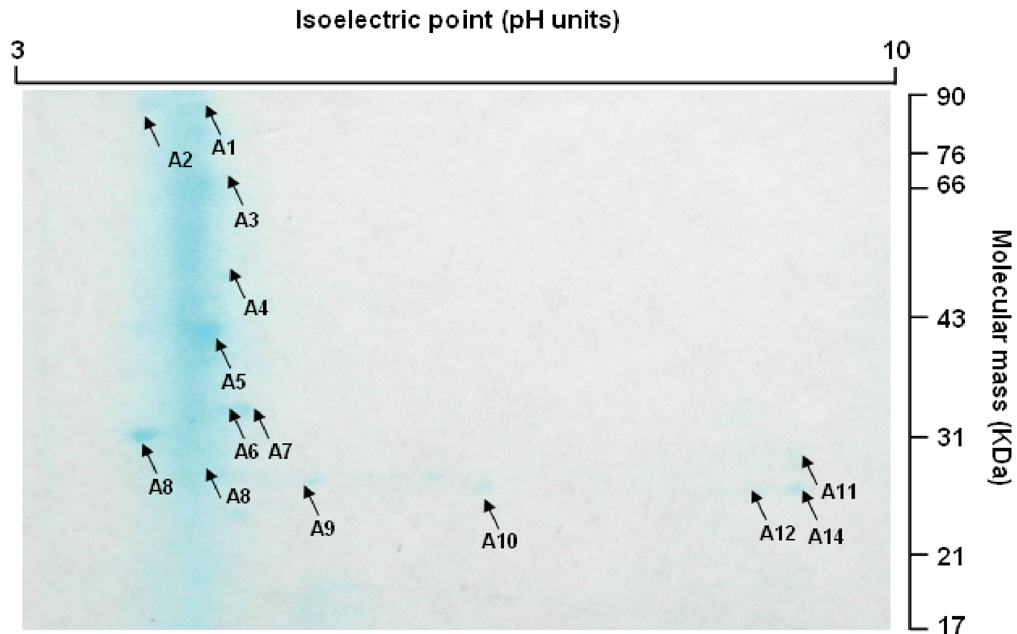



Figure 4.4. 2DE gel pattern of pine at 6 months indicating 14 proteins detected.

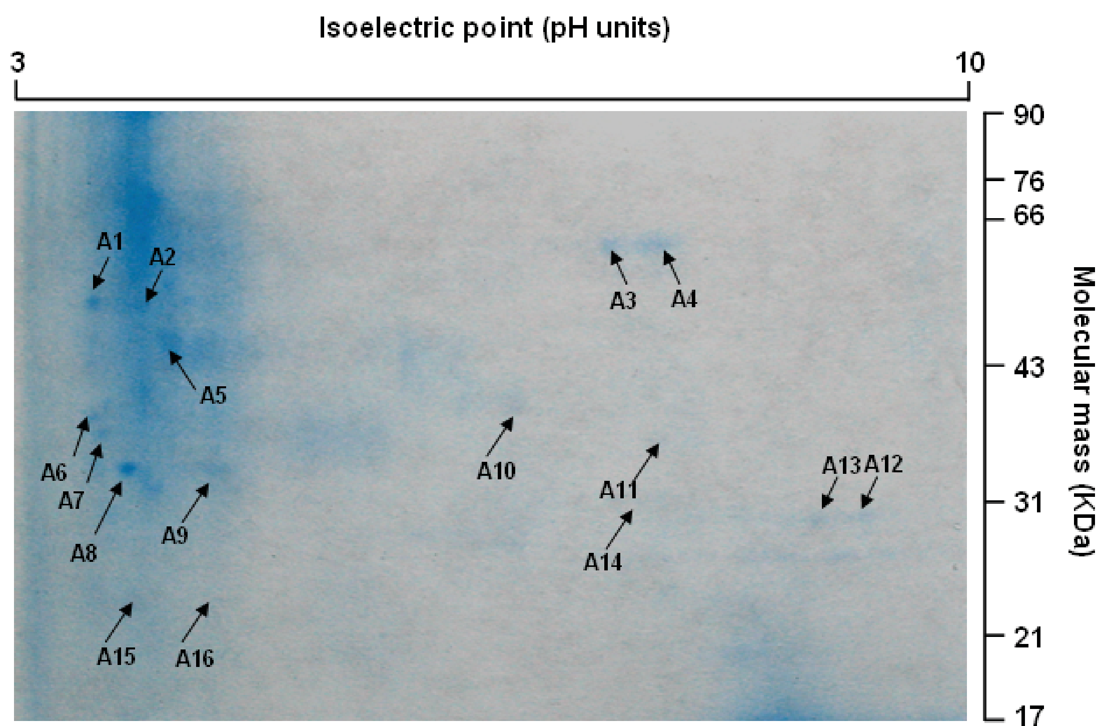



Figure 4.5. 2DE gel pattern of pine at 8 months indicating 16 proteins detected.

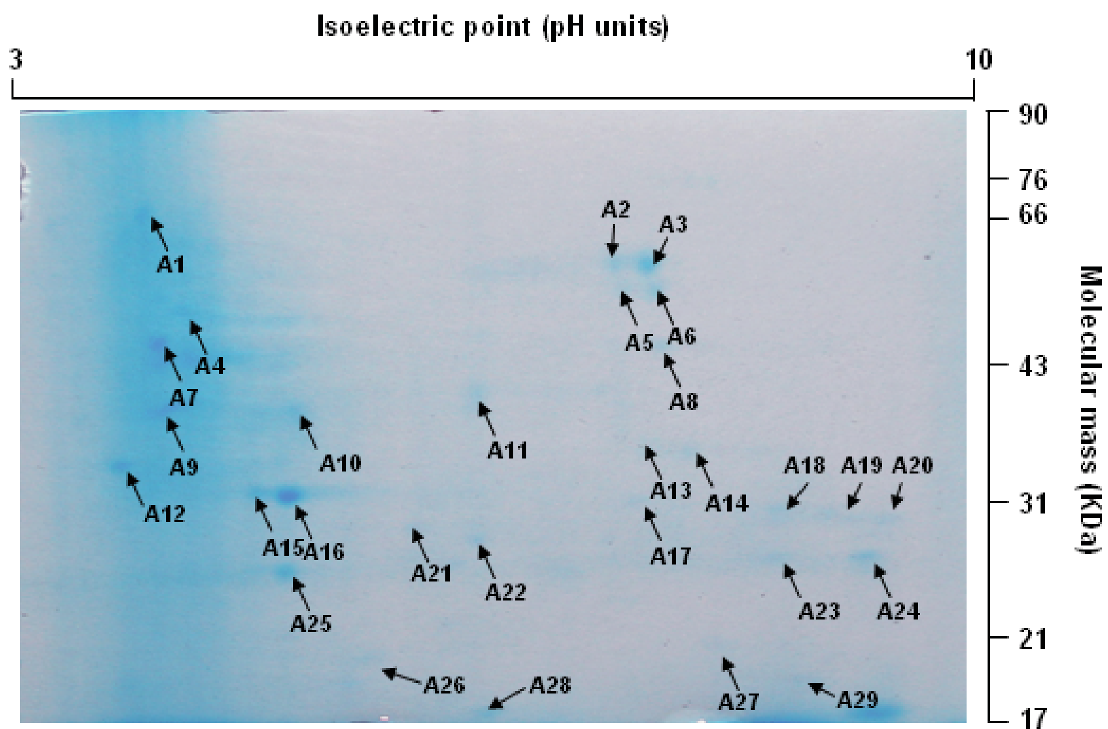



Figure 4.6. 2DE gel pattern of pine at 10 months indicating 29 proteins detected.

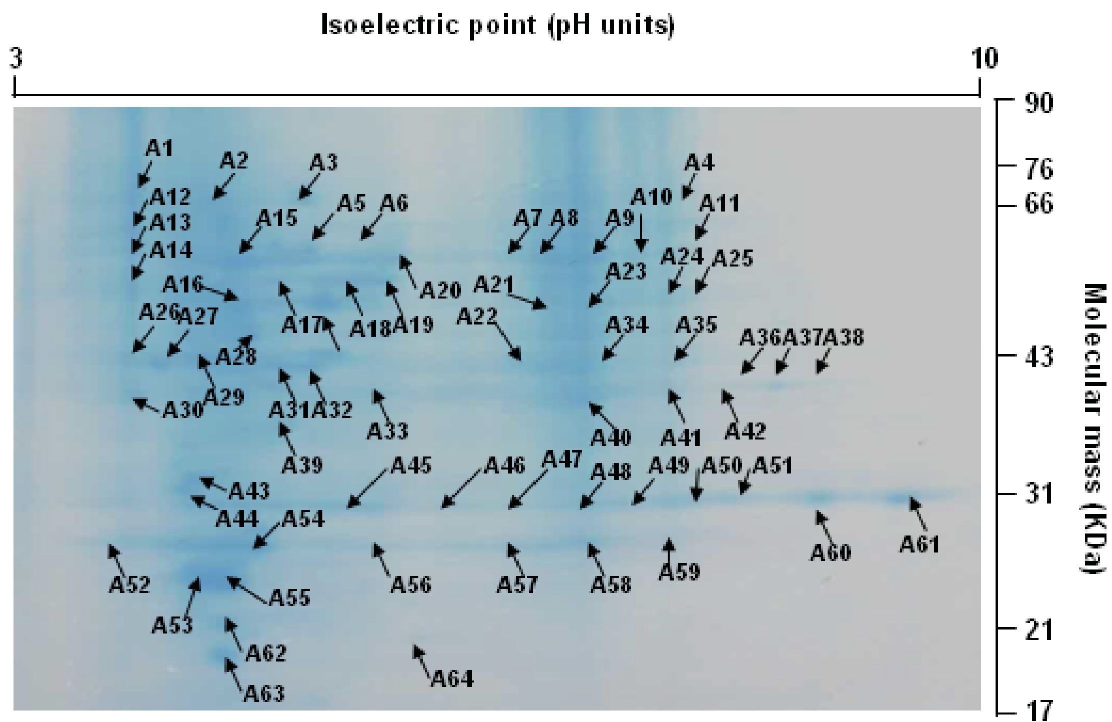



Figure 4.7. 2DE gel pattern of pine at 14 months indicating 64 proteins detected.

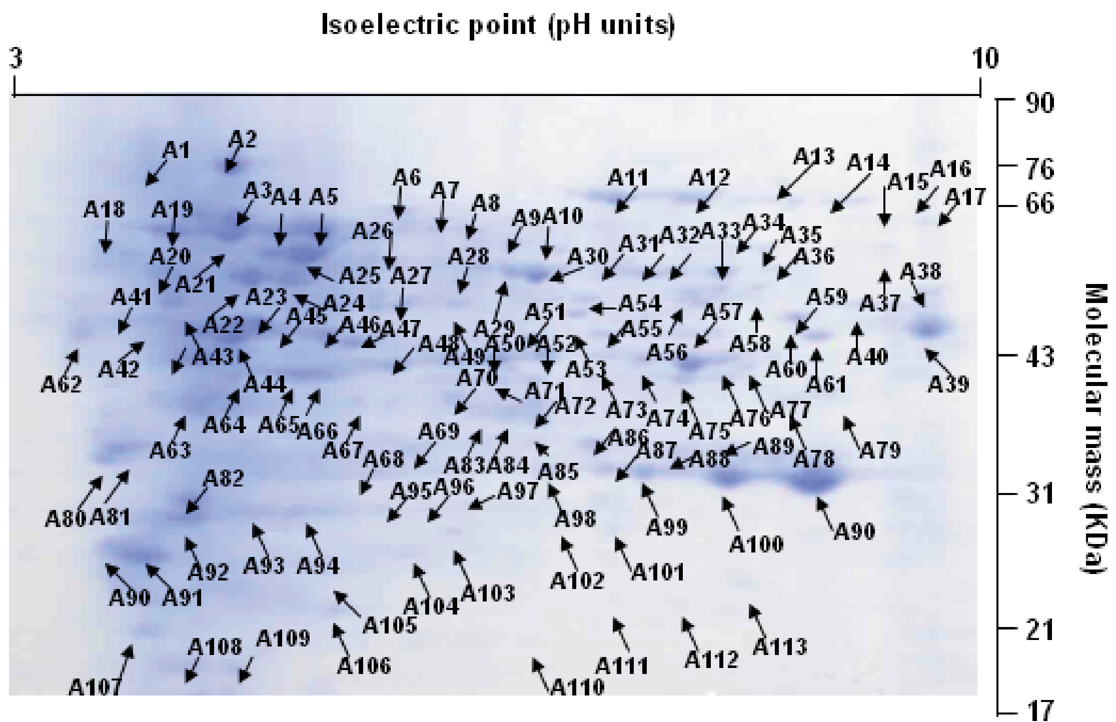



Figure 4.8. 2DE gel pattern of pine at 16 months indicating 113 proteins detected.

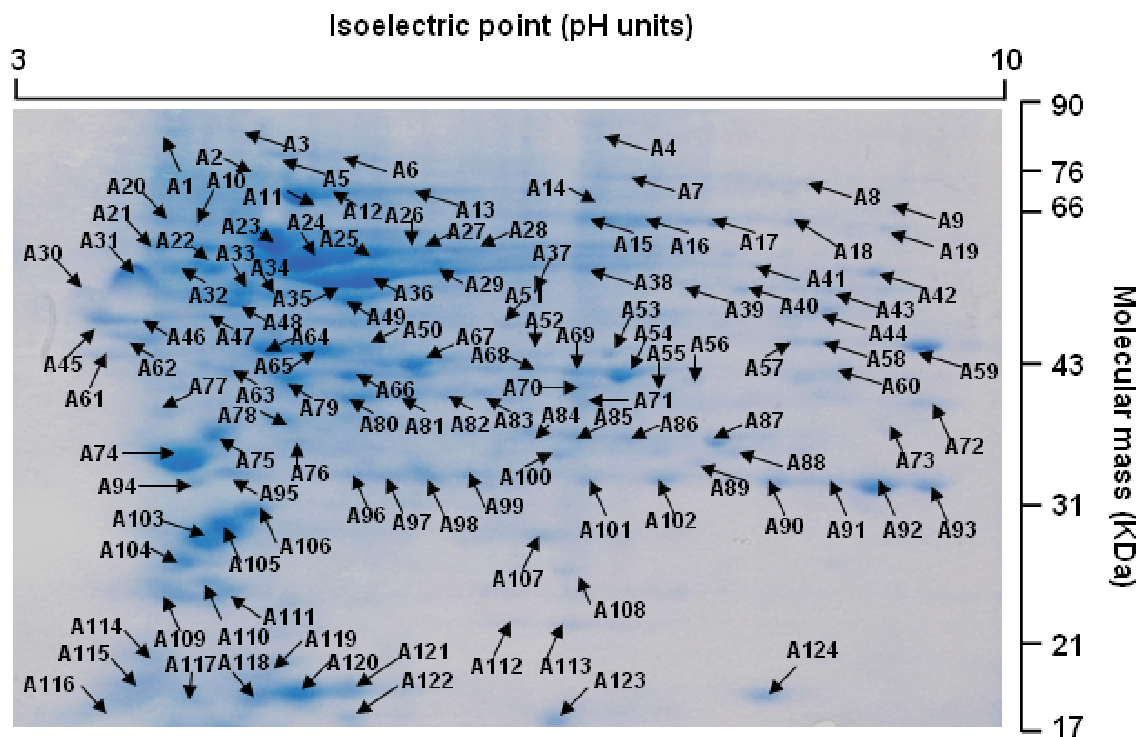



Figure 4.9. 2DE gel pattern of pine at 18 months indicating 124 proteins detected.

Proteins on the cedar were first detected at 14 months. Microorganisms could be detected on cedar before 14 months, but there was not enough biomass to detect protein spots until 14 months. At this time, fungal mycelia were visually present on the surface of cedar. From untreated cedar, three gels were run and 2 protein spots detected at 14 months (Figure 4.10), 3 protein spots at 16 months (Figure 4.11), and 16 protein spots at 18 months (Figure 4.12). The proteins on gels from 14, 16, and 18 months equaling 21 protein spots were selected for spot cutting and identification.

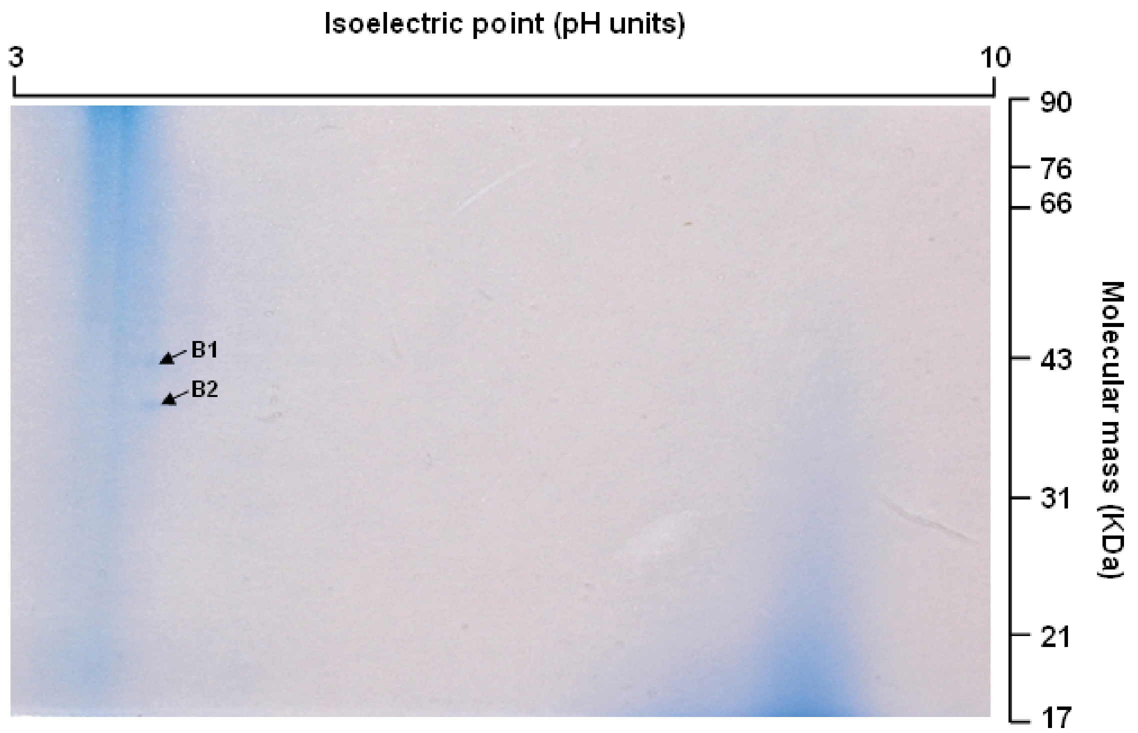



Figure 4.10. 2DE gel pattern of cedar at 14 months indicating 2 proteins detected.

