INVESTIGATING THE PHENOLIC DEFENSE

MECHANISMS OF LOBLOLLY AND WHITEBARK PINE

by

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(Under the Direction of Caterina Villari)

ABSTRACT

The chemical defense response of pines includes both terpenes and phenolic compounds. Phenolic compounds are secondary metabolites that are ubiquitous, but highly variable, among plant species. The focus of this thesis is the phenolic component of the defense mechanisms of two north American pine species, loblolly pine (*Pinus taeda*) and whitebark pine (*P. albicaulis*). Chapter 2 aims to characterize the phenolic profile of loblolly pine, which had yet to be identified, and investigate how the compounds may change after inoculation with root feeding beetle-associated blue stain fungi. I identified and quantified 25 compounds and measured their significant variation, both as increases or decreases, after induction. Chapter 3 investigates whether the phenolic composition of whitebark pine can be used to predict potentially resistant individuals to mountain pine beetle. While I found significant differences in single compound concentrations between resistant phenotypes, the overall profile was not a good predictor for resistance.

INDEX WORDS: Loblolly pine, Phenolics, Secondary metabolites, Whitebark pine

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DEDICATION

Dedicated to my parents: Terry and Nancy Parker. Although y'all never fully understood the direction I decided to take my life, or logic, both of you supported me either way. I feel very blessed by the life I was provided and know that the completion of this chapter of my life will make you both very proud.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

THESIS INTRODUCTION AND LITERATURE REVIEW

1.1 Defense Responses in Pines

Conifers consist of some of the oldest living species of trees and fill many different ecological and economic niches. One of the more popular families of conifers is the Pinaceae, which include lobolly pine (*Pinus taeda L.*) and whitebark pine (*Pinus albicaulis* Engelm.), the two species of focus for this thesis. Due to its fast growth and straight form, loblolly pine is the dominant timber species grown commercially in the southeastern forests of the United States and is a major source of softwood products worldwide, making it a significant species for the economy (Fox et al. 2004). Fortunately, loblolly pine is currently not facing any major threat from a pathology point of view, even though selection for faster growth often comes with the tradeoff of being less able to defend itself against pests and pathogens (Loehle and Namkoong 1987). Conversely, whitebark pine is a slow-growing species found in high-elevation ecosystems in the western United States, where it is considered a keystone species. Unfortunately, whitebark populations are currently declining, and it is listed as endangered on the IUCN Red List (Goeking and Izlar 2018).

Like all plants, pines must protect themselves from biotic stressors through defense mechanisms that can be of different nature: from those directly damaging the attacking agent to those attracting/favoring natural enemies; or from those that constitute physical barriers for the agents to those that are of chemical nature (Franceschi et al. 2000). Because of limited resources

within the plant, however, there can be a tradeoff between growing and defending, as predicted by the growth-differentiation balance hypothesis (GDBH) (Herms and Mattson 1992). Functions to do with growing and other basic living needs, such as photosynthesis and respiration, are referred to as primary metabolism. Secondary metabolites, on the other hand, are regarded as compounds found in specialized cells that are not required for essential metabolic processes related to cell maintenance, photosynthesis, or respiration but are still necessary for surviving in respective environments (Lattanzio et al. 2006). When a plant needs to defend itself, limited resources might need to be diverted from primary metabolic functions to secondary metabolism, which comprises the production of defense related metabolites, among others. In pine species, defense related secondary metabolites primarily consist of compounds classified as phenolics and terpenes (Hofstetter et al. 2005). The role of these metabolites is to repel the invading organism by manipulating behavior and/or impeding growth (Raffa et al. 2017). Terpenes are recognized as having a strong association with herbivores, either aiding in tree defense (Keeling and Bohlmann 2006) or being exploited by herbivores to locate a suitable host (Raffa 2014). When a tree is wounded, a viscous oleoresin comprised of monoterpenoids, sesquiterpenoids, and diterpene acids is secreted that can physically push out or entomb invading insects and clean and seal the wound (Keeling and Bohlmann 2006). Phenolics, on the other hand, are well known for having antifungal and antioxidant properties, as well as being activators of plant defense related metabolic pathways (Hammerschmidt 2005). Metabolites used for plant defense from both classes can be either constitutive or induced. Constitutive defenses are always present, while induced defenses are developed only in response to a stressor and are usually of chemical nature because of their need to be rapid and sometimes highly specialized, though physical defenses can also be induced (Young and Okello 1998). Resin ducts, for instance, always contain

some amount of constitutive secondary metabolites (in this case, terpenoids) ready to defend the tree, but once a duct is stressed or exposed to oxygen, this can rapidly induce the production of more secondary metabolites (Berryman 1972). Conifers under stress can also produce traumatic resin ducts (TRD) near the site of stress or in remote areas of the tree (Franceschi et al. 2000), which is a good example of an induced physical defense mechanism. In Norway spruce (*Picea abies* (L.) Karst), it was found that wounding and fungal inoculation led to the production of TRDs in the secondary xylem and they were often associated with radial ray cells (Nagy et al. 2000). This same study also observed differences in the resin produced by the TRDs as there were compositional changes in the terpene content as well as the presence of phenolics. A later study on Norway spruce found that TRDs could be induced without wounding and by applying methyl jasmonate (Martin et al. 2002). This was an interesting finding because methyl jasmonate is known to be a signaling molecule in the defense response of many plant species (Farmer et al. 1992).

Typically, conifer defenses, and plants in general, vary depending on the causal agent of stress, but in some cases, different pathogens induce the same response (Koricheva et al. 2004, Villari et al. 2012). Plants rely on microbe-associated molecular patterns (MAMPs), also called pathogen-associated molecular patterns (PAMPs) in more specific cases, to recognize the attacking agent (Bolton 2009). For example, a MAMP could be a unique structure possessed by an attacking agent, such as fungal chitin, and that information can induce a response specific to fungal infection (Kaku et al. 2006). However, plants may also produce several types of defense in response to an attack and in certain environments that can be advantageous (Koricheva et al. 2004).

This thesis explores the phenolic component of the of the defense mechanisms of both loblolly and whitebark pine to fungal pathogens and beetle colonizers. In the case of loblolly pine, we characterized the phenolic compounds present in the tree phloem, as this information was lacking in current loblolly literature. We also investigated whether the constitutive properties of loblolly phenolics differ from induced properties when trees are inoculated with ophiostomatoid fungal species associated with collar and root feeding beetles commonly found in the Southeast.

In the case of whitebark pine, we analyzed whether the phenolic profile could discriminate between survivors and potentially susceptible trees within a stand that experienced ~95% mortality that is primarily due to mountain pine beetle (*Dendroctonus ponderosae* Hopkins).

1.2 Role of Phenolics

The term phenolic was defined by Harborne (1989) as "a substance which possesses an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives". Phenolic compounds are one of the most prevalent groups of antioxidative secondary metabolites produced by plants that arise from the shikimate-phenylpropanoids-flavonoids pathways (Lattanzio et al. 2006, Pereira et al. 2009). In other words, there is high variability among existing phenolics even though they share a common origin from the amino acids phenylalanine or tyrosine (Seabra et al. 2006). Phenolics are needed for a wide range of various functions for plant survival like pigmentation, growth, reproduction, resistance to pests and pathogens, and many more (Hammerschmidt 2005, Lattanzio et al. 2006, Witzell and Martín 2008, Pereira et al. 2009). Regarding plant defense, phenolics participate in both constitutive and induced defenses. The roles of constitutive phenolics in plant defense,

however, are still poorly understood and may not have primary functions but rather contribute to resistance indirectly (Witzell and Martín 2008). On the other hand, the roles of induced phenolics are well established as phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, and signaling molecules (Hammerschmidt 2005).

There are several classes of phenolic compounds with thousands of unique structures, and they can vary greatly depending on the taxon of plant and its respective environment. (Lattanzio et al. 2006, Seabra et al. 2006, Moore et al. 2014). The main classes of phenolics are: derivates of both benzoic and cinnamic acid, flavonoids, anthocyanidins, tannins, coumarins, stilbenes, lignans, and lignins (Seabra et al. 2006). While compounds belonging to each class can be found in conifers, stilbenes and flavonoids are most associated with conifers' induced responses to pathogens, with the former being the most frequently studied in this context (Witzell and Martín 2008). The stilbenes pinosylvin and pinosylvin monomethyl ether and some flavonoids were found to accumulate in the reaction zone to fungal infection in Scots pine (*Pinus sylvestris* L.) (Lieutier et al. 1996) and these same stilbenes showed antifungal properties against *Heterobasidion annosum* (Fr.) Bref in Scots pine (Bonello et al. 1993). However, a later study observed no negative affects to *H. annosum* by any concentration of Norway spruce phenolics, and higher concentrations even stimulated fungal growth (Evensen et al. 2000). In a separate study on Norway spruce, infection by *Gremmeniella abietina* (Lagerberg) Morelet caused an accumulation of lignin and cell-wall-bound phenolics in bark tissues at the infection site, as well as accumulation of benzoic acid derivatives in regions not in contact with the pathogen (Cvikrová et al. 2006). Lignification was also observed by Bonello and Blodget (2003) in Austrian pine (*Pinus nigra* Arnold) when inoculated with *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, but only seemed to have a moderate defensive effect at the infection site. Other phenolic

compounds involved in conifer defense may just serve as precursors for larger molecules (Witzell and Martín 2008). These were but a few examples of the role phenolics play in conifer defense, but current literature supports that there is considerable variation among compound concentrations, both constitutive and induced, and their effect on tree resistance to pathogens (Witzell and Martín 2008).