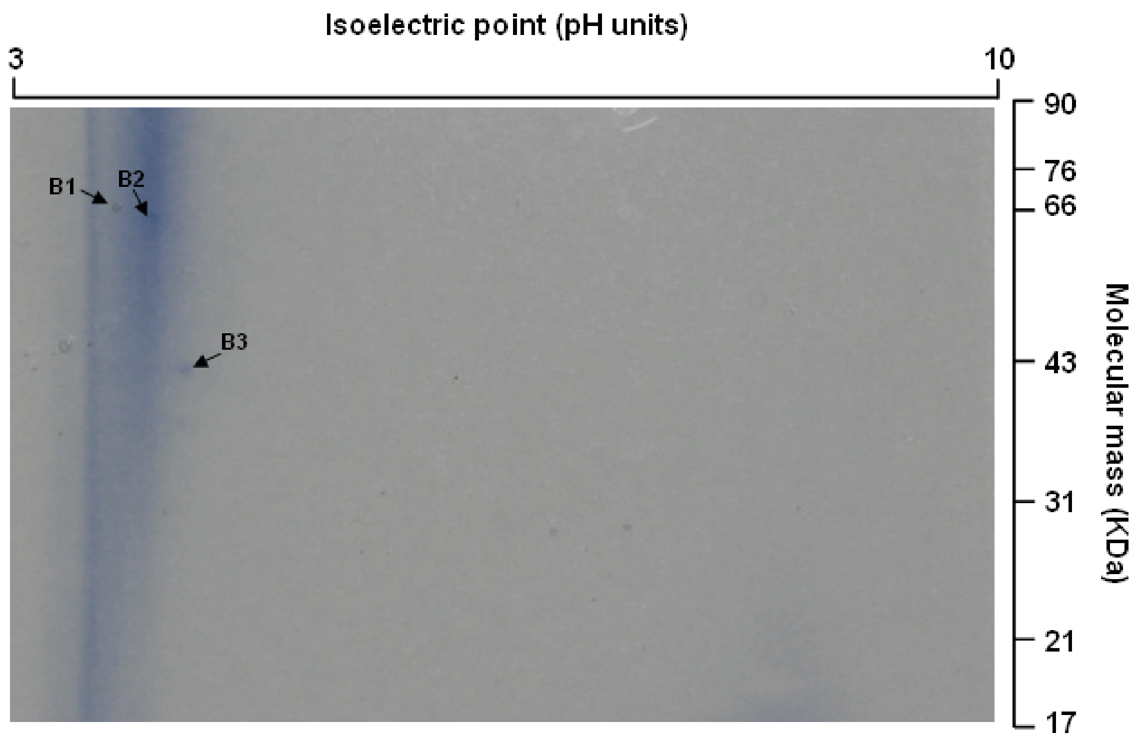



Figure 4.11. 2DE gel pattern of cedar at 16 months indicating 3 proteins detected.

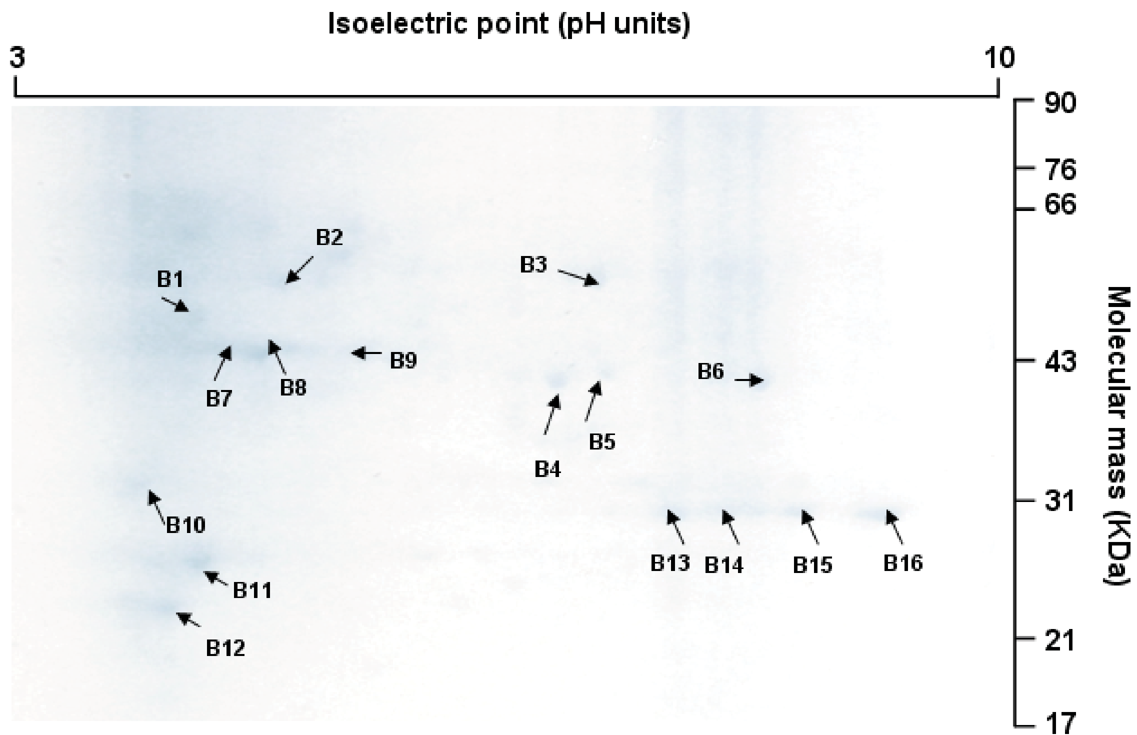



Figure 4.12. 2DE gel pattern of cedar at 18 months indicating 16 proteins detected.

Proteins were first detected on ACQ-treated pine at 6 months and the numbers continued to increase through 18 months. ACQ-treated pine produced 3 protein spots at 6 months (Figure 4.13), 6 protein spots at 8 months (Figure 4.14), 8 protein spots at 10 months (Figure 4.15), 34 protein spots at 14 months (Figure 4.16), 40 protein spots at 16 months (Figure 4.17), and 47 protein spots at 18 months (Figure 4.18). The proteins on gels from 6, 8, 10, and 18 months equaling 138 proteins were selected for spot cutting and identification.

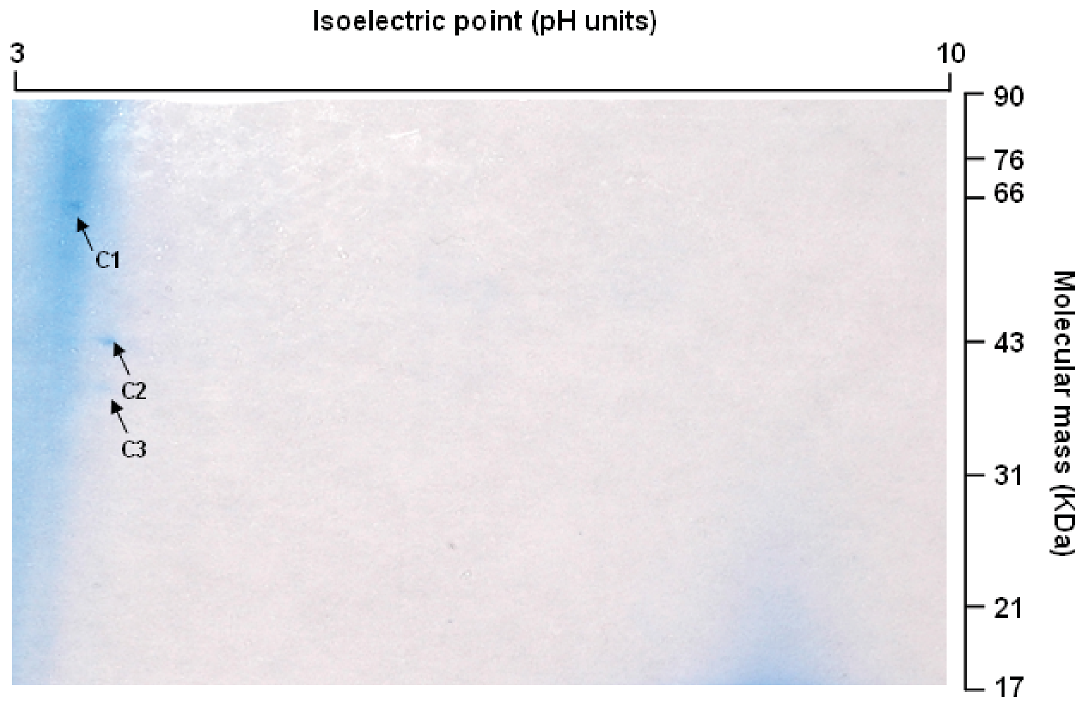



Figure 4.13. 2DE gel pattern of ACQ-treated pine at 6 months indicating 3 proteins detected.

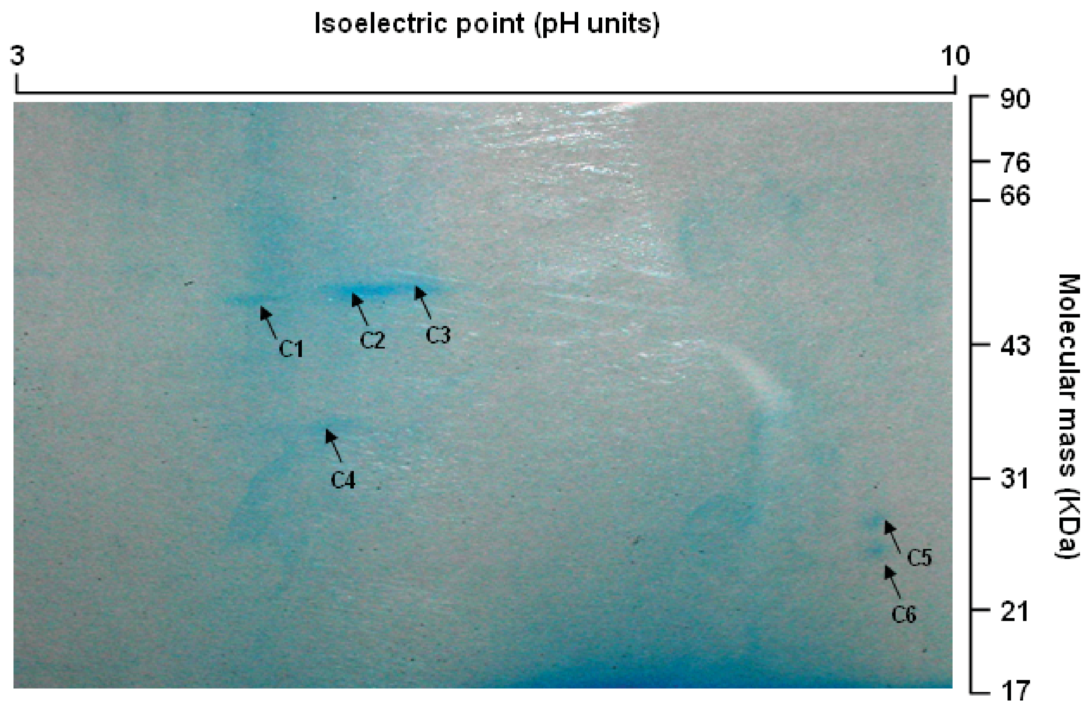



Figure 4.14. 2DE gel pattern of ACQ-treated pine at 8 months indicating 6 proteins detected.

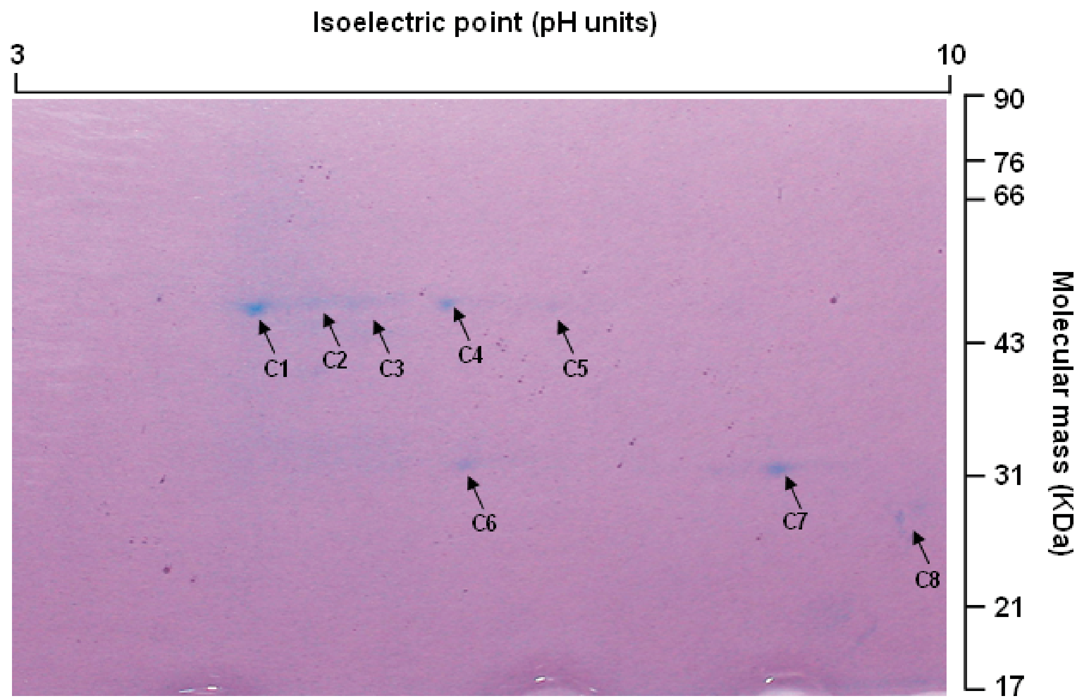



Figure 4.15. 2DE gel pattern of ACQ-treated pine at 10 months indicating 8 proteins detected.

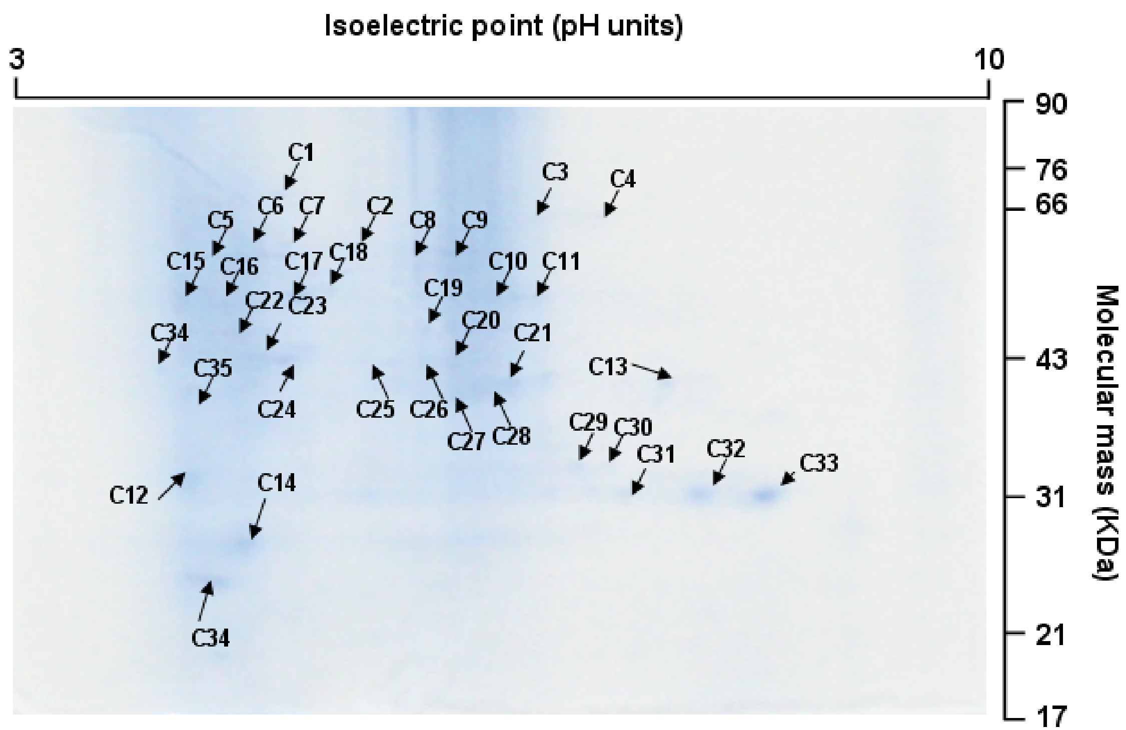



Figure 4.16. 2DE gel pattern of ACQ-treated pine at 14 months indicating 34 proteins detected.

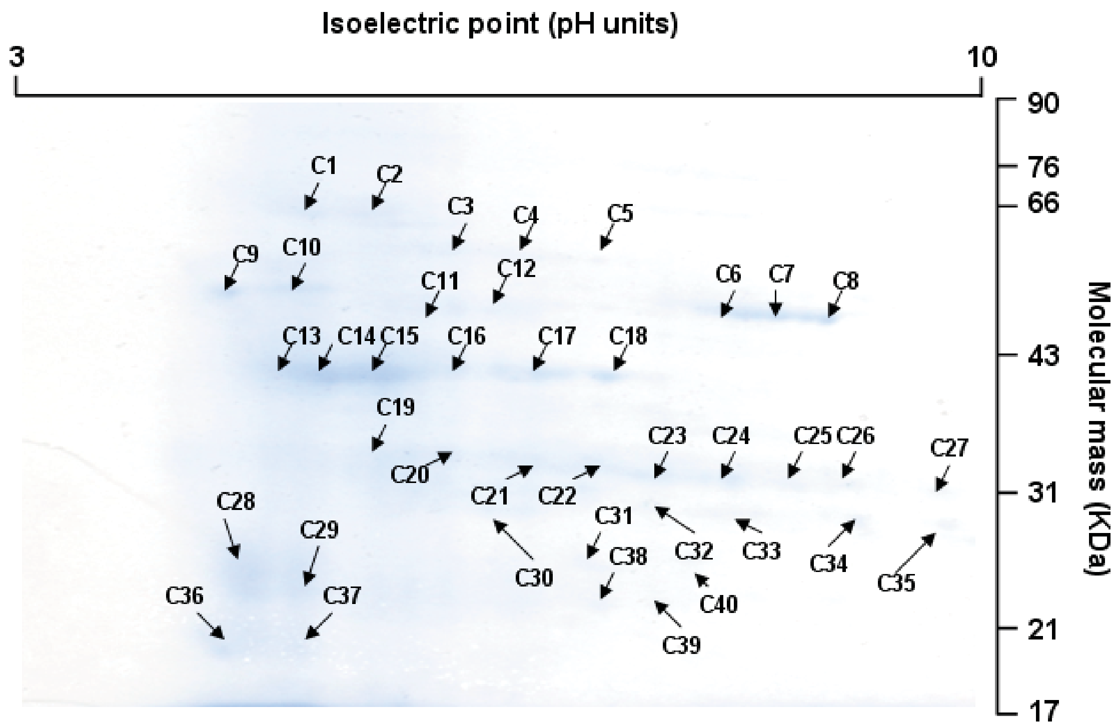



Figure 4.17. 2DE gel pattern of ACQ-treated pine at 16 months indicating 40 proteins detected.

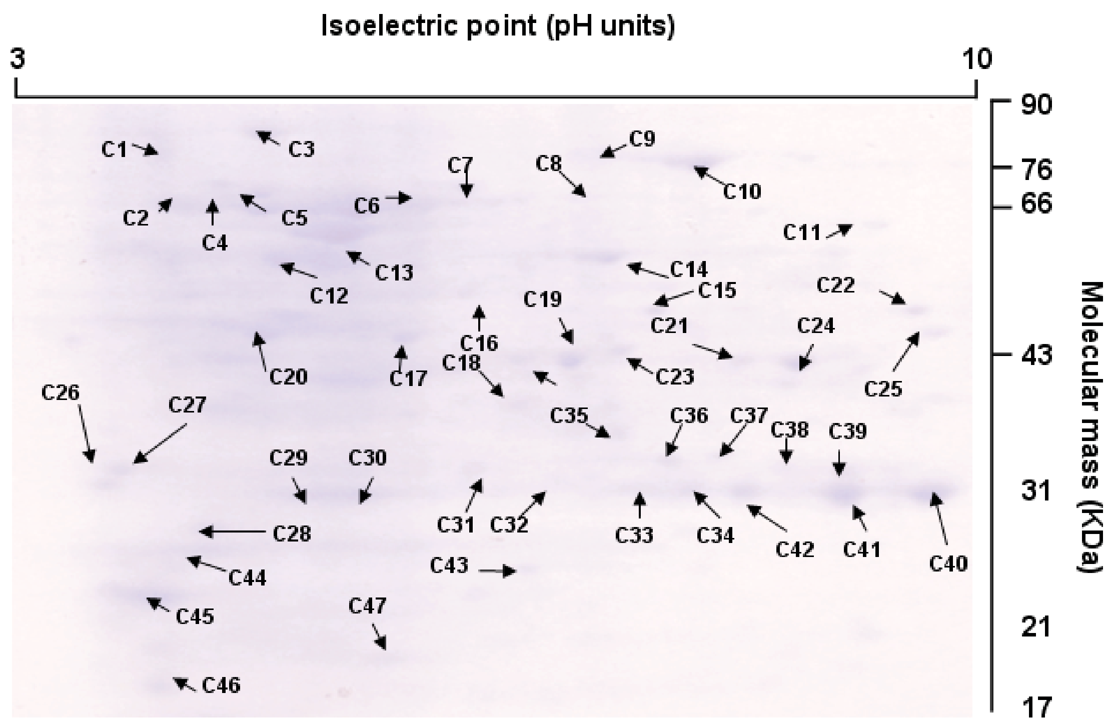



Figure 4.18. 2DE gel pattern of ACQ-treated pine at 18 months indicating 47 proteins detected.

**MALDI-TOF/TOF/MS analysis and protein identification**

The tryptic peptide products from the 2DE gels were analyzed by MALDI-TOF/TOF/MS and the identified and tentatively identified proteins are listed in Tables 4.2 through Table 4.12. Proteins were identified from basidiomycetes, fungi, and bacteria in NCBI known databases. Proteins with a MASCOT high cross confidence interval (C.I. % > 95) score were considered identified. Proteins that were matched with a lower confidence score were considered tentative.

From untreated pine at 6 months, a total of 14 proteins were identified and only 2 proteins matched a protein score greater than 95% (Table 4.2). These were two cellulose 1,4-beta cellobiosidases from *Pleurotus* sp. in the basidiomycete database. This enzyme acts on β-1,4-linked glucose units of cellulose.

Table 4.2. Results of BLAST homology search of protein identification from pine samples at 6 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| A1 (6m) | 0 | a 1-2 protein - inky cap (*Coprinus cinereus*) | S52540 | 547908 | 9.82 |
| A2 (6m) | 0 | acyl-CoA dehydrogenase (*Streptomyces avermitilis*) | NP_823091 | 44099.3 | 5.51 |
| A3 (6m) | 0 | ComE operon protein 4 (*Bacillus cereus*) | ZP_00240008 | 29608.3 | 6.00 |
| A4 (6m) | 0 | metallo-beta-lactamase family protein, putative (uncultured marine bacterium) | AAR38150 | 35888.1 | 5.60 |

Table 4.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A5 (6m) | 0 | cytochrome oxidase subunit 2 (*Trimorphomyces papilionaceus*) | CAA52099 | 12476.3 | 4.97 |
| A6 (6m) | 0 | methyltransferase (*Schizosaccharomyces pombe*) | NP_588543 | 29670.1 | 5.82 |
| A7 (6m) | 99.99 | cellulose 1,4-beta-cellobiosidase precursor (*Pleurotus* sp.) | CAK18800 | 55465.4 | 4.69 |
| A8 (6m) | 99.58 | cellulose 1,4-beta-cellobiosidase precursor (*Pleurotus* sp.) | CAK18800 | 55465.4 | 4.69 |
| A9 (6m) | 0 | diaminopimelate epimerase (*Burkholderia mallei*) | YP_104734 | 30879.6 | 5.99 |
| A10 (6m) | 0 | chitinase (uncultured bacterium) | AAV39439 | 31548.1 | 4.81 |
| A11 (6m) | 0 | NADH dehydrogenase type 1 subunit (uncultured bacterium) | AAC44353 | 27484.3 | 7.63 |
| A12 (6m) | 0 | HD domain-containing protein (*Geobacter sulfurreducens*) | NP_952736 | 20324.7 | 6.55 |
| A13 (6m) | 0 | alkaline serine protease (*Bionectria ochroleuca*) | AAO65478 | 24806.9 | 10.4 |
| A14 (6m) | 0 | glycosyl transferase, group 1 family protein (*Desulfovibrio vulgaris*) | YP_009094 | 49916.5 | 9.69 |

At 8 months, a total of 16 proteins were identified and the highest protein score was 100% beta-glucosidase-like protein of *Magnaporthe grisea* from the fungi database. This protein is a very common enzyme in glucose metabolism. One protein had 78%

protein score as a mitochondrial precursor from *Gibberella zeae* (Table 4.3). This enzyme assists ATP transfer in mitochondria.

Table 4.3. Results of BLAST homology search of protein identification from pine samples at 8 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| A1 (8m) | 100 | beta-glucosidase-like protein (*Magnaporthe grisea*) | AAX07690 | 94605.8 | 5.30 |
| A2 (8m) | 0 | glycosyl transferase family protein (*Prochlorococcus marinus*) | NP_895691 | 27502.5 | 9.95 |
| A3 (8m) | 0 | Activator 140 kDa subunit (*Cryptococcus neoformans*) | AAW44676 | 38575 | 6.52 |
| A4 (8m) | 0 | mitochondrial matrix chaperone Hsp78 (predicted) (*Schizosaccharomyces pombe*) | NP_596117 | 90098.7 | 7.16 |
| A5 (8m) | 0 | NADPH nitrite reductase (*Laccaria bicolor*) | XP_001887050 | 118850.1 | 6.17 |
| A6 (8m) | 0 | hobase (*Ustilago maydis*) | ABD17352 | 168051.9 | 9.00 |
| A7 (8m) | 0 | Glutamyl tRNA Synthetase (*Encephalitozoon cuniculi*) | NP_584646 | 73917.2 | 6.73 |
| A8 (8m) | 0 | leucyl aminopeptidase (*Bartonella quintana*) | YP_031901 | 49929.8 | 7.78 |

Table 4.3(continued)

| | | | | | |
|---|---|---|---|---|---|
| A9 (8m) | 0 | ribosomal protein S3 (Podospora anserine) | NP_074911 | 53749.6 | 10.26 |
| A10 (8m) | 78 | ATP synthase beta chain, mitochondrial precursor (*Gibberella zeae*) | EAU88709 | 54851.6 | 5.40 |
| A11 (8m) | 0 | HD domain-containing protein (*Geobacter sulfurreducens*) | NP_952736 | 20324.7 | 6.55 |
| A12 (8m) | 0 | farnesyltranstransferase (*Cryptococcus neoformans*) | XP_572774 | 36830.9 | 5.37 |
| A13 (8m) | 0 | DNA polymerase IV (*Lactococcus lactis*) | NP_268186 | 40413.2 | 6.73 |
| A14 (8m) | 0 | topoisomerase I (*Cryptococcus bacillisporus*) | BAB72193 | 31141.7 | 9.52 |
| A15 (8m) | 0 | putative 3B (*Simian enterovirus*) | AAA35308 | 40913.6 | 10.46 |
| A16 (8m) | 0 | Fructose-1,6-bisphosphatase (*Nostoc* sp.) | NP_488061 | 38494.4 | 5.10 |

At 10 months, a total of 29 proteins were identified and methanol dehydrogenase from *Methylococcus capsulatus* matched with 99% score from bacteria database (Table 4.4). This enzyme is a methanol NAD+ oxidoreductase (Saham 1977). Methanol dehydrogenase produced by bacteria and fungi reduces $O_2$ to $H_2O_2$ during wood decay (Saham 1977). Methanol dehyrogenase catalyzes the oxidation reaction of methanol to formaldehyde with subsequent reduction of oxygen to water. Beta-glucosidase-like protein from *Magnaporthe grisea* matched with 76% score from fungi database. An

endoxylanase from *Cellvibrio mixtus* matched with 0% score from the bacteria database. Endoxylanase is a hemicellulose degrading enzyme.

Table 4.4. Results of BLAST homology search of protein identification from pine samples at 10 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| A1 (10m) | 0 | HD domain-containing protein (*Geobacter sulfurreducens*) | NP_952736 | 20324.7 | 6.55 |
| A2 (10m) | 99.99 | methanol dehydrogenase protein, large subunit (*Methylococcus capsulatus*) | YP_112833 | 67563.4 | 5.94 |
| A3 (10m) | 0 | hesp-417-like protein (*Melampsora occidentalis*) | ABS86214 | 2101 | 5.91 |
| A4 (10m) | 3.4 | maltose-binding protein (*Burkholderia pseudomallei*) | YP_109207 | 44273.8 | 9.15 |
| A5 (10m) | 0 | endoxylanase (*Cellvibrio mixtus*) | AAD09439 | 42886 | 7.22 |
| A6 (10m) | 0 | chaperone/heat shock protein (*Emericella nidulans*) | AAB69701 | 8825.4 | 6.56 |
| A7 (10m) | 0 | kinesin domain-containing protein (*Laccaria bicolor*) | EDR14885 | 224223.6 | 5.49 |
| A8 (10m) | 0 | pyridoxal reductase (*Schizosaccharomyces pombe*) | NP_594584 | 36791.1 | 6.77 |
| A9 (10m) | 0 | 30S ribosomal protein S8 (*Clostridium perfringens*) | NP_563307 | 14584.9 | 9.48 |
| A10 (10m) | 0 | transposase for insertion sequence element (*Lactobacillus delbrueckii*) | AAQ06907 | 7767.9 | 6.81 |

Table 4.4 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A11 (10m) | 76.43 | beta-glucosidase-like protein (*Magnaporthe grisea*) | AAX07690 | 94605.8 | 5.30 |
| A12 (10m) | 0 | macrolide-binding protein (*Cryptococcus neoformans*) | XP_569051 | 14644.6 | 9.34 |
| A13 (10m) | 0 | argininosuccinate lyase (*Laccaria bicolor*) | EDR14244 | 51834.6 | 5.66 |
| A14 (10m) | 0 | orotidine 5'-phosphate decarboxylase (*Xanthomonas oryzae*) | YP_198886 | 25486.3 | 5.48 |
| A15 (10m) | 0 | carboxy-lyase UbiD-like protein (*Candida albicans*) | XP_718068 | 57551.3 | 5.84 |
| A16 (10m) | 0 | 50S ribosomal protein (*Staphylococcus epidermidis*) | NP_765366 | 20222.8 | 9.20 |
| A17 (10m) | 0 | isopentenyl-diphosphate delta-isomerase, putative (*Cryptococcus neoformans*) | AAW40822 | 29840 | 4.95 |
| A18 (10m) | 0 | ABC transporter ATP-binding protein (*Fusobacterium nucleatum*) | NP_603777 | 38931.6 | 7.62 |
| A19 (10m) | 0 | likely mitochondrial ribosome protein S18 (*Candida albicans*) | XP_713862 | 24441 | 10.00 |
| A20 (10m) | 0 | isopentenyl-diphosphate delta-isomerase (*Cryptococcus neoformans*) | XP_566641 | 29840 | 4.95 |
| A21 (10m) | 0 | HD domain-containing protein (*Geobacter sulfurreducens*) | NP_952736 | 20324.7 | 6.55 |
| A22 (10m) | 0 | enoyl reductase (*Schizosaccharomyces pombe*) | NP_595365 | 34617.6 | 10.20 |

Table 4.4 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A23 (10m) | 0 | tRNA pseudouridine synthase (*Sinorhizobium meliloti*) | NP_384527 | 27709.2 | 8.93 |
| A24 (10m) | 0 | 50S ribosomal protein L29 (*Rhodopseudomonas palustris*) | NP_948581 | 7976.2 | 10.61 |
| A25 (10m) | 0 | Malic enzyme (*Neocallimastix frontalis*) | P78715 | 65503.5 | 6.44 |
| A26 (10m) | 0 | tyrosine type recombinase (*Bacteroides fragilis*) | YP_100318 | 30749 | 10.18 |
| A27 (10m) | 0 | enoyl reductase (*Schizosaccharomyces pombe*) | NP_595365 | 34617.6 | 10.20 |
| A28 (10m) | 0 | Dehydrogenases (*Magnetospirillum magnetotacticum*) | ZP_00049915 | 22704.4 | 6.32 |
| A29 (10m) | 0 | macrolide-binding protein (*Cryptococcus neoformans*) | AAD16171 | 11600.9 | 5.69 |

There is no data for 12 months because 2D-PAGE was not performed. At 14 months and 16 months, 64 proteins and 113 proteins were detected, respectively although these samples were not analyzed due to costs. At 18 months, 124 proteins were detected (Table 4.5). Sample A109 failed to provide any meaningful spectrum and is therefore removed from the results. Aconitate hydratase, matched 100% matched to *Laccaria bicolor*, is a large complex enzyme with multiple subunits involved in the citric acid cycle (Nelson and Cox. 2005). Additionally, fructose 1,6-bisphosphate of *Laccaria bicolor* was also matched with a 100% protein score. This enzyme is in the glycolysis

metabolic pathway and is produced by phosphorylation of fructose 6-phosphate. It is in turn broken down into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Many forms of fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate were identified from pine at 18 months. There was a greater number of high protein matches at 18 months, compared to the other sampling dates.

Table 4.5. Results of BLAST homology search of protein identification from pine samples at 18 months.

| **Sample Name** | **Protein Score (%)** | **Protein Name** | **Accession Number** | **MW** | **pI** |
|---|---|---|---|---|---|
| A1 (18m) | 92.32 | hypothetical protein (*Malassezia globosa*) | 164662098 | 34457.2 | 6.61 |
| | 75 (similarity) | pyruvate dehydrogenase (*Cryptococcus neoformans*) | | | |
| A2 (18m) | 100 | predicted protein (*Laccaria bicolo*r) | 170087590 | 89973.6 | 4.92 |
| | 98 (similarity) | cell division control protein (*Neosartorya fischeri* ) | | | |
| A3 (18m) | 100 | hypothetical protein (*Coprinopsis cinerea*) | 16984894 | 89678.5 | 4.91 |
| | 61 (similarity) | endopeptidase (*Cryptococcus neoformans*) | | | |
| A4 (18m) | 100 | aconitate hydratase (*Laccaria bicolor*) | 170094674 | 82291.7 | 6.02 |
| A5 (18m) | 100 | aconitate hydratase (*Laccaria bicolor*) | 170094674 | 82291.7 | 6.02 |
| A6 (18m) | 100 | predicted protein (*Laccaria bicolor*) | 170084893 | 84352.1 | 6.22 |
| | 100 (similarity) | methyltransferase (*Cryptococcus neoformans*) | | | |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A7 (18m) | 0 | ParaA family ATPase (*Bacteroides sp*) | 262384045 | 28405.1 | 5.14 |
| A8 (18m) | 100 | predicted protein (*Laccaria bicolor*) | 170087204 | 71129.2 | 5.12 |
| | 99 (similarity) | heat shock protein 70 (*Cryptococcus curvatus*) | | | |
| A9 (18m) | 100 | heat shock protein (*Saccharomyces cerevisiae*) | 6320950 | 69608.6 | 5.03 |
| A10 (18m) | 100 | chaperone (*Cryptococcus neoformans)* | 58264778 | 69575.3 | 5.04 |
| A11 (18m) | 99.604 | molecular chaperone DnaK (*Nostoc punctiforme*) | 186685947 | 68115.2 | 5.06 |
| A12 (18m) | 100 | heat shock protein (*Cryptococcus neoformans*) | 58262484 | 88062.4 | 5.48 |
| A13 (18m) | 100 | chaperone (*Cryptococcus neoformans)* | 58264778 | 69575.3 | 5.04 |
| A15 (18m) | 100 | predicted protein (*Laccaria bicolor*) | 170097912 | 71650.2 | 5.7 |
| | 99 (similarity) | heat shock protein (*Cryptococcus neoformans*) | | | |
| A16 (18m) | 99.99 | predicted protein (*Laccaria bicolo*r) | 170094206 | 68050.6 | 5.46 |
| | 100 (similarity) | endodeoxyribonuclease (*Cryptococcus neoformans)* | | | |
| A17 (18m) | 79.32 | hypotheticalprotein (*Moniliophthora perniciosa*) | 238599737 | 39882.4 | 7.74 |
| | 95 (similarity) | RNA-directed RNA polymerase (*Laccaria bicolor*) | | | |
| A18 (18m) | 0 | conserved hypothetical protein (*Prevotella bergensis*) | 270339888 | 5488.9 | 8.15 |
| A19 (18m) | 100 | fumarate reductase (*Laccaria bicolor*) | 170097978 | 62652.3 | 7.71 |
| A20 (18m) | 89.87 | predicted protein (*Postia placenta*) | 242211502 | 60478.5 | 10.16 |
| | 14 (similarity) | apocytochrome b (*Ustilago maydis*) | | | |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A21 (18m) | 0 | ATP-binding protein (*Lactobacillus gasser*i) | 282851459 | 32780.3 | 8.7 |
| A22 (18m) | 100 | chaperone (*Cryptococcus neoformans*) | 58264778 | 69575.3 | 5.04 |
| A23 (18m) | 99.99 | heat shock protein (*Cryptococcus neoformans*) | 58258689 | 67085.5 | 5.37 |
| A24 (18m) | 98.77 | SNF2 family helicase/ATPase (*Talaromyces stipitatus*) | 242793727 | 189138.8 | 9.29 |
| A25 (18m) | 0 | GTP pyrophosphokinase (*Aggregatibacter actinomycetemcomitans*) | 261867309 | 84374.3 | 6.29 |
| A26 (18m) | 0 | peptidase, U32 family (S*elenomonas sputigena*) | 260887348 | 47530.7 | 5.7 |
| A27 (18m) | 87.25 | pyruvate kinase (*Laccaria bicolor*) | 170086043 | 57833.9 | 6.29 |
| A28 (18m) | 95.10 | KLTH0A02618p (*Lachancea thermotolerans*) | 255710575 | 66964.6 | 9.57 |
| A29 (18m) | 78.76 | Conserved hypothetical protein (*Prevotella melaninogenica )* | 288803574 | 17548.2 | 7.77 |
| | 29 (Similarity) | DNA topoisomerase II (*Pleurotus ostreatus*) | | | |
| A30 (18m) | 99.64 | tubulin beta chain (*Coprinopsis cinerea*) | 169863355 | 49882.2 | 4.79 |
| A31 (18m) | 0 | beta-tubulin (*Laccaria bicolor)* | 170104184 | 50017.3 | 4.79 |
| A32 (18m) | 99.99 | tubulin beta chain (*Coprinopsis cinerea okayama7*) | 169863355\| | 49882.2 | 4.79 |
| A33 (18m) | 52.44 | glycosyl transferase (*bacterium Ellin*) | 223934770 | 44382.3 | 9.44 |
| A34 (18m) | 98.38 | 26S proteasome regulatory protein (*Candida Dubliniensis)* | 241954580 | 61703.1 | 7.95 |
| A35 (18m) | 66.47 | ATP synthase beta chain (*Coprinopsis cinerea*) | 169852676 | 57891.2 | 5.58 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A36 (18m) | 0 | N-terminal acetyltransferase (*Aspergillus flavus*) | 238486130 | 94817.3 | 6.73 |
| A37 (18m) | 0 | fatty acid desaturase (*Ruegeria pomeroyi*) | 56697186 | 37678.4 | 9.28 |
| A38 (18m) | 100 | mitochondrial processing peptidase (*Uncinocarpus reesii* ) | 258578259 | 53005 | 5.79 |
| A39 (18m) | 95.56 | 23S rRNA (guanosine-2-O-) -methyltransferase (*Photobacterium damselae*) | 269103599 | 26639 | 6.97 |
| A40 (18m) | 100<br>99 (similarity) | predicted protein (*Laccaria bicolo* )<br>t-complex protein (*Cryptococcus neoformans*) | 170097289 | 56782.8 | 5.55 |
| A41 (18m) | 99.55 | NAD-aldehyde dehydrogenase (*Laccaria bicolor*) | 170091726 | 53866.4 | 5.61 |
| A42 (18m) | 95.45 | alanine--tRNA ligase (*Leptotrichia hofstadii*) | 260891458 | 98704.4 | 5.12 |
| A43 (18m) | 31.268 | IstB domain protein ATP-binding protein *(Dehalococcoides sp)* | 269920211 | 43759.2 | 6.97 |
| A44 (18m) | 99.932 | acetylglutamate kinase (*Laccaria bicolor*) | 170089063 | 96861.1 | 9.06 |
| A45 (18m) | 0 | transposase family protein (*Brevibacterium linens*) | 260905857 | 50057.9 | 9.33 |
| A46 (18m) | 97.50 | light-independent protochlorophyllide reductase, (*Rhodopseudomonas palustris*) | 283842419 | 33806.3 | 5.72 |
| A47 (18m) | 99.99 | translation elongation protein (*Cryptococcus neoformans*) | 58262104 | 50247.3 | 9.1 |
| A48 (18m) | 0 | glycosyltransferase family 32 protein *(Laccaria bicolor*) | 170086041 | 87542.6 | 9.45 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A49 (18m) | 92.76 | acyl-CoA:6-aminopenicillanic-acid-acyltransferase (*Ajellomyces dermatitidis*) | 261192468 | 38784.8 | 5.39 |
| A50 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolor)* | 170085829 | 47209.6 | 5.55 |
| A51 (18m) | 0 | cellulose synthase subunit (*Escherichia coli*) | 215488810 | 99685.4 | 9.54 |
| A52 (18m) | 99.99 | mitochondrial processing peptidase (*Aspergillus flavus*) | 238488967 | 53138 | 5.48 |
| A53 (18m) | 0 | RNA ligase (*Pyramidobacter piscolens*) | 282855979 | 20953 | 9.87 |
| A54 (18m) | 100 | ATP synthase (*Coprinopsis cinerea*) | 169852676 | 57891.2 | 5.58 |
| A55 (18m) | 100 | ATP synthase (*Coprinopsis cinerea*) | 169852676 | 57891.2 | 5.58 |
| A56 (18m) | 100 | ATP synthase (*Coprinopsis cinerea*) | 169852676 | 57891.2 | 5.58 |
| A57 (18m) | 29.66 | short-chain dehydrogenase (*Acidovoraxavenae subsp*) | 270492556 | 28131.5 | 6.38 |
| A58 (18m) | 29.95 | chitin synthase 5 (*Ustilago maydis*) | 71014456 | 137203.3 | 29.9 |
| A59 (18m) | 0 | pyruvate dehydrogenase (*Azospirillum sp*) | 288961016 | 36145.1 | 5.52 |
| A60 (18m) | 100 | actin (*Coprinopsis cinerea*) | 169865532 | 41585.9 | 5.3 |
| A61 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolor*) | 170085829 | 47209.6 | 5.55 |
| A62 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolo*r) | 170085829 | 47209.6 | 5.55 |
| A63 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolo*r) | 170085829 | 47209.6 | 5.55 |
| A64 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolor*) | 170085829 | 47209.6 | 5.55 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A65 (18m) | 100 | phosphoglycerate kinase (*Laccaria bicolor*) | 170094220 | 44624.5 | 5.66 |
| A66 (18m) | 99.84 | predicted protein (*Postia placenta*) | 242208024 | 26752.8 | 5.52 |
| A67 (18m) | 100 | 26S proteasome (*Laccaria bicolor*) | 170107177 | 44502.6 | 9.02 |
| A68 (18m) | 100 | glutamine synthetase (*Laccaria bicolor*) | 170091848 | 39254.2 | 5.95 |
| A69 (18m) | 0 | Transposase (*Lactobacillus crispatus*) | 262048049 | 31284.8 | 10.26 |
| A70 (18m) | 94.01 | SmpA/OmlA domain protein (*Zymomonas mobilis*) | 283856577 | 16030.1 | 8.85 |
| A71 (18m) | 99.62 | ketol-acid reductoisomerase (*Uncinocarpus reesii*) | 258564795 | 44769.9 | 9.18 |
| A72 (18m) | 45.63 | glutamine synthetase (*Laccaria bicolor*) | 170091848 | 39254.2 | 5.95 |
| A73 (18m) | 100 | Ketol-acid reductoisomerase (*Talaromyces stipitatus*) | 242822237 | 44035.7 | 9.2 |
| A74 (18m) | 69.29 | hypothetical protein (*Candidatus Desulforudis*) | 169831751 | 33433.4 | 5.58 |
| A75 (18m) | 93.87 | DNA gyrase (*Slackia exigua*) | 269216532 | 90744.4 | 6.31 |
| A76 (18m) | 0 | low molecular weight protein (Vibrio coralliilyticus) | 260777111 | 16854.6 | 5.63 |
| A77 (18m) | 86.21 | glutaminyl-tRNA synthetase (*Uncinocarpus reesii*) | 258573053 | 72984.8 | 6.61 |
| A78 (18m) | 100 | Fructose 1,6-bisphosphate aldolase (*Laccaria bicolor*) | 170106499 | 38915.8 | 5.54 |
| A79 (18m) | 99.91 | malate dehydrogenase (*Cryptococcus neoformans*) | 58269764 | 35636.9 | 8.92 |
| A80 (18m) | 93.43 | phosphoribosylformylglycinamidine synthase (*Clostridium botulinum*) | 226949935 | 140636.2 | 5.75 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A81 (18m) | 86.59 | coproporphyrinogen III oxidase (*Aeromonas salmonicida*) | 145298845 | 49659.9 | 6.31 |
| A82 (18m) | 91.69 | conserved hypothetical protein (*Uncinocarpus reesii*) | 258563926 | 92636.2 | 8.31 |
| A83 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A84 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolo* ) | 170096991 | 37070.1 | 7.64 |
| A85 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A86 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A87 (18m) | 55.62 | Rha family phage regulatory protein (*Aeromonas hydrophila*) | 117619787 | 28184.3 | 9.02 |
| A88 (18m) | 99.98 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A89 (18m) | 80.07 | Zinc metalloendopeptidase (*Pichia pastoris*) | 254565745 | 83467.3 | 5.75 |
| A90 (18m) | 99.99 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A91 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A92 (18m) | 99.96 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A93 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor)* | 170096991 | 37070.1 | 7.64 |
| A94 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| A95 (18m) | 100 | DNA damage checkpoint protein (*Coprinopsis cinerea*) | 169861744 | 28941.3 | 4.76 |
| A96 (18m) | 100 | DNA damage checkpoint protein (*Coprinopsis cinerea*) | 169861744 | 28941.3 | 4.76 |
| A97 (18m) | 100 | DNA damage checkpoint protein (*Coprinopsis cinerea*) | 169861744 | 28941.3 | 4.76 |
| A98 (18m) | 0 | 6-phosphogluconate dehydrogenase (*Laccaria bicolor*) | 170097731 | 53324.5 | 6.58 |
| A99 (18m) | 99.99 | DNA damage checkpoint protein (*Coprinopsis cinerea*) | 169861744 | 28941.3 | 4.76 |
| A100 (18m) | 100 | phosphoglyceromutase 1 (*Escherichia coli*) | 218699120 | 28538.8 | 5.85 |
| A101 (18m) | 91.92 | hypothetical protein (*Citrobacte youngae*) | 283835802 | 85333.1 | 5.68 |
| | 25 (similarity) | transcription elongation protein (*Laccaria bicolor*) | | | |
| A102 (18m) | 91.92 | glucose-1-phosphate adenylyltransferase (*Citreicella* sp) | 260427150 | 47969.9 | 5.79 |
| A103 (18m) | 24.25 | Cytosolic NADP-specific isocitrate dehydrogenase (*Pichia pastoris*) | 254568772 | 48724.1 | 5.56 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A104 (18m) | 0 | sensory transduction histidine kinase (*Clostridium botulinum*) | 188588135 | 60361.9 | 8.84 |
| A105 (18m) | 0 | Acyl-CoA dehydrogenase type 2 domain protein (*Micromonospora urantiaca*) | 270502954 | 45614.4 | 6.17 |
| A106 (18m) | 99.32 | excision endonuclease (*Prevotella bergensis*) | 261878713 | 111537.4 | 8.22 |
| A107 (18m) | 96.11 | 26S proteasome regulatory subunit putative (*Candida Dubliniensis*) | 241954580 | 61703.1 | 7.95 |
| A108 (18m) | 98.24<br>8 (similarity) | hypothetical protein (*Malassezia globosa*)<br>ER to Golgi transport-related protein (*Cryptococcus neoformans*) | 164663081 | 192221.2 | 5.35 |
| A109 (18m) | - | - | - | - | - |
| A110 (18m) | 68.58<br>56 (similarity) | hypothetical protein (*Campylobacter jejuni*)<br>ATPase (*Ustilago hordei*) | 283956115 | 27710.5 | 5.6 |
| A111 (18m) | 100 | phosphoglyceromutase (*Escherichia coli*) | 218699120 | 28538.8 | 5.85 |
| A112 (18m) | 99.99 | translationally-controlled tumor protein (*Laccaria bicolor*) | 170090958 | 18955.3 | 4.61 |
| A113 (18m) | 81.5 | regulator of virG protein (*Escherichia coli*) | 218702987 | 36889.1 | 9.51 |
| A114 (18m) | 100 | alkyl hydroperoxide reductase (*Escherichia coli*) | 218694047 | 20748.4 | 5.03 |
| A115 (18m) | 9.39 | polyphosphate kinase (*Thioalkalivibrio* sp). | 289208129 | 77987.3 | 5.74 |
| A116 (18m) | 0 | putative periplasmic protein (Neisseria cinerea) | 269213925 | 20329.7 | 10.03 |

Table 4.5 (continued)

| | | | | | |
|---|---|---|---|---|---|
| A117 (18m) | 99.98<br>99 (similarity) | hypothetical protein (*Moniliophthora perniciosa*)<br>actin filament-coating protein tropomyosin (*Laccaria bicolor*) | 238606531 | 18935.8 | 4.99 |
| A118 (18m) | 0 | ATP/GTP-binding site domain-containing protein A (*Brucella* sp) | 261758253 | 31756.1 | 0 |
| A119 (18m) | 46.37 | THO complex component (*Talaromyces stipitatus*) | 242818308 | 262450.8 | 4.92 |
| A120 (18m) | 25.97 | Heat repeat protein (*Talaromyces stipitatus*) | 242777438 | 295769.5 | 6.39 |
| A121 (18m) | 0 | kinesin heavy chain (*Laccaria bicolor*) | 170088352 | 107597.4 | 5.61 |
| A122 (18m) | 100 | phosphoglycerate kinase (*Laccaria bicolor*) | 170094220 | 44624.5 | 5.66 |
| A123 (18m) | 100<br>100 (similarity) | hypothetical protein (*Ustilago maydis*)<br>phosphoglycerate kinase (*Cryptococcus neoformans*) | 71021575 | 44705.2 | 5.74 |
| A124 (18m) | 99.997 | aconitate hydratase (*Laccaria bicolor*) | 170094674 | 82291.7 | 6.02 |

From cedar, 2 proteins were indentified from 14 months (Table 4.6) and 3 proteins from 16 months (Table 4.7). Most identified proteins were general metabolic enzymes and the protein score was low.

Table 4.6. Results of BLAST homology search of protein identification from cedar samples at 14 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| B1 (14m) | 0 | predicted protein (*Coprinopsis cinerea*) | 169851887 | 16723.5 | 4.38 |
| | 68 (similarity) | ectomycorrhiza-regulated small secreted protein (*Laccaria bicolor*) | | | |
| B2 (14m) | 0 | predicted protein (*Coprinopsis cinerea*) | 169843457 | 29637.1 | 5.38 |
| | 86 (similarity) | predicted protein (*Postia placenta*) | | | |

Table 4.7. Results of BLAST homology search of protein identification from cedar samples at 16 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| B1 (16m) | 35.52 | kinesin family protein (*Ajellomyces dermatitidis*) | 261200713 | 94783.8 | 8.77 |
| B2 (16m) | 94.92 | hypothetical protein (*Moniliophthora perniciosa*) | 238569069 | 24613.4 | 5.04 |
| | 100 (Similarity) | pre-mRNA splicing factor (*Cryptococcus neoformans*) | | | |
| B3 (16m) | 79.13 | peroxisomal membrane protein | 242793703 | 57988.8 | 5.07 |

Sixteen proteins were found on cedar at 18 months (Table 4.8). Phosphoglycerate kinase matched to *Laccaria bicolo*r as 100% protein score. It is a part of pathway in the glycolysis pathway. Other proteins were general metabolic enzymes. There was a

greater number of high protein matches at 18 months, compared to the other sampling dates.

Table 4.8. Results of BLAST homology search of protein identification from cedar samples at 18 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| B1 (18m) | 99.81 | molecular chaperone (*Aspergillus flavus*) | 238490041 | 69662.6 | 5.11 |
| B2 (18m) | 95.02 | serine/threonine protein kinase (*Micrococcus luteus*) | 281413629 | 35564.4 | 5.26 |
| B3 (18m) | 100 | hypothetical protein (*Cryptococcus neoformans*) translation elongation factor protein (*Cryptococcus neoformans)* | 134118367 | 50247.3 | 100 |
| B4 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolo*r) | 170085829 | 47209.6 | 5.55 |
| B5 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolo*r) | 170085829 | 47209.6 | 5.55 |
| B6 (18m) | 100 | phosphoglycerate kinase (*Laccaria bicolor*) | 170094220 | 44624.5 | 5.66 |
| B7 (18m) | 100 | phosphopyruvate hydratase (*Laccaria bicolo*r) | 170085829 | 47209.6 | 5.55 |
| B8 (18m) | 100 | phosphoglycerate kinase (*Laccaria bicolor*) | 170094220 | 44624.5 | 5.66 |
| B9 (18m) | 99.93 | phosphopyruvate hydratase (*Salmonella enterica*) | 194735542 | 45570.3 | 5.25 |
| B10 (18m) | 95.22 | translational activator, (*Talaromyces stipitatus*) | 242776149 | 316516.2 | 8.39 |
| B11 (18m) | 58.58 | polysaccharide transport protein (*Bifidobacterium longum*) | 189440436 | 46985.4 | 4.78 |

Table 4.8 (continued)

| | | | | | |
|---|---|---|---|---|---|
| B12 (18m) | 99.09 | outer membrane efflux protein (*Pseudomonas putida*) | 167034073 | 57387.4 | 5.97 |
| B13 (18m) | 58.58 | flagellar protein (*Clostridium Botulinum*) | 188588315 | 47486.9 | 5.46 |
| B14 (18m) | 88.05 | tryptophan synthase (*Ruegeria pomeroyi*) | 56695710 | 45508.6 | 5.5 |
| B15 (18m) | 0 | DNA-directed RNA polymerase (*Catenulispora acidiphila*) | 256390127 | 143920.8 | 7.81 |
| B16 (18m) | 76.16 | glyceraldehyde-3-phosphate dehydrogenase (*Cronobacter turicensis*) | 260597595 | 35649.4 | 6.33 |

In ACQ-treated pine, the first proteins were detected at 6 months. All three proteins detected at 6 months were from the bacteria database with protein scores of 0% (Table 4.9).

Table 4.9. Results of BLAST homology search of protein identification from ACQ-treated pine samples at 6 months.

| **Sample Name** | **Protein Score (%)** | **Protein Name** | **Accession Number** | **MW** | **pI** |
|---|---|---|---|---|---|
| C1 (6m) | 0 | HD domain-containing protein (*Geobacter sulfurreducens*) | NP_952736 | 20324.7 | 6.55 |
| C2 (6m) | 0 | mobilization protein BmpH (*Bacteroides fragilis*) | YP_098519 | 48296.8 | 8.11 |
| C3 (6m) | 0 | N-acetylneuraminate lyase (*Fusobacterium nucleatum*) | ZP_0014345( | 32925.2 | 6.26 |

At 8 months, six proteins were identified from the fungi and bacteria database but all proteins had a 0% match (Table 4.10). Malate dehydrogenase of *Neurospora crassa* was identified from the fungi database. Malate dehydrogenase catalyzes the conversion of malate into oxaloacetate an enzyme in the citric acid cycle (Banaszak and Bradshaw 1975).

Table 4.10. Results of BLAST homology search of protein identification from ACQ-treated pine samples at 8 months.

| **Sample Name** | **Protein Score (%)** | **Protein Name** | **Accession Number** | **MW** | **pI** |
|---|---|---|---|---|---|
| C1 (8m) | 0 | peptidyl-tRNA hydrolase (*Streptococcus agalactia*) | NP_687043 | 21595.1 | 9.08 |
| C2 (8m) | 0 | malate dehydrogenase (*Neurospora crassa*) | XP_958408 | 35495.8 | 8.80 |
| C3 (8m) | 0 | colicin A (*Escherichia coli*) | CAA51711 | 2080.9 | 6.47 |
| C4 (8m) | 0 | macrolide-binding protein (*Cryptococcus neoformans*) | XP_569051 | 14644.6 | 9.34 |
| C5 (8m) | 0 | 50S ribosomal protein L5 (*Staphylococcus epidermidis*) | NP_765366 | 20222.8 | 9.20 |
| C6 (8m) | 0 | malate dehydrogenase (*Neurospora crassa*) | XP_958408 | 35495.8 | 8.80 |

At 10 months, 8 proteins with 0% protein score were identified from bacteria, fungi, and basidiomycete databases (Table 4.11). Nitrate reductase of *Botryotinia fuckeliana* was identified from the fungi database. This enzyme is involved in the reduction of nitrate to ammonia.

Table 4.11. Results of BLAST homology search of protein identification from ACQ-treated pine samples at 10 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| C1 (10m) | 0 | nitrate reductase (*Botryotinia fuckeliana*) | AAC02633 | 101832 | 5.47 |
| C2 (10m) | 0 | NifU domain protein (*Campylobacter lari*) | ZP_00368592 | 9711 | 5.19 |
| C3 (10m) | 0 | phosphoglycerate kinase (*Agaricus bisporus*) | CAA62559 | 44690.7 | 7.71 |
| C4 (10m) | 0 | ribonuclease Z (*Clostridium perfringens*) | NP_562279 | 35464.2 | 5.97 |
| C5 (10m) | 0 | palmitoyltransferase (*Schizosaccharomyces pombe*) | NP_595701 | 38065.6 | 8.11 |
| C6 (10m) | 0 | gamma-tubulin complex component (*Laccaria bicolor*) | EDR12438 | 96272.5 | 7.01 |
| C7 (10m) | 0 | reverse transcriptase (*Crinipellis perniciosa*) | AAT97974 | 14985.9 | 9.74 |
| C8 (10 m) | 0 | Nima-Like Ser/Thr Protein Kinase (*Encephalitozoon cuniculi*) | NP 586456 | 34560 | 8.98 |

At 18 months, 47 proteins were detected on ACQ-treated pine (Table 4.12). Similar to pine, ACQ-treated pine contained aconitate hydratase, fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate, and phosphglycerate kinase with high protein scores. There was a greater number of high protein matches at 18 months, compared to the other sampling dates.

Table 4.12. Results of BLAST homology search of protein identification from ACQ-treated pine samples at 18 months.

| Sample Name | Protein Score (%) | Protein Name | Accession Number | MW | pI |
|---|---|---|---|---|---|
| C1 (18m) | 100 | cell division cycle protein (*Uncinocarpus reesii*) | 258566938 | 88677 | 5.00 |
| C2 (18m) | 100 | aconitate hydratase (*Laccaria bicolor*) | 170094674 | 82291.7 | 6.02 |
| C3 (18m) | 99.84 | heat shock protein (*Cryptococcus neoformans*) | 58264706 | 69310.2 | 4.94 |
| C4 (18m) | 98.50 | heat shock protein (*Cryptococcus neoformans*) | 58264706 | 69310.2 | 4.94 |
| C5 (18m) | 100 | chaperone (*Cryptococcus neoformans*) | 58264778 | 69575.3 | 5.04 |
| C6 (18m) | 100<br>98 (Similarity) | hypothetical protein (*Coprinopsis cinerea*)<br>heat shock protein (*Cryptococcus neoformans*) | 169847838 | 72040.2 | 5.67 |
| C7 (18m) | 97.71 | heat shock protein (*Candida tropicalis*) | 255731870 | 70355.5 | 5.97 |
| C8 (18m) | 0 | ribonucleotide-diphosphate (*Brucella suis*) | 261753310 | 80521.4 | 5.81 |
| C9 (18m) | 100<br>93 (Similarity) | hypothetical protein (*Coprinopsis cinerea*)<br>heat shock protein (*Cryptococcus neoformans*) | 169867040 | 63304.9 | 5.62 |
| C10 (18m) | 86.28 | ATP-dependent Clp protease (*Streptococcus pneumoniae*) | 15900708 | 83762.2 | 5.48 |

Table 4.12 (continued)

| | | | | | |
|---|---|---|---|---|---|
| C11 (18m) | 100 | ATP synthase (*Ajellomyces dermatitidis*) | 261187936 | 55784 | 5.27 |
| C12 (18m) | 100 | ATP synthase beta chain (*Clavispora lusitaniae*) | 260949645 | 44570.1 | 4.95 |
| C13 (18m) | 99.89 | ATP synthase (*Coprinopsis cinerea*) | 169852676 | 57891.2 | 5.58 |
| C14 (18m) | 0 | two-component osmosensing histidine kinase (*Talaromyces stipitatus*) | 242786803 | 144825.8 | 5.07 |
| C15 (18m) | 92.06 | cytochrome P450, putative (*Aspergillus flavus*) | 238497457 | 27763.3 | 8.93 |
| C16 (18m) | 95.93 | predicted protein (*Postia placenta*) | 242206742 | 65484.6 | 5.86 |
| | 98.6 (similarity) | predicted protein (*Coprinopsis cinerea*) | | | |
| C17 (18m) | 99.79 | fructose 1,6-bisphosphate aldolase (*Laccaria bicolor*) | 170106499 | 38915.8 | 5.54 |
| C18 (18m) | 0 | C6 transcription factor protein (*Ajellomyces dermatitidis*) | 261197858 | 97239.5 | 5.93 |
| C19 (18m) | 99.83 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C20 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C21 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C22 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C23 (18m) | 100 | protein BMH2 (*Candida tropicalis*) | 255731125 | 29405.7 | 4.76 |

Table 4.12 (continued)

| | | | | | |
|---|---|---|---|---|---|
| C24 (18m) | 20.68 | Rad2-like endonuclease (*Aspergillus flavus*) | 238489679 | 97202 | 8.96 |
| C25 (18m) | 99.98 | hypothetical protein (*Moniliophthora perniciosa*) | 238594627 | 11729.8 | 5.95 |
| | 73.2 (Similarity) | nascent polypeptide-associated complex protein (*Melampsora medusae*) | | | |
| C26 (18m) | 100 | DNA damage checkpoint protein (*Coprinopsis cinerea*) | 169861744 | 28941.3 | 4.76 |
| C27 (18m) | 0 | alkaline serine protease (*Ajellomyces dermatitidis*) | 261199199 | 14727.9 | 10.33 |
| C28 (18m) | 54.58 | VWA containing CoxE family protein (*Streptosporangium roseum*) | 271964348 | 42113 | 10.79 |
| C29 (18m) | 0 | gluconate 5-dehydrogenase (*Aspergillus flavus*) | 238506183 | 27533.1 | 5.57 |
| C30 (18m) | 0 | serine/theronine-protein kinase (*Ajellomyces dermatitidis*) | 261206360 | 96659.7 | 6.18 |
| C31 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C32 (18m) | 40.13 | DNA topoisomerase (*uncultured bacterium*) | 31795143 | 74822.5 | 8.67 |
| C33 (18m) | 99.84 | chaperone protein DnaK (*Pyramidobacter piscolens*) | 282857129 | 65167.5 | 5.07 |
| C34 (18m) | 100 | predicted protein (*Laccaria bicolor*) | 170098056 | 62414.9 | 5.81 |
| | 92 (Similarity) | heat shock protein (*Cryptococcus neoformans*) | | | |
| C35 (18m) | 100 | ATP synthase (*Clavispora lusitaniae*) | 260949645 | 44570.1 | 4.95 |
| C36 (18m) | 0 | conserved domain protein (*Lactobacillus gasseri*) | 282850944 | 6083.3 | 9.43 |

Table 4.12 (continued)

| | | | | | |
|---|---|---|---|---|---|
| C37 (18m) | 91.73 | ATP-dependent Clp protease, (*Streptococcus sp*) | 270293062 | 83943.2 | 5.26 |
| C38 (18m) | 100 | phosphoglycerate kinase (*Laccaria bicolor* ) | 170094220 | 44624.5 | 5.66 |
| C39 (18m) | 99.67 | phosphopyruvate hydratase (*Uncinocarpus reesii*) | 258564696 | 47359.5 | 5.57 |
| C40 (18m) | 0 | gluconate 5-dehydrogenase, putative (*Aspergillus flavus*) | 238506183 | 27533.1 | 5.57 |
| C41 (18m) | 99.20 | protein BMH2 (*Candida tropicalis*) | 255731125 | 29405.7 | 4.76 |
| C42 (18m) | 99.99 | BMH1 protein (*Clavispora lusitaniae*) | 260945016 | 29017.5 | 4.68 |
| C43 (18m) | 98.39 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C44 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C45 (18m) | 100 | glyceraldehyde 3-phosphate dehydrogenase (*Laccaria bicolor*) | 170096991 | 37070.1 | 7.64 |
| C46 (18m) | 0 | signal recognition particle docking protein (*Prochlorococcus marinus*) | 123965244 | 45987.2 | 5.3 |
| C47 (18m) | 0 | gluconate 5-dehydrogenase (*Aspergillus flavus*) | 238506183 | 27533.1 | 5.57 |

**Comparison of microbial proteins using functional and taxonomical grouping**

In order to determine how many proteins were involved in different metabolic functions and how protein groups changed over time, comparison analysis of functional grouping was performed. Proteins with a low protein score were un-confirmed but were analyzed for functional grouping based on a given protein family in the NCBI database. From the untreated pine at 6 months, proteins were grouped as 7% in protein metabolism, 7% in DNA & RNA metabolism, 7% in pathogenesis, 7% in control of gene expression, 14% in electron transport, 21% in glycolysis, and 37% in other (Table 4.13).

At 8 months, proteins were grouped as 13% in protein metabolism, 13% in DNA & RNA metabolism, 6% in pathogenesis, 6% in control of gene expression, 13% in electron transport, 24% in glycolysis, and 25% in other. At 10 months, proteins grouped as 17% in protein metabolism, 10% in DNA & RNA metabolism, 10% in pathogenesis, 17% in electron transport, 25% in glycolysis, and 21% in other. At 18 months, the largest functional group of proteins on pine was glycolysis at 29% with 4% in control of gene expression, 7% in other, 11% in pathogenesis, 15% in DNA & RNA metabolism, 18% in electron transport, and 19% in protein metabolism. Proteins listed in the other function were involved in lipid metabolism, intracellular vesicular traffic, protein sorting, and cell division.

Table 4.13. Comparison of microbial proteins of pine samples in functional grouping at given time.

| Months | Group (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Protein metabolism | DNA&RNA metabolism | Pathogenesis | Control of gene expression | Electron transport | Glycolysis | Other |
| 6 | 7 | 7 | 7 | 7 | 14 | 21 | 37 |
| 8 | 13 | 13 | 6 | 6 | 13 | 24 | 25 |
| 10 | 17 | 10 | 10 | 0 | 17 | 25 | 21 |
| 18 | 19 | 15 | 11 | 4 | 18 | 29 | 7 |

From the untreated cedar at 14 months, proteins were grouped as 50% in DNA & RNA metabolism and 50% in other (Table 4.14). At 16 months, proteins were grouped as 33% protein metabolism and 67% in DNA & RNA metabolism (Table 4.14). At 18 months, proteins were variably grouped as 4% in control of gene expression, 15% in protein metabolism, 17% DNA & RNA metabolism, 24% in other, 26% electron transport, and 38% in glycolysis.

Table 4.14. Comparison of microbial proteins of cedar samples in functional grouping at given time.

| Months | Group (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Protein metabolism | DNA&RNA metabolism | Pathogenesis | Control of gene expression | Electron transport | Glycolysis | Other |
| 14 | 0 | 50 | 0 | 0 | 0 | 0 | 50 |
| 16 | 33 | 67 | 0 | 0 | 0 | 0 | 0 |
| 18 | 6 | 13 | 0 | 0 | 38 | 19 | 24 |

In comparison to pine, the protein functional group from ACQ-treated pine varied widely. From the ACQ-treated pine at 6 months, proteins were grouped as 33% in

DNA & RNA metabolism and 67% in other (Table 4.15). At 8 months, proteins were grouped as 17% in control of gene expression, 33% in glycolysis, and 50% in other. At 10 months, proteins were grouped as 38% in DNA & RNA metabolism, 25% in electron transport, and 37% in glycolysis. At 18 months, proteins were grouped as 4% control of gene expression, 15% protein metabolism, 17% DNA & RNA metabolism, 26% in electron transport, and 38% in glycolysis. The largest functional group of proteins on ACQ-treated pine at 18 months was glycolysis.

Table 4.15. Comparison of microbial proteins of ACQ-treated pine samples in functional grouping at given time.

| Months | Group (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Protein metabolism | DNA&RNA metabolism | Pathogenesis | Control of gene expression | Electron transport | Glycolysis | Other |
| 6 | 0 | 33 | 0 | 0 | 0 | 0 | 67 |
| 8 | 0 | 0 | 0 | 17 | 0 | 33 | 50 |
| 10 | 0 | 38 | 0 | 0 | 25 | 37 | 0 |
| 18 | 15 | 17 | 0 | 4 | 26 | 38 | 0 |

Protein taxonomical grouping was performed among three wood types from each collection time. Each protein was placed in either bacteria, fungi, basidiomycetes or others. At 6 months from untreated pine, 8 proteins were from bacteria, 2 from fungi, and 4 from basidiomycetes (Table 4.16). At 8 months, there were 4 proteins from bacteria, 5 proteins from fungi, 6 proteins from basidiomycetes, and 1 protein from other. The one protein in the other group was a putative virus designated as *Simian enterovirus*. At 10 months, there were 14 proteins from bacteria, 9 proteins from fungi, and 6 proteins from basidiomycetes. At 18 months, 124 proteins were grouped as 30 proteins from bacteria, 17 proteins from fungi, 77 proteins from basidiomycetes. The proteins from

basidiomycetes on pine increased from 6 to 18 months while the proteins from bacteria on pine continually decreased.

Table 4.16. Protein taxonomical grouping for pine samples at given time.

| Months | Group(s) | | | | Total |
|---|---|---|---|---|---|
| | Bacteria | Fungi | Basidiomycetes | Other(s) | |
| 6 | 8 (58%) | 2 (14%) | 4 (28%) | 0 (0%) | 14 (100%) |
| 8 | 4 (25%) | 5 (31%) | 6 (38%) | 1 (6%) | 16 (100%) |
| 10 | 14 (48%) | 9 (31%) | 6 (21%) | 0 (0%) | 29 (100%) |
| 18 | 30 (24%) | 17 (14%) | 77 (62%) | 0 (0%) | 124 (100%) |

Proteins from cedar were grouped taxonomically (Table 4.17). The 2 proteins, identified on cedar at 14 months were from basidiomycetes. At 16 months, there were 2 proteins from fungi and 1 protein from basidiomycetes. The first proteins from bacteria were found on cedar at 18 months. At 18 months, there were 8 proteins from bacteria, 2 proteins from fungi, and 6 proteins from basidiomycetes. Most proteins on cedar were from basidiomycetes.

Table 4.17. Protein taxonomical grouping for cedar samples at given time.

| Months | Group(s) | | | | Total |
|---|---|---|---|---|---|
| | Bacteria | Fungi | Basidiomycetes | Other(s) | |
| 14 | 0 (0%) | 0 (0%) | 2 (100%) | 0 (0%) | 2 (100%) |
| 16 | 0 (0%) | 2 (67%) | 1 (33%) | 0 (0%) | 3 (100%) |
| 18 | 8 (50%) | 2 (12.5%) | 6 (37.5%) | 0 (0%) | 16 (100%) |

Proteins from ACQ-treated pine were grouped taxonomically (Table 4.18). At 6 months, all 3 proteins were from bacteria. At 8 months, 3 proteins were from bacteria and

3 proteins from fungi. None were found from basidiomycetes. At 10 months, there were 2 proteins from bacteria, 3 proteins from fungi, and 3 proteins from basidiomycetes. A total 47 proteins from ACQ-treated pine at 18 months were grouped as 8 from bacteria, 16 from fungi, and 23 from basidiomycetes.

Table 4.18. Protein taxonomical grouping for ACQ-treated pine samples at given time.

| Months | Group(s) | | | | Total |
|---|---|---|---|---|---|
| | Bacteria | Fungi | Basidiomycetes | Other(s) | |
| 6 | 3 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 3 (100%) |
| 8 | 3 (50%) | 3 (50%) | 0 (0%) | 0 (0%) | 6 (100%) |
| 10 | 2 (25%) | 3 (37.5%) | 3 (37.5%) | 0 (0%) | 8 (100%) |
| 18 | 8 (17%) | 16 (34%) | 23 (49%) | 0 (0%) | 47(100%) |

## DISCUSSION

Understanding microbial wood degradation in a forest ecosystem is very difficult because environmental conditions vary greatly over the long period of time needed for decay to occur. Within this ecosystem, many different microbes live on or around wood and may deteriorate wood as a food source. Metaproteomics can be used to identify the numerous proteins secreted by the microbial community at a given time. In this study, there was a significant level of decay detected visually and by dynamic MOE for untreated pine. However, there was only a small level of decay for cedar and ACQ-treated pine with no significant difference between these two wood types.

Over 18 months, proteins on untreated pine increased from 14 to 124. The largest functional group at each time point was glycolysis. The only decay enzymes found were cellulose 1, 4-beta cellobiosidase of *Pleurotus* sp. at 6 months and methanol

dehydrogenase of *Methylococus capsulatus* at 10 months. Degradation of cellulose in pine might occur from cellulose 1, 4-beta cellobiosidase, also called exo-1,4-beta-D-glucanase or avicelase, through the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose (Edwards *et al.* 2008). Vorob'ev *et al.* (2009) reported that the methylotrophic bacterium, *Methylovirgula ligni*, degraded complex carbon compounds using methanol dehydrogenase.

Over 18 months, the numbers of proteins on cedar increased from 2 at 14 months to 35 at 18 months. Fungal mycelia were visually present on surface of cedar at 4 months through the end of study however there was apparently not enough biomass to detect protein spots until 14 months. Numerous studies have reported that proteins could be missed by this type of protein analysis because the concentration was not sufficient for detection (Gromov and Celis 2000; Phillips and Bogyo 2005; Kang *et al.* 2009; Keller and Hettich 2009). Cedar strongly reduced the wood decay community and its activities in comparison to untreated pine so fewer microbial proteins were detected and less decay occurred. The natural chemicals in cedar most likely reduced the microbial growth and prevented the production of enzymes.

Over 18 months on ACQ-treated pine, the number of proteins increased from 3 to 92. Most proteins were involved in DNA & RNA metabolism or in other functions (which included lipid metabolism, intracellular vesicular traffic, protein sorting, and cell division). The number of proteins involved in glycolysis increased from 33% at 8 months to 38% at 18 months. Many bacterial and fungal proteins were detected but most proteins were involved in metabolic metabolism. Although more proteins were detected on ACQ-treated pine than cedar, the level of decay was the same. The ACQ preservative protected

the wood from decay by chemical protection but did not inhibit the colonization or activity of microbes. Therefore, many fewer proteins were found on ACQ-treated pine compare to untreated pine.

Interestingly, there were different microbial proteins found between untreated pine and ACQ-treated pine. The largest functional group of proteins on untreated pine at all sampling times was glycolysis. On the other hand, the protein functional group from ACQ-treated pine varied widely. No proteins from glycolysis were detected at 6 month but did appear at 8 and 10 months. Proteins in DNA & RNA metabolism, gene expression, and electron transfer appeared and disappeared. The largest percentage of proteins was involved in other functions. The taxonomical group detected on untreated pine varied at all sampling times although the numbers in each taxonomic group increased over time. The total number of bacteria proteins at 6 months was greater than both fungi and basidiomycete proteins however the total number of fungi and basidiomycete proteins surpassed bacterial proteins at 8 and 10 months. In comparison, the number of proteins on ACQ-treated pine was less than untreated pine. At 6 months, only bacteria proteins were detected. Non-basidiomycete proteins appeared at 8 months, while basidiomycete proteins did not appear until 10 months. Bacteria are thought to be the first colonizers of wood and then followed by molds or basidiomycetes (Rayner and Boddy 1998). Although basidiomycetes were detected on wood beginning at 4 months, it was 6 months later before their proteins were detected.

Interestingly, higher protein scores were found all three wood types at 18 months than other sampling periods. Because these are environmentally exposed samples, the proteins extracted are from many sources. Some decay related enzymes were likely

missed because they are being produced in such small quantities. Another limitation of this type of analysis is the extraction of proteins is based on weight. When biomass of the microorganism is extracted from decayed wood, the tissue is mostly wood material not biomass of microorganisms. Thus some proteins were likely missed because the concentration or the efficiency of protein extraction was not sufficient for detection and identification. A third limitation is the lack of well annotated protein sequence data on the public databases. Identification of proteins is only as good as the existing searchable databases. This limitation equals that which occurred in many years past with the lack of DNA sequences on GenBank. As more scientists sequence proteins and use proteomic approaches, more proteins will be characterized and placed in public databases.

## CONCLUSION

Proteomics has become a vital tool in the field of functional genomics, because only proteins are directly related to specific metabolic functions. The microorganisms on non-resistant pine produced more proteins, more decay enzymes, and had a higher percentage of MOE loss than cedar or ACQ-treated pine. Few proteins were produced on naturally durable cedar and these were produced late in this study. There were fewer proteins produce on chemically-resistant ACQ-pine than on untreated pine, but more than on cedar. The type of proteins produced on ACQ-treated wood was very different from untreated pine as was the microbial source of proteins. Results from the study indicate that different resistant woods have different effects on the microbial communities and its enzymatic production during decay. To our knowledge, this study is the first report of

comparative metaproteomics from microbial communities on different decay resistant woods and therefore is projected to produce a baseline for subsequent studies.

# BIBLIOGRAPHY

Abbas, A., H. Koc, F. Liu, and M. Tien. 2005. Fungal degradation of wood: initial proteomic analyses of extracellular proteins of *Phanerochaete chrysosporium* grown on oak substrate. Current Genetics 47: 49-56.

Banaszak, L.J. and R.A. Bradshaw.1975. Malate dehydrogenase. The enzymes. 3rd ed., New York: Academic Press. pp. 369–396.

Beguin, P. and J.P. Aubert. 1994. The biological degradation of cellulose. FEMS Microbiology Letters. 13:25-58.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 72: 248-254.

Edwards, I.P., R.A. Upchurch, and D.R. Zak, 2008. Isolation of fungal cellobiohydrolase I genes from sporocarps and forest soils by PCR. Applied and Environmental Microbiology 74(11):3481-3489.

Eriksson, K.E.L., R.A. Blanchette, and P. Andr. 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin Heidelberg New York.

Fryksdale, B.G., P.T. Jedrzejewski, D.L. Wong, A.L. Gaertner, and B.S. Miller. 2002. Impact of deglycosylation methods on two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization–time of flight-mass spectrometry for proteomic analysis. Electrophoresis 23:2184-2193.

Gromov, P. S. and Celis, J. E. 2000. From genomics to proteomics. Molecular Biology 34:508-520.

Hackl, E., M. Pfeffer, C. Donat, G. Bachmann, and S. Zechmeister-Boltenstern. 2005. Composition of the microbial communities in the mineral soil under different types of natural forest. Soil Biology & Biochemistry 37: 661-671.

Kang, Y.M., M.L. Prewitt and S.V. Diehl. 2009. Proteomics for biodeterioration of wood (Pinus taeda L.): Challenging analysis by 2-DE and MALDI-TOF-MS. International Biodeterioration & Biodegradation 63(8):1036-1044.

Karas, M., D. Bachmann and F. Hillenkamp. 1985. Influence of the wavelength in high-irradiance ultraviolet laser desorption mass spectrometry of organic molecules.

Analytical Chemistry 57: 2935–2939.

Keller, M. and R. Hettich. 2009. Environmental proteomics: a paradigm shift in characterizing microbial activities at the molecular level. Microbiology and Molecular Biology Reviews 73(1):62-70.

Koker, T. H. and P.J. Kersten. 2002. Characterization of wood decay enzymes by MALDI-MS for post-translation modification and gene identification. International Research Group on Wood Protection IRG/WP 02-10442.

Liebler, D.C. 2002. Introduction to proteomics: Tools for the new biology. Humana Press, Totowa, NJ. USA.

Markowitz, V.M. 2007. Microbial genome data resources. Current Opinion in Biotechnology 18: 267-272.

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C.P., Ferreira, P., Ruiz-Duenas, F.J., Martinez, A.T., Kersten, P., Hammel, K.E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., Splinter BonDurant, S., Larrondo, L.F., Canessa, P., Vicuna, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavin, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kües, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R., and Cullen, D. 2009. Genome, transcriptome, and secretome analysis of wood decay fungus Postia placenta supports unique mechanisms of lignocellulose conversion. Proceedings of the National Academy of Sciences 106(6):1954-1959.

Martinez, D., Larrondo, L.F., Putnam, N., Gelpke, M.D.S., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D., Rokhsar, and D. 2004. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nature Biotechnology 22: 695-700.

Mathewson, P.R. 1998. Enzymes. An Eagan Press Handbook, American Association of Cereal Chemists, Inc. St. Paul, MN, USA.

Millsa, D.K., J.A. Entry, P.M. Gillevertc, and K. Matheed. 2007. Assessing microbial community diversity using amplicon length heterogeneity polymerase chain reaction. Soil Science Society of America Journal 71: 572-578.

Nelson, D. and M. Cox. 2005. Lehninger Principles of Biochemistry. Fourth edition. W.H.

Freeman publisher.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry 250(10):4007-4021.

Pappin, D.J.C., P. Hojrup, and A.J. Bleasby. 1993. Rapid identification of proteins by peptide mass fingerprinting. Current Biology 3:327-332.

Pechanova, O., W.D. Stone, W. Monroe, T.E. Nebeker, K.D. Klepzig, K.D., and C. Yuceer. 2008. Global and comparative protein profiles of the pronotum of the southern pine beetle, Dendroctonus frontalis Zimmermann (Coleoptera: Scolytidea). Insect Molecular Biology 17(3):261-277.

Pevsner, J. 2003. Bioinformatics and functional genomics. John Wiley & Sons, Inc. Hoboken. NY. USA.

Phillips, C. and M. Bogyo. 2005. Proteomics meets microbiology: technical advances in the global mapping of protein expression and function. Cellular Microbiology 7: 1061-1076.

Pointing, S., M.M. Parungao, and K.D. Hyde. 2003. Production of wood-decay enzymes, mass loss and lignin solubilization in wood by tropical Xylariaceae. Mycological Research 107: 231–235.

Prabakaran, P., An, J., Gromiha, M., Selvaraj, S., Uedaira, H., Kono, H., and Sarai, A. 2001. Thermodynamic database for protein-nucleic acid interactions (ProNIT). Bioinformatics 17: 1027-1034.

Rayner, A.D.M. and L. Boddy. 1998. Fungal decomposition of wood: its biology and ecology. John Wiley, Chichester Publisher.

Saham, H. 1977. Metabolism of methanol by yeasts. Advances in Biochemical Engineering/Biotechnology 6:77-103.

Vanden Wymelenberg, A., Sabat, G., Martinez, D., Rajangam, S., Teeri, T.T., Gaskell, P., Kersten, P.J., and Cullen, D. 2005. The Phanerochaete chrysosporium secretome: database predictions and initial mass spectrometry peptide identification in cellulose-grown medium. Journal of Biotechnology 118:17-34.

Vorob'ev, A.V., W. Boer, L. B. Folman, P.L. E. Bodelier, N.V. Doronina, N.E. Suzina, Y. A. Trotsenko, and S. N. Dedysh. 2009. Methylovirgula ligni gen. nov., sp. nov., an obligately acidophilic, facultatively methylotrophic bacterium with a highly

divergent mxaF gene. International Journal of Systematic and Evolutionary Microbiology 59: 2538–2545.

Zabel, R.A., C.J.K.Wang, and S.E. Anagnost. 1991. Soft-rot capabilities of the major micro fungi, isolated from Douglas-fir poles in the North-East. Wood Fiber Science 23:220-237.

Zhang, H.B., M.X. Yang, R.Tu, L. Gao, and Z.W. Zhao. 2008. Fungal communities in decaying sapwood and heartwood of a conifer Keteleeria evelyniana. Current Microbiology 56:358-362.

Zhou, X.W., Blackman, M.J., Howell, S.A., Carruthers, V.B., 2004. Proteomic analysis of cleavage events reveals a dynamic two-step mechanism for proteolysis of a key parasite adhesive complex. Molecular and Cellular Proteomics 3:565-576.

Zwolinski, M.D. 2007. DNA sequencing strategies for soil microbiology. Soil Science Society of America Journal 71:592-600.

# CHAPTER V

# SUMMARY OF RESEARCH

## SUMMARY

The objective of this study was undertaken to characterize decay genes and proteins that are expressed on three different wood types undergoing decay over 18 months. Variation in expression pattern of decay genes and proteins were determined for pine (non-resistant), cedar (naturally durable), and ACQ-treated pine (chemically resistant) in a soil decay bed.

### 0 and 2 months

Tracheid fibers for all wood types were free from fungal attack (microbial presence) and well organized at 0 and 2 months. There was no significant difference in MOE loss and visual decay rating among the three wood types. BS decay genes were detected on untreated pine and ACQ-treated pine but not on cedar. No BS decay genes were being expressed at this time.

### 4 months

Visual decay ratings of the three wood types were significantly different beginning at 4 months between pine and cedar and between pine and ACQ-treated pine, but there was no significant difference in MOE loss. Pine stakes developed distinct discoloration at 4 months, while, cedar and ACQ-treated pine showed only mild discoloration. Cell wall degradation was observed only in pine. Basidiomycetes were present on all samples. BS decay genes were detected on all samples, however, only on pine and on ACQ-treated pine were they expressed. The first detection of Pr-Mnp was found on pine and ACQ-treated pine at 4 months. Although both pine and ACQ-treated pine had similar expression levels of Pr-Mnp, only pine showed visual decay. There were several Basidiomycete species identified on all three wood types: *Blastosporella zonata*, *Boletaceae sp.*, and *Phlebia radiata*. In total, there were four species of basidiomycete detected on cedar, six on ACQ treated pine, and twelve on pine.

### 6, 8, and 10 months

A significant decrease in MOE and presence of visual decay was seen between pine and cedar and between pine and ACQ-pine from 6 to 10 months. At 6 months, fungal mycelia covered the surface of pine stakes. Fungal mycelia were not observed on either cedar or ACQ-treated pine stakes. Discoloration occurred at 8 and 10 months on pine, cedar, ACQ-treated pine. Fungal hyphae were detected in the secondary cell wall and middle lamella of pine stakes at 8 months exposure. However, no fungi were observed in cell lumens or cell walls of the cedar and ACQ-treated pine stakes at either 8

or 10 months. Although basidiomycetes were present on cedar, no enzymes were expressed. Decay fungi can be present on wood but may or may not produce decay enzymes. The expression level of Lcc in ACQ-treated pine was significantly higher than in pine. *P. radiata* appeared to colonized ACQ-treated pine but did not cause decay.

No proteins were detected from any of the three wood types until 6 months. From 6 to 10 months on pine, protein numbers increased from 14 to 29. No proteins were detected on cedar. Proteins were first detected on ACQ-treated pine at 6 months and the numbers increased from 3 to 8. The largest functional group of proteins on untreated pine at all sampling times was glycolysis. The only decay enzymes found were cellulose 1, 4-beta cellobiosidase matched to *Pleurotus* sp. at 6 months and methanol dehydrogenase matched to *Methylococus capsulatus* at 10 months. In comparison, the protein functional group from ACQ-treated pine varied widely. No glycolysis related proteins were detected at 6 months but did appear at 8 and 10 months.

14, 16, and 18 months

A significant decrease in MOE was found between pine and cedar and between pine and ACQ-pine. During this time, the BS gene was continually found on all three wood types indicating that Basidiomycetes were continuously present. There were no significant differences in the decrease in MOE between cedar and ACQ-treated pine, but both cedar and ACQ-treated pine were degraded up to 19%.

The 14 month pine samples contained no fungal hyphae in the S2 region. However, at18 months, numerous fungal hyphae were observed in the cell lumen and the

cell wall structure was degraded. At 14 months and 18 months, many microorganisms colonized the exterior of pine stakes and caused discoloration. A few microbes visually colonized the exterior of cedar stakes at 14 months and 18 months. During the same time, some microbes colonized the exterior surface of ACQ-treated pine stakes causing minor visual discoloration. At 18 months, six species of basidiomycetes were detected on pine, three on cedar, and five on ACQ treated pine. *Trametes elegans* and *Trametes lactinea* were found all three wood types. *Gloeophyllum sepiarium* was only found on pine. *Gloeophyllum trabeum* was found on both cedar and ACQ treated pine at 18 months. *Phlebia radiata* was found on pine and ACQ-treated pine but not on cedar. The expression of Pr-Lip and the expression of Pr-Mnp in pine over 18 months were not significantly different in ACQ-treated pine. However, the expression of Pr-Lcc in ACQ-treated pine at 16 months and 18 months were significantly higher than pine. No enzymes were expressed on cedar.

Proteins spots on pine continually increased from 64 at 14 months to 124 at 18 months. Proteins on cedar were first detected at 14 months. From cedar, 2 protein spots were detected at 14 months, 3 protein spots at 16 months, and 16 protein spots at 18 months. From ACQ-treated pine, there were 34 protein spots at 14 months which increased to 47 protein spots at 18 months. The largest functional group of proteins on pine at 18 months was glycolysis. Basidiomycete proteins dramatically increased on pine at 18 months. The number of proteins from cedar involved in electron transport and glycolysis increased at 18 months. However, no decay enzymes were found on cedar. Bacteria proteins were detected on cedar at 18 months. Fungi and basidiomycetes proteins were detected from 14 months to 18 months, but most proteins were involved in

the metabolism. The proteins of basidiomycetes on ACQ-treated pine continually increased up to 18 months. The largest protein functional group on ACQ-treated pine at 18 months was glycolysis but no decay enzymes were found. The percentage of proteins from basidiomycetes increased continually on pine and ACQ-treated pine, but not on cedar.

## CONCLUSION

This study compared microbial colonization and gene expression on woods with two different durabilities (natural vs chemical). These results indicate that different resistant woods have different effects on the microbial communities and their enzymatic activities during decay. Western red cedar negatively influenced the colonization of fungi probably because of toxic chemicals such as thujaplicin and thujic acid (Van der Kamp 1986). When the fungus is starved, it produces the decay enzymes required to degrade wood to provide nutrients for survival (Maheshwari *et al*. 2000). When a decay enzymes like Lcc is produced by a fungus, copper ions can increase the transcription of Lcc because the cofactor of Lcc is the copper ion (Solomon *et al*. 1996). Copperions in ACQ-treated pine might provide the particular condition for activating Lcc gene.

In natural forest soil, there are many fungal species that degrade wood. Although only *P. radiata* quantitive gene expression was measured, this study also screened for the presence (genomic DNA) and expression (cDNA) of lignin modifying enzymes in basidiomycetes from three wood types. Such new information could lead to a better understanding of wood decay. The results showed that the naturally durable cedar reduced the wood decay community and its activities in comparison to untreated pine and

ACQ-treated pine. ACQ-treated wood did not stop the production of the laccase decay enzymes but did inhibit the effectiveness of the enzymes. Metaproteomics provided functional, and taxonomical grouping from a mixed environment from different wood types during biodeterioration. The microorganisms on non-resistant pine produced more proteins, more decay enzymes, and had a higher percentage of MOE loss than cedar or ACQ-treated pine. Few proteins were produced on naturally durable cedar and these were produced late in this study. There were fewer proteins produce on chemically-resistant ACQ-pine than on untreated pine, but more than on cedar. The type of proteins produced on ACQ-treated wood was very different from untreated pine as was the microbial source of proteins. To our knowledge, this is the first report that describes expression of decay genes and proteins on different wood types in forest soils. The study also provides a decay comparison of natural durable wood versus chemical protected wood.

BIBLIOGRAPHY

Van der Kamp, B.J. 1986. Effects of heartwood inhabiting fungi on thujaplicin content and decay resistance of western red cedar (*Thuja plicata* Donn). Wood and Fiber Science 18(3):421-427.

Maheshwari, R., G. Bharadwaj, and M.K. Bhat. 2000. Thermophilic fungi: their physiology and enzymes. Microbiology and Molecular Biology Reviews 64:461-488.

Solomon, E. I., U.M. Sundaram, and T.E. Machonkin. 1996. Multicopper oxidases and oxygenases. Chemical Reviews 96: 2563-2606.

# APPENDIX

# CURRICULUM VITAE

# CURRICULUM VITAE

**Youngmin Kang Ph.D**

## *Education*

**Ph.D., Forest Resources (Forest products)-May 2010**
**(Minor in Biochemistry)**
June 2007 – May 2010, Dep. of Forest Products, Mississippi State University.
August 2005 – May 2007, Dep.of Forestry, Mississippi State University, USA.
*Dissertation*: Molecular approaches for characterization of biodegradation genes expressed during microbial colonization of decay resistant and non-resistant woods in forest soil.

**M. S., Forest Resources (Forest production and protection)-February 2005**
March 2003 – February 2005, Department of Forest Resources,
Gyeongsang National University, Republic of Korea.
*Thesis*: In vitro propagation and metabolic engineering by tropane alkaloids biosynthesis genes of *Scopolia parviflora.*

**B. S., Forest Resources (Forestry)-February 2003**
**(Minor in Horticulture)**
March 1998 – February 2003, Department of Forest Resources,
Gyeongsang National University, Republic of Korea.
*Thesis*: In vitro propagation of junos orange (*Citrus junos*) through nucellar polyembryoid cultures and genetic analysis of identical polymorphism by RAPD markers.

## *Professional experience*

1. **June 2007 to May 2010**: Graduate Research Assistant (Doctoral degree) and student worker, Laboratory of Wood Microbiology and Biotechnology (Co-PI: Dr. Lynn Prewitt and Dr. Susan Diehl), Department of Forest Products, Mississippi State University, USA.

2. **August 2005 to May 2007**: Graduate Research Assistant (Doctoral degree) and student worker, Laboratory of International Molecular Forest Technology (Co-PI: Dr. Emily Schultz and Dr. Cetin Yuceer), Department of Forestry, Mississippi State University, USA.

3. **March 2003 to February 2005**: Graduate Research Assistant (Master degree), Forest biotechnology & Tree breeding laboratory (PI: Dr. Myung-Suk Choi), Department of Forest Resources, Gyeongsang National University, Republic of Korea.

4. **March 2000 to February 2003**: Technical Assistant (Bachelor degree), Forest biotechnology & Tree breeding laboratory (PI: Dr. Myung-Suk Choi), Department of Forest Resources, Gyeongsang National University, Republic of Korea.

***Publication (peer-reviewed articles)***


1. **Y. Kang,** S.V. Diehl. D.D. Nicholas, and M.L. Prewitt.Gene expression of selected lignin modifying enzymes (LMEs) and screening of Basidiomycetes during biodeterioration of three different wood types. **(Submitting in 2010).**

2. **Y. Kang,** M.L. Prewitt, T. Pechan. and S.V. Diehl. Comparative protein profiling for biodeterioration of non-decay resistant and decay resistant woods. **(Submitting in 2010).**

3. Y.D. Kim, **Y. Kang**, N.Y. Ahn, J.C. Kim, K.H. Cho, S.H. Lee, M.J. Jeong, C.S. Karigar, M.S. Choi., Transformation of *Populus alba* with the SCOF-1 gene from *Soybean* improves cold tolerance. **Tree physiology (Submitted, 2010). (co-first author)**

4. **Y. Kang**, D.J. Park, J.Y. Min, H.J. Song, M.J. Jeong, Y.D. Kim, S.M. Kang, C.S. Karigar, M.S. Choi, Enhanced production of tropane alkaloids in transgenic *Scopolia parviflora* hairy root cultures over-expressing putrescine N-methyl transferase and hyoscyamine-6β-hydroxylase and their responses to growth regulators **In vitro cellular development biology – Plant (In review, 2010).**

5. **Y. Kang,** M.L. Prewitt. S.V. Diehl, Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-DE and MALDI-TOF-MS. **International Biodeterioration & Biodegradation 63(8):1036-1044 (2009).**

6. J.-Y. Min, D.-J. Park, M.-J. Jeong, H.-J. Song, S.-M. Kang, **Y. Kang**, M.-S. Choi. Selection of tropane alkaloids high-producing lines by single cell cloning of *Hyoscyamus niger* L. root cultures. **Journal of Korean Forestry Society 98(2):142~147 (2009).**

7. Y.-D. Kim, J.-Y. Min, W.-J. Kim, **Y. Kang**, H.-S. Moon, C.-H. Lee, D.T. Prasad, M.-S. Choi. High frequency plant regeneration and accumulation of tropane alkaloids in regenerated plants of *Scopolia parviflora.* **In vitro cellular development biology – Plant 42: 203–208 (2008).**

8. J.-Y. Min, H.-Y. Jung, S.-M. Kang, Y.-D. Kim, **Y. Kang**, D.-J. Park, D.T. Prasad, Myung-Suk Choi. Production of tropane alkaloids by small-scale bubble column bioreactor cultures of *Scopolia parviflora* adventitious roots. **Bioresource Technology 98:1748-1753 (2007).**

9. S.-M. Kang, J.-Y. Min, Y.-D. Kim, **Y. Kang**, D.-J. Park, H.-N. Jung, S.-W. Kim and

M.-S. Choi. Effects of methyl iasmonate and salicylic acid on the production of bilobalide and ginkgolides in cell cultures of *Ginkgo biloba*. **In vitro cellular development biology – Plant, 42:44-49 (2006).**

10. D.-J. Park, **Y. Kang**, H.-N. Jung, J.-Y. Min, Y.-D. Kim, C.S. Karigar and M.-S. Choi. Rapid micropropagation of *Hovenia dulcis* Thunb. through in vitro stem nodal cultures. **Journal of Korean Forestry Society 95(2):155-159 (2006).**

11. S.-M. Kang, H.-Y. Jung, **Y. Kang**, J.-Y. Min, C.S. Karigar, J.-K. Yang, S.-W. Kim, Y.-R. Ha, S.-H. Lee, and M.-S. Choi. Biotransformation and impact of ferulic acid on phenylpropanoid and capsium annuum L. Cv.P1482 cell suspension cultures. **Journal of Agricultural and Food Chemistry 53(9):3449-3453 (2005).**

12. O.-S. Lee, **Y. Kang**, H.-Y. Jung, J.-Y. Min, S.-M. Kang, C.S. Karigar, D.T. Prasad, J.-D. Bahk, and M.-S. Choi. Enhanced production of tropane alkaloids in *Scopolia parviflora* by introducing PMT (putrescine N-methyltransferase) gene. **In Vitro Cellular and Developmental Biology–Plant 41:167-172 (2005). (co-first author)**

13. **Y. Kang,** O.-S. Lee, H.-Y. Jung, S.-M Kang, B.-H Lee, C. Karigar, T. Prasad, J.-D. Bahk, and M.-S. Choi. Overexpression of hyoscyamine 6β-hydroxylase (*h6h*) gene and enhanced production of tropane alkaloids in *Scopolia parviflora* hairy root lines. **Journal of Microbiology and Biotechnology 15(1):91-98 (2005).**

14. **Y. Kang**, J.-Y. Min, H.-S. Moon, C.S. Karigar, D.T. Prasad, C.-H. Lee, M.-S. Choi. Rapid in vitro adventitious shoot propagation of *Scopolia parviflora* through rhizome cultures for enhanced production of tropane alkaloids. **Plant Cell Reports 23:128-133 (2004).**

15. Y.-G.. Park, S.-J. Kim, **Y. Kang**, H.-Y. Jung, D.T. Prasad, S.-W. Kim, Y.-G. Chung, M.-S. Choi. Production of Ginkgolides and Bilobalide from Optimized the *Ginkgo biloba* Cell Culture. **Biotechnology and Bioprocess Engineering 9:41-46 (2004).**

16. Y.-G. Park, S.-J. Kim, H.-Y. Jung, **Y. Kang**, S.-M. Kang, D.T. Prasad, S.-W. Kim, and M.-S. Choi. Variation of Ginkgolides and Bilobalide Contents in Leaves and Cell Cultures of in *Ginkgo biloba* L. **Biotechnology and Bioprocess Engineering 9:35-40 (2004)**.

17. S.-M. Kang, H.-Y. Jung**, Y. Kang**, D.-J. Yun, J.-D. Bahk, J.-K.Yang, M.-S. Choi. Effects of Methyl Jasmonate and Salicylic Acid on the Production of Tropane Alkaloids and the Expression of PMT and H6H in Adventitious Hairy Root Cultures of *Scopolia parviflora.* **Plant Science 166:745-751 (2004)**.

18. W.-J. Park , **Y. Kang**, J.-Y. Min, D.-J.Park, Y.-D. Kim, C.S. Karigar, M.-S. Choi. *In vitro* propagation of junos orange (*Citrus junos* Sieb.) through nucellar polyembryoid cultures**. Korean Journal of Medicinal Crop Science 12(5):384-390 (2004)**.

19. W.-J. Kim, **Y. Kang**, D.-J. Park, G.-H. Huh, B.-H. Lee, M.-S. Choi. Optimal culture condition for in vitro propagation of *Orostachys japonicus* and enhancement of polysaccharides production. **Korean Journal of Medicinal Crop Science 12(2):129-134 (2004).**

20. H.-Y. Jung, W.- J. Kim, S.-M. Kang, D.-J. Park, **Y. Kang**, M.-S. Choi. Effects of elicitors on scopolamine production of *Scopolia parviflora* Nakai. Adventitious roots in bubble column bioreactor. **Korean Journal of Medicinal Crop Science 12(5):378-383 (2004)**.

21. W.-J. Kim, H.-Y. Jung, J.-Y. Min, D.-J. Park, Y.-D. Kim**, Y. Kang**, and M.-S. Choi. Effects of growth regulators on shoot regeneration and polysaccharide production of *Orostachys japonicus* Berger. **Korean Journal of Medicinal Crop Science 12(5):391-396 (2004)**.

22. **Y. Kang**, H.-Y. Jung, S.-M. Kang, B.-R. Jin, B.-H. Lee, and M.-S. Choi. Production of monoclonal antibody about the specific key enzyme of hyoscyamine 6β-hydroxylase in *Scopolia parviflora.* **Korean Journal of Medicinal Crop Science 12(2):135-140 (2004)**.

23. **Y. Kang**, J.-Y. Min, W.-J. Kim, Y.-D. Kim, B.-H. Lee, and M.-S. Choi. Growth pattern and content of tropane alkaloids of metabolic engineered *Scopolia parviflora* hairy lines. **Korean Journal of Medicinal Crop Science 12(2):123-128 (2004)**.

24. H.-Y. Jung, S.-M. Kang, **Y. Kang**, M.-J. Kang, D.-J. Yun, J.-D. Bahk, M.-S. Choi. Enhanced production of scopolamine by bacterial elicitors in adventitious hairy root cultures of *Scopolia parviflora***. Enzyme and Microbial Technology 33:987-990 (2003).**

25. H.-Y. Jung, S.-M. Kang, **Y. Kang**, Y.-D. Kim, J.-K. Yang, Y.-G. Chung, and M.-S. Choi. Selection of Optimal Biotic Elicitor on Tropane Alkaloid Production in *Scopolia parviflora* Adventitious Hairy Root Cultures. **Korean Journal of Medicinal Crop Science 5:358-363 (2003).**

26. W.-T. Seo, J.-K. Yang, B.-K. Kang, W.-J. Park, S.-C. Hong, **Y. Kang**, H.-Y. Jung, Y.-D. Kim, S.-M. Kang, S.-W. Kim, M.-S. Choi. Leaves Extraction and Biological Activities of Essential Oil from *Thuja occidental*. **Korean Journal of Medicinal Crop Science 5:364-370 (2003).**

27. H.-Y. Jung, M.-J. Kang, **Y. Kang**, D.-J. Yun, J.-D. Bahk, Y.-G. Chung, M.-S. Choi. Optimal culture coditions and XAD resin on Tropane alkaloid production in Scopolia parviflora hairy root cultures. **Korean Journal of Biotechnology Bioengineering 6:525-530 (2002)**.

***Non-peer-reviewed articles***

**1. Y. Kang** S. Diehl, L., and D. Nicholas. Structural changes, basidiomycete richness, enzyme activity and proteomic profiling of decay resistant and non-resistant woods over 18 months in soil contact. International Research Group on Wood Protection Document **No.IRG/2010.**

**2. Y. Kang,** L. Prewitt, and S. Diehl. Gene expression of selected decay enzymes produced during biodeterioration of three wood types. International Research Group on Wood Protection Document **No.IRG/2009/10702.**

**3.** S. Diehl, L. Prewitt, **Y. Kang,** L. Mangum, J. Tang. Wood Decay Research Using Molecular Procedures, What Can It Tell Us. International Research Group on Wood Protection Document **No.IRG/2008/10678**.

**4. Y. Kang**, S. Diehl, and L. Prewitt. Characterization of protein patterns from decayed wood of loblolly pine (*Pinus taeda* L.) by proteomic analysis. International Research Group on Wood Protection Document **No.IRG/2008/10654**.

***Book Chapters***

**1.** S.-M. Kang, **Y. Kang**, M.-S. Choi. Biotechnological Approach on Production of Ginkgolides from Cell Cultures of Ginkgo Biloba. **Recent Advances in Plant Biotechnology and its Applications, I.K. International Publishing House Pvt. Ltd. New Delhi, India. pp 631-644 (2008) – ISBN: 8189866095.**

**2. Y. Kang**, H.-Y. Jung, M.-S. Choi. Production of tropane alkaloids by in vitro cultures of Korean endangered medicinal plant, *Scopolia parviflora*. **Genetic Resources and Biotechnology, Vol.2.Regency Publications, New Delhi, India. pp 248-268 (2005)- ISBN : 8189233157.**

***Acknowledgement***

1. Bacterial protection of beetle-fungus mutualism. **Science, 322, 63. 2008.** We thank A. Adams, S. Adams, C. Booth, E. Caldera, J. Ensign, C. Hsu, **Y. Kang**, A. Lawrence,W.Monroe, P. Jeffreys,M. Palmisano, A. Pinto, M. Poulsen, K. Raffa, D. Stone, and E. Vallery for assistance.

***Oral presentation***

**1. Youngmin Kang et al.,** structural changes, basidiomycete richness, enzyme activity and proteomic profiling of decay resistant and non-resistant woods over 18 months in soil contact. The International Research Group on Wood Protection, the 41st Annual Meeting Biarritz, France 9-13 May 2010.

2. **Youngmin Kang et al.,** "Gene expression of selected decay enzymes produced during biodeterioration of three wood types", The International Research Group on Wood Protection, 40th annual Meeting, Beijing, China, May 25-29, 2009.

3. **Youngmin Kang et al.,** "Gene expression of wood decay enzymes during biodeterioration of decay-resistant and non-resistant wood", The Forest Products Society 62nd International Convention, Hyatt Regency St. Louis at Union Station, St. Louis, Missouri, USA, June 22-24, 2008.

4. **Youngmin Kang et al.**, "Characterization of protein patterns from decayed wood of loblolly pine (*Pinus taeda* L.) by proteomic analysis", The International Research Group on Wood Protection, 39th annual Meeting, Istanbul, Turkey, May 25-29, 2008.

5. **Youngmin Kang et al.,** "Proteomic profiling of decay fungi on loblolly pine (*Pinus taeda* L.)",
   Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS), Starkville, Mississippi, USA, March 26-28, 2008.

6. **Youngmin Kang et al.,** "Cloning and characterization of the tropane alkaloids biosynthesis genes from *Scopolia parviflora*, a new family of proteins transport into transgenic hairy root lines", The Korean Society of Plant Biotechnology, Republic of Korea, May 08, 2004.

7. **Youngmin Kang et al.,** "Metabolic Engineering of Putrescine N-Methyltransferase (PMT) Gene Introduced into *Scopolia parviflora* for Production of Tropane Alkaloids", Korean Forestry Society, Republic of Korea, June 26, 2003.

8. **Youngmin Kang et al.**," Production of tropane alkaloids thought hairy root culture of Korea native *Scopolia parviflora*", The paper presentation of Korean university students, Seoul National University, Republic of Korea. December 01, 2001.

***Poster presentation & Proceeding papers***

1. **Youngmin Kang**, Lynn Prewitt, Susan Diehl, Darrel Nicholas, "Characterization of biodeterioration genes and proteins expressed during decay of different wood types in forest soil" The Forest Products Society 4th International Convention. June 20-22, 2010, Monona Terrace Community & Convention Center, Madison, Wisconsin, USA

2. Juliet Tang, **Youngmin Kang**, Lee Mangum, Santosh Kumar, Lynn Prewitt, Susan Diehl. "Using Wood for Biofuels: Biological Barriers to the Decomposition of Wood", The 2009 Biofuels Conference, Marriott Hotel, Jackson, Mississippi, USA, August 6-7, 2009.

3. **Youngmin Kang**, Lynn Prewitt, Susan Diehl, Darrel Nicholas, Tor Schultz, "Structural changes of resistant and non-resistant woods during decay", International

Conference on Woody Biomass Utilization, Bost Conference Center, Mississippi State University, Starkville, Mississippi, USA, August 4-5, 2009.

4. **Youngmin Kang,** Lynn Prewitt, and Susan Diehl, "Expression of selected decay enzymes during biodeterioration of different resistant woods", the 2009 US-Korea Conference on Science, Technology and Entrepreneurship, Raleigh Convention Center in Raleigh, North Carolina, USA, July 16-19, 2009.

5. **Youngmin Kang**, Susan Diehl, and Lynn Prewitt, "Comparative proteomics of three wood types during biodeterioration: , the 40th Annual Meeting of the International Research Group on Wood Protection, Jiuhua Spa & Resort, Beijing , China , May 24-28, 2009.

6. **Youngmin Kang**, Lynn Prewitt, Susan Diehl, Darrel Nicholas, Tor Schultz, " Comparison of microscopic examination and Modulus of Elasticity (MOE) of chemically treated and naturally durable wood during decay", Annual meeting of American Wood Protection Association (AWPA), San Antonio, Texas, USA, April 19-21.2009.

7. **Youngmin Kang**, Susan Diehl, Lynn Prewitt, "The comparison of proteomic pattern on decay non-resistant and decay resistant wood during biodeterioration", Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS), Mississippi, USA, March 25-27. 2009.

8. **Youngmin Kang,** Lynn Prewitt, Susan Diehl, "Identification of basidiomycetes and detection of decay enzymes on three different wood types in soil bed decay test during biodeterioration , Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS), Mississippi, USA, March 25-27. 2009.

9. **Youngmin Kang,** Lynn Prewitt, and Susan Diehl, "Gene expression of wood decay enzymes during biodeterioration of decay-resistant and non-resistant wood", The Forest Products Society 62$^{nd}$ International Convention, Hyatt Regency St. Louis at Union Station, St. Louis, Missouri, USA, June 22-24, 2008.

10. **Youngmin Kang**, Susan Diehl, Lynn Prewit. "Characterization of proteins patterns for decay fungi growing on loblolly pine (*Pinus taeda* L.)", the Forest Products Society 62$^{nd}$ International Convention, Hyatt Regency St. Louis at Union Station, St. Louis, Missouri, USA, June 22-24, 2008.

11. **Youngmin Kang**, Lynn Prewitt, and Susan Diehl. "Gene expression of decay enzymes during biodeterioration of wood" The International Research Group on Wood Protection, 39$^{th}$ annual Meeting, Istanbul, Turkey, May 25-29, 2008.

12. **Youngmin Kang,** Lynn Prewitt, and Susan Diehl. "Characterization of selected biodegradation wood decay genes expressed during microbial colonization of decay-resistant and non- resistant woods", Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS), Mississippi, USA, March 26-28. 2008.

13. **Youngmin Kang,** Susan Diehl, and Lynn Prewitt, "Proteomic profiling of decay fungi on loblolly pine (*Pinus taeda* L.)", Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS), Starkville, Mississippi, USA, March 26-28, 2008.

14. M. Lynn Prewitt, Susan V. Diehl, T.C. McElroy, Walter J. Diehl and **Youngmin Kang**. Comparison of general fungal and Basidiomycete specific ITS primers for identification of wood decay fungi. Forest Products Society 61st International Convention. Nashville, TN, USA, June 11, 2007.

15. **Youngmin Kang**, Cetin Yuceer, Kier Klepzig, Development of a submerged culture system for mapping the proteome of *Entomocorticium* sp. *A*, a mutualistic fungal associate of the southern pine beetle. The North American Forest Insect Work Conference, Asheville, NC, USA, May 23, 2006.

16. Kier Klepzig, Olga Pechanova, **Youngmin Kang**, Cetin Yuceer. Proteomic and microscopic examination of the southern pine beetle-fungus symbiosis. Proceedings of the North American Forest Insect Work Conference. Asheville, NC, USA, May 23, 2006.

17. **Youngmin Kang**, Ji-Yun Min, Yong-Duck Kim, Seung-Mi Kang, Dong-Jin Park, Ha-Na Jung, Su-Jin Lee, Myung-Suk Choi, "Metabolic Engineering of *Scopolia parviflora* through Tropane Alkaloids biosynthesis genes", Korean Forestry Society, June 23, 2005.

18. Yong-Duck Kim, **Youngmin Kang**, Seung-Mi Kang, Ji-Yun Min, Dong-Jin Park, Ha-Na Jung, Su-Jin Lee, Byung-Hyun Lee, Myung-Suk Choi, "Transformation of the chloroplast-localized small heat shock protein(smHSP) gene in *Populous glandulosa*", Korean Forestry Society, June 23, 2005.

19. Dong-Jin Park, **Youngmin Kang**, Ha-Na Jung, Ji-Yun Min, Yong-Duck Kim, Seung-Mi Kang, Myung-Suk Choi, "Propagation of *Hovenia dulcis* Thumb, through In vitro cultures", Korean Forestry Society, June 23, 2005.

20. **Youngmin Kang**, Ji-Yun Min, Yong-Duck Kim, Seung-Mi Kang, Dong-Jin Park, Ha-Na Jung, Sun-Won Kim, Byung-Hyun Lee, Myung-Suk Choi, "Metabolic Engineering of Transgenic Spearmint (*Mentha spicata*) through Limonene Synthase Gene for Useful Terpenoids Production", The Korean Society of Plant Biotechnology, April. 23, 2005.

21. Ji-Yun Min, Yong-Duck Kim, **Youngmin Kang**, Seung-Mi Kang, Dong-Jin Park, Ha-Na Jung, Myung -Suk Choi, "Optimal Culture Conditions for In Vitro Propagation of

Hinoki Cypress (*Chamaecyparis obtusa* Sieb. et Zucc.)", The Korean Society of Plant Biotechnology, April 23, 2005.

22. Seung-Mi Kang, Ji-Yun Min, Yong-Duck Kim, **Youngmin Kang**, Dong-Jin Park, Ha-Na Jung, Sun-Won Kim, Myung-Suk Choi, "Effects of Methyl Jasmonate and Salicylic Acid on the Production of Bilobalide and Ginkgolides in Cell Suspension Culture of *Ginkgo biloba*", The Korean Society of Plant Biotechnology, April 23, 2005.

23. Dong-Jin Park, **Youngmin Kang**, Ha-Na Jung, Ji-Yun Min,Young- Duck Kim, Seung-Mi Kang, Myung-Suk Choi, "Propagation of *Hovenia dulcis* Thumb. through In Vitro Cultures", The Korean Society of Plant Biotechnology, April 23, 2005.

24. Seung-Mi Kang, Ji-Yun Min, Ha-Na Jung, **Youngmin Kang**, Yong-Duck Kim, Won-Jung Kim, Dong-Jin Park, Myung-Suk Choi, "The influence of methyl jasmonate and salicylic acid on the production of ginkgolides and bilobalide in cell suspension cultures of *G. biloba*", The Korean Society of Plant Biotechnology, October 22, 2004.

25. Yong-Duck Kim, Won-Jung Kim, **Youngmin Kang**, Seung-Mi Kang, Ji-Yun Min, Dong-Jin Park, Ha-Na Jung, Myung-Suk Choi, "Changes of tropane alkaloids contents based on shoot differentiation stage of *Scopolia parviflora* callus", The Korean Society of Plant Biotechnology, October 22, 2004.

26. **Youngmin Kang** and Myung-Suk Choi, "Production of tropane alkaloids from transgenic hairy root lines of *Scopolia parviflora*", World Congress on In Vitro Biology, San Francisco, California USA. May 22, 2004.

27. Won-Jung Kim, Hee-Young Jung, Dong-Jin Park, **Youngmin Kang**, Yong-Duck Kim, Ji-Yun Min, and Myung-Suk Choi, "Production of Scopolamine by Elicitors of *Scopolia parviflora* roots in bubble column bioreactor", The Korean Society of Plant Biotechnology, May 08. 2004.

28. Ji-Yun Min, Hee-Young Jung, **Youngmin Kang**, Yong-Duck Kim, Seung-Mi Kang, Won-Jung Kim, Dong-Jin Park, Ha-Na Jung, Myung-Suk Choi, "Selection of tropane alkaloids high-producing root lines through the Dragendorff's reagent method in adventitious root culture of solanaceae plant", The Korean Society of Plant Biotechnology, May 08. 2004.

29. Yong-duck Kim, **Youngmin Kang**, Hee-Young Jung, Seung-Mi Kang, Won-Jung Kim, Ji-Yun Min, Byung-Hyun Lee, Myung-Suk Choi" Transformation of the chloroplast-localized small heat shock(smHSP) gene in *Populus alba*", The Korean Society of Plant Biotechnology, May 08, 2004.

30. Seung-Mi Kang, **Youngmin Kang**, Yong-Duck Kim, Hee-Young Jung, Won-Jung Kim, Ji-Yun Min, Dong-Jin Park, Myung-Suk Choi, "Mass propagation of

C*amptotheca acuminata* through auxiliary bud culture and Camptothecin analysis", The Korean Society of Plant Biotechnology, May 08, 2004.

31. **Youngmin Kang**, Eun Sil Lee, Hee Young Jung, Seung Mi Kang, Yong-Duck Kim, Won Jung Kim, Ji Yun Min, Dong Jin Bahk, Jung Dong Bahk, Myung Suk Choi. "Cloning and characterization of the tropane alkaloids biosynthesis genes from *Scopolia parviflora*, a new family of proteins transport into transgenic hairy root lines" The Korean Society of Plant Biotechnology, May 08, 2004.

32. Won Jung Kim, **Youngmin Kang**, Dong Jin Park, Gyung Hye Huh, Byung Hyun Lee, Myung Suk Choi. "Optimal culture condition for in vitro propagation of *Orostachys japonicus* and enhancement of polysaccharides production", Korean Journal of Medicinal Crop Science. May 07, 2004.

33. **Youngmin Kang**, Ji Yun Min, Won Jung Kim, Yong Duck Kim, Byung Hyun Lee and Myung Suk Choi. "Growth pattern and content of tropane alkaloids of metabolic engineered *Scopolia parviflora* hairy lines" Korean Journal of Medicinal Crop Science. May 07, 2004.

34. **Youngmin Kang**, Hee Young Jung, Seung Mi Kang, Byung Rae Jin, Byung Hyun Lee and Myung Suk Choi."Production of monoclonal antibody about the specific key enzyme of hyoscyamine 6β- hydroxylase in *Scopolia parviflora.* Korean Journal of Medicinal Crop Science. May 07, 2004.

35. Seung-Mi Kang, **Youngmin Kang**, Yong-Duck Kim, Hee-Young Jung, Won-Jung Kim, Ji-Yun Min, Dong-Jin Park, Myung-Suk Choi, "Mass propagation of C*amptotheca acuminata* through auxiliary bud culture and Camptothecin analysis", Korean Forestry Society, Feburary 12. 2004.

36. Ji-Yun Min, **Youngmin Kang**, Yong-Duck Kim, Hee-Young Jung, Seung-Mi Kang, Won-Jung Kim, , Dong-Jin Park, Myung-Suk Choi, "Selection of tropane alkaloid high producing cell lines by Dragendorff's reagent method from *Scopolia parviflora* root cultures", Korean Forestry Society, Feburary 12, 2004.

37. Jae Kyung Yang, Won Taek Seo, Hee Young Jung, **Youngmin Kang**, Seung Mi Kang, and Myung Suk Choi, Seon Won Kim, Biological activities and terpenoid composition from Essential Oil of *Thuja orientalis,* The Korean Society of Plant Biotechnology, November 01, 2003.

38. Won-Jung Kim, **Youngmin Kang**, Hee-Young Jung, Seung-Mi Kang, Dong-Jin Park, Yong-Duck Kim, Ji-Yun Min, Myung-Suk Choi . Plant regeneration from hairy root derived calli in *Scopolia parviflora,* The Korean Society of Plant Biotechnology, November 01, 2003.

39. **Youngmin Kang**, Hee Young Jung, Seung Mi Kang, Yong-Duck Kim, Won Jung Kim, Ji Yun Min, Dong Jin Bahk, Dae Jin Yun, Jung Dong Bahk, Myung Suk Choi .

Differences of tropane alkaloids content, growth and morphology in metabolic engineered *Scopolia parviflora* hairy root lines, The Korean Society of Plant Biotechnology, November 01, 2003.

40. Seung-Mi Kang, Hee-Young Jung, **Youngmin Kang**, Yong-Duck Kim, Won-Jung Kim, Ji-Yun Min, Dong-Jin Park, Seon-Won Kim, Myung-Suk Choi, Biotransformation of ferulic acid to vanillin and capsaicin in suspension cell cultures of *Capsicum annuum*, The Korean Society of Plant Biotechnology, November 01, 2003.

41. Hee-Young Jung, Seung-Mi Kang, Won-Jung Kim, **Youngmin Kang**, Dong-Jin Park, Yong-Duck Kim, Ji-Yun Min, and Myung-Suk Choi, The Small-Scale Bioreactor Culture for the Production of Tropane Alkaloids, The Korean Society of Plant Biotechnology, November 01, 2003.

42. **Youngmin Kang**, Ok-Sun Lee, Hee-Young Jung, Won-Jung Kim, Seung-Mi Kang, Ji-Yun Min, Dong-Jin Park, Myung-Suk Choi ,"Metabolic engineering of Putrescine N- Methyltransferase(PMT) gene introduced into *Scopolia parviflora* for production of tropane alkaloids ", Korean Forestry Society, June 26, 2003.

43. Seung-Mi Kang, Hee-Young Jung, **Youngmin Kang**, Won-Jung Kim, Ji-Yun Min, Dea- Jin Yun, Jung-Dong Bahk, Myung-Suk Choi, "Enhanced Production of Scopolamine by Bacterial Elicitors in Adventitious Hairy Root Cultures of *Scopolia parviflora"* , Korean Forestry Society, June 26, 2003.

44. Hee-Young Jung, Seung-Mi Kang, **Youngmin Kang**, Won-Jung Kim, Ji-Yun Min, Dong-Jin Park, Dae-Jin Yun, Jung-Dong Bahk, and Myung-Suk Choi, " Elicitation of Tropane Alkaloid Production by Use of Signaling Agents in *Scopolia parviflora* Adventitious Hairy Root Culture" , Korean Forestry Society, June 26, 2003.

45. Ji-Yun Min, Min-Jung Kang, Hee-Young Jung, **Youngmin Kang**, Seung-Mi Kang, Won-Jung Kim, Dong-Jin Park, Myung-Suk Choi, "Selection of tropane alkaloids high-producting cell lines through efficient selection method in *Hyoscyamus niger* root culture", Korean Forestry Society, June 26, 2003.

46. **Youngmin Kang,** Ji-Yun Min, Won-Jung Kim, Hee-Young Jung, Seung-Mi Kang, Dong-Jin Park, Dae-Jin Yun, Jung-Dong Bahk, Myung-Suk Choi "Rapid propagation and tropane alkaloid production of *Scopolia parviflora* through rhizome cultures" The Korean Society of Plant Biotechnology, April 25, 2003.

47. Hee Young Jung, Seung Mi Kang, **Youngmin Kang**, Won Jung Kim, Ji Yun Min, Dong Jin Bahk, Dae Jin Yun, Jung Dong Bahk, Myung Suk Choi," Selection of Optimal Biotic Elicitor on Tropane Alkaloids Production in *Scopolia parviflora* Adventitious Hairy Root Cultures", The Korean Society of Plant Biotechnology, April 25, 2003.

48. Seung Mi Kang, Hee Young Jung, **Youngmin Kang**, Won Jung Kim, Ji Yun Min, Dong Jin Bahk,Dae Jin Yun, Jung Dong Bahk, Myung Suk Choi," The Expression Level of PMT and H6H and the Accumulation of Tropane Alkaloids by Signaling Compounds in *Scopolia parviflora* Adventitious Hairy Root Cultures", The Korean Society of Plant Biotechnology, April 25, 2003.

49. Jae Kyung Yang, Byung Kook Kang, Seong Cheol Hong, Woo Jin Park, Min Jung Kang, **Youngmin Kang**, Hee Young Jung, Seung Mi Kang, Won Jung Kim, Ji Yun Min, Dong Jin Bahk, Won Taek Seo and Myung Suk Choi ," Efficient Extraction Methods and Analysis of Essential Oil from Softwood Leaves", The Korean Society of Plant Biotechnology, April 25, 2003.

50. Hee Young Jung, Seung Mi Kang**, Youngmin Kang**, Won Jung Kim, Ji Yun Min, Dong Jin Bahk, Dae Jin Yun, Jung Dong Bahk, Myung Suk Choi,"Effects of Conditioning Factors on Production of Tropane Alkaloids in *Scopolia parviflora* Adventitious Hairy Root Cultures", The Korean Society of Plant Biotechnology, April 25, 2003.

51. **Youngmin Kang**, Ok-Sun Lee, Hee-Young Jung, Won-Jung Kim, Seung-Mi Kang, JI-Yun Min, Dong-Jin Bahk, Dae-Jin Yun, Jung-Dong Bahk, Myung-Suk Choi," Production of tropane alkaloids by metabolic engineering of *Hyoscyamus niger* H6H (hyoscyamine 6β-hydroxylase) gene introduced *Scopolia parviflora* hairy root", The Korean Society for Biotechnology and Bioengineering, April 12, 2003.

52. Hee-Young Jung, Seung-Mi Kang, **Youngmin Kang**, Won-Jung Kim, Ji-Yun Min, Dae-Jin Yun, Jung-Dong Bahk, Myung-Suk Choi," The effects of various biotic elicitors on the accumulation of scopolamine and hyoscyamine by adventitious hairy root cultures of *Scopolia parviflora",* The Korean Society for Biotechnology and Bioengineering, April 12, 2003.

53. **Youngmin Kang**, Woo-Jin Park, Byung-Hyun Lee, Myung-Suk Choi."Efficient propagation by Poly-embryogenesis of *Citrus junos*", The Korean Society of Plant Biotechnology, November 01, 2002.

54. Seung-mi Kang, Hee-Young Jung, Min-Jung Kang, **Youngmin Kang**, Won-Jung Kim, Jin-Yun Min, Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi, "The influence of Salicylic acid and Methyl jasmonate on the production of Tropane alkaloids in adventitious hairy root cultures of *scopolia parviflora*,The Korean Society of Plant Biotechnology, November 01, 2002.

55. Hee-Young Jung, Seung-mi Kang, **Youngmin Kang**, Min-Jung Kang, Won-Jung Kim, Jin-Yun Min, Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi "Elicitors to increase Tropane alkaloid production in *scopolia parviflora* adventitious hairy root culture". The Korean Society of Plant Biotechnology, November 01, 2002.

56. Hee-Young Jung, Seung-Mi Kang, Min-Jung Kang, **Youngmin Kang**, Jin-Yun Min,

Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi, Enhanced production of tropane alkaloids by elicitation in adventitious hairy root cultures of *scoplia praviflora* ",The Korean Society for Biotechnology and Bioengineering, October 25, 2002.

57. **Youngmin Kang**, Ok-Sun Lee, Hee-Young Jung, Min-Jung Kang, Seung-mi Kang, Won-Jung Kim, Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi, "Production of Tropane alkaloids by metabolic engineering of putrescene N-methyltransferase(PMT) gene of scopolia *parviflora*."The Korean Society for Biotechnology and Bioengineering, October 25, 2002.

58. Hee-Young Jung, **Youngmin Kang**, Min-Jung Kang, Jung-Dong Bahk, Dae-Jin Production of scopolamine and hyoscyamine by elicitation of adventitious hairy root of *Scopolia parviflora,* The Korean Society of Plant Biotechnology, April 26, 2002.

59. Seung-mi Kang, Hee-Young Jung, Min-Jung Kang, **Youngmin Kang**, Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi, "Enhanced production of tropane alkaloid in *scopolia parviflora* hairy root cultures by optimization of micro and macro components" The Korean Society of Plant Biotechnology , April 26, 2002.

60. Hee-Young Jung, Min-Jung Kang, **Youngmin Kang,** Seung-mi Kang, Woo-Jin Park, Jung-Dong Bahk, Dae-Jin Yun, Myung Suk Choi "Production of Tropane alkaloids from *scopolia parviflora* of hairy roots." The Korean Society for Biotechnology and Bioengineering, July 11. 2001.

***Invited speech (Guest presentation)***

**1.** Forest and wood protection by biotechnology, College of agriculture and life science, **Gyeongsang National University, Republic of Korea, May 12. 2009.** (Host: Dr. Myung-Suk Choi in department of Environmental Forest Resources in GNU).

***Teaching experience and related works***

**1.** Guest Lecturer, Environmental Issues in Forest Products (FP 8133), Mississippi State University, USA, **21, October, 2008** (Thank-you letter provided by **Dr. Hamid Borazjani**).

**2.** Attending in General Teaching Assistant Work Shop in studies of graduate school at Mississippi State University, USA. **13-14, August, 2007.**

**3.** Attending in International Teaching Assistant Work Shop in studies of graduate school at Mississippi State University, USA. **06-10, August, 2007.**

***Awards & Fellowships***

1. **Travel Assistance Grant for Graduate Students (TAGGS) Award ($1,250)**, pleased to award by the Office of the Graduate School at Mississippi State University, April, 2010.

2. **Commendable Student Chapter Performance Award** (2008-2009 Events Coordinator of Mississippi State University Student Chapter) by Forest Products Society (It was announced during the official Luncheon at the 2009 International convention in Boise, Idaho by the President Anthony Weatherspoon), July 21, 2009.

3. **The second Place in Poster presentation in Southeastern Natural Resources-Graduate Student Symposium (SNR-GSS)**, (This award consists of an engraved plaque and a cash honorarium of **US$150**) Mississippi, USA, March 25-27, 2009.

4. **The second Place in the 2008 FPS Wood Award competition** (This award consists of an engraved plaque and a cash honorarium of **US$500** sponsored by ARCLIN plus free conference registration fee) by Forest Product Society, USA, June 22-24, 2008.

5. **The Ron Cockcroft Award (RCA) by the International Research Group on Wood Protection (IRG-WP)** in 39th Annual Conference of IRG-WP in Istanbul, Turkey, May 25-29, 2008 (Swedish Kronor 13,460 [**=US$1,800** ]plus free conference registration fee).

6. **The Fellowship of the Graduate Studies Abroad Program** (Doctoral degree) by Korea Science and Engineering Foundation (KSEF) in South Korea, 2005 – 2007 (Fellowship: **US$60,000**).

7. **The Best Poster Presentation Award,** the Korean Society of Plant Biotechnology, April 23, 2005 (Cash honorarium of **US$100**).

8. **The Best Poster & Oral Presentation Award**, the Korean Society of Plant Biotechnology, May 08, 2004 (cash honorarium of **US$100**).

9. **The Best Poster Presentation Award**, Development of Core Plant Biotechnology for Next Generation, the Korean Society of Plant Biotechnology, November 01. 2003 (Cash honorarium of **US$100**).

10. **The Best Poster Presentation Award**, Plant Bio-venture Festival, Jinju city hall, The Korean Society of Plant Biotechnology, April 25, 2003 (Cash honorarium of **US$100**).

11. **The Second place award,** Students paper presentation of Gyeongsang National University, Gyeongsang National University, October 1, 2002 (Cash honorarium of **US$100**).

12. **The Second place award, the competition of research presentation in Korean universities**, Seoul National University, December 01, 2001 (Cash honorarium of **US$500**).

***Specialized technical Skills***

- Molecular / biochemistry techniques (genomics, transcriptomics, and proteomics)
- Bioinformatics & Computational biology (PERL, BLAST, SEQ, and NCBI database)
- Cell & tissue cultures and propagation (plant, microbe, and insect bearing)
- Analytical techniques (LC and GC)
- Microscopic techniques (LM, DM, SEM, ESEM, and TEM)

***Patent***

- **Submitting application**: Production method of tropane alkaloids by pmt, h6h genes introduced hairy root cultures of *Scopolia parviflora* Nak. Korean Intellectual Property Office (code 10-2003-0014928)

***Licenses & Certifications***

1. **Certification of Woodshop Operator** by Certifying instructors (Dr. Rubin Shmulsky & Mr. David Butler) in department of Forest Products at Mississippi State University. 2007.

2. **License of English Story Teller for Children**, Korea National University's Lifelong Education Center, Association for Certification of Qualifications. 2005.

3. **License of English Teacher for Children**, Korea National University's Lifelong Education Center, Association for Certification of Qualifications. 2004.

4. **Secondary school teacher (middle and high school grade)**, Plant Resources and Landscaping, The delegation of authority from the Korea Minister of Education & Resources Development. 2003.

5. **Secondary school teacher (middle and high school grade)**, Forestry, The delegation of authority from the Korea Minister of Education & Resources Development. 2003.

***Social Service Activities***

**1.** Member of Forest Products Society (FPS), USA, 2007- present.

**2**. Member of Korean-American Science Engineering Association (KSEA), USA, 2009- present.

**3.** Member of Forest Products Society at Mississippi State University, USA, 2007 - 2010.

**4.** Events Coordinator, Forest Products Society Chapter at Mississippi State University, 2008-2009.

**5.** Representative Graduate Student Ambassador in Department of Forest Products (nominated by Department Head), Mississippi State University graduate ambassador program, USA, 2009- present.

**6.** SNRGSS Travel Award Committee, Southeastern Natural Resources-Graduate Student Symposium (SNRGSS), Mississippi, USA, February 25-27. 2009.

**7.** Student member of Korean Student Association at Mississippi State University, USA, 2005-2010.

**8.** President of Korean Student Association at Mississippi State University, USA, 2005-2006. (Advisor: Dr. Hyena Lim in department of mathematics and statistics).

***References***

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**2. Dr. Susan Diehl**
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**3. Dr. Darrel Nicholas**
- Position: Professor
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