1.3 The Loblolly System

1.3.1 The Host: *Pinus taeda*

The success of plantation forestry in the southeastern United States has allowed the region to become the "woodbasket" of the United States (Schultz 1997) and due to continued success, this region may even be the woodbasket of the world (Fox et al. 2004). In these plantations, loblolly pine is the dominant species grown and in 2010, planted pine accounted for 19 percent of southern forests (Wear 2013, McKeand 2019). It is expected that by the year 2060, planted pine will account for 24-36% of forested area even though the amount of all forested areas is expected to decrease (Wear 2013). Thus, loblolly pine has a significant role in the economy of the southeastern United States as well as the timber market worldwide.

Much of the success of growing loblolly pine in the region is because it is a native species that was selected particularly for its fast growth and straight form. Loblolly pine is a three-needle pine that reaches maturity at age 80 and can live up to 300 years, growing up to 18-28 meters (Schultz 1997). Needles are 15-25 centimeters long and are dark green in color. As the tree grows, older branches will self-prune, leaving a rounded crown (Schultz 1997). This species underwent intensive genetic improvement and benefitted tremendously from better silviculture in the region adhering to best management practices (Fox et al. 2004). Improvement efforts were

primarily aimed at enhancing volume growth, tree form, and wood quality but they also sought to improve disease resistance to fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*) (Dorman 1976, Zobel and Talbert 1984). While genetic improvement has proven to have economic and ecological benefits (Aspinwall et al. 2012, McKeand 2019), there are likely biological tradeoffs worthy of investigation (Herms and Mattson 1992).

1.3.2 Biotic Agents: Root-feeding Beetles and their Associated Fungi

Loblolly pine is a potential host for many different diseases and insects and stressed trees are often defending against a multitude of agents concurrently. In loblolly plantations, the three most common and relevant diseases are fusiform rust, pitch canker (*Fusarium circinatum* Nirenberg & O'Donnell), and root rot caused by *H. annosum* (Baker and Langdon 1990). While all three can damage trees at any point in their life cycle, seedlings tend to be most susceptible to pitch canker and fusiform rust whereas saplings and mature trees tend to be affected mostly by fusiform rust and *H. annosum* (Baker and Langdon 1990). Fusiform rust, by and large, causes the most economic damage (Cubbage et al. 2000), hence the importance for genetic improvement of fusiform rust resistance.

Of the different insect pests to loblolly pine, the most damaging group is the bark beetles (Coleoptera: Curculionidae) (Baker and Langdon 1990). Bark beetles are a diverse order of insects that colonize all parts of woody tissue in loblolly pine. The best studied species are those that cause the most economic damage, such as the southern pine beetle (*Dendroctonus frontalis* Zimmerman [SPB]) (Cook and Hain 1986, Harrington 2005, Knebel et al. 2008), pine engraver beetles (*Ips* spp.), and the black turpentine beetle (*D. terebrans* Olivier) (Stephen 2011). Other

loblolly insect pests of note include pine tip moths (*Rhyacionia* spp.) and cone and seed feeders (*Dioryctria* spp. and *Leptoglossus* spp.) (Baker and Langdon 1990).

While it is certainly important to investigate the economically significant species, other species should not be overlooked as contributing factors to tree mortality. Recently, the interactions among root-feeding beetles—such as those in the genera *Hylobius*, *Hylastes*, and *Pachylobius*, their associated fungi, and loblolly pine have received more attention in the southeast. It has been determined that these beetles are generally secondary invaders, attempting to colonize after tree defenses are already weakened (Eckhardt et al. 2007, Matusick et al. 2013). Additionally, root-feeding beetles are associated with phoretic, ophiostomatoid fungi in the *Leptographium* complex that have been found to cause fine root mortality and root staining on loblolly pine (Eckhardt et al. 2007). These fungi, commonly referred to as blue-stain fungi, belong to the Ophiostomataceae family in the Ascomycota phylum and are adapted for colonization of bark and wood in live trees and dispersal by insects, as evidenced by their long conidiophores producing spores in sticky droplets (Wingfield et al. 1993). It is also hypothesized that all bark beetles feed on fungal colonized plant tissue, even if just briefly (Harrington 2005), suggesting there may be a nutritional benefit for the beetles. While the suite of possible fungal species found on root-feeding beetles in a region may be relatively static, species collected from individual beetles can vary (Matusick et al. 2013). This suggests no obligate relationship exists between the beetle and associated fungi (Zanzot et al. 2010, Matusick et al. 2013). With such a loose association, the potential exists for local fungal communities associated with root-feeding beetles to be disrupted or displaced.

In Alabama pine forests, a correlation was found between root-feeding beetles and their associated fungi and stands exhibiting tree mortality (Eckhardt et al. 2007). Throughout the

literature, this tree mortality has been referenced as a phenomenon called Southern Pine Decline (SPD) (Eckhardt et al. 2007, Zanzot et al. 2010, Matusick et al. 2013). However, there are many factors that influence the health of a tree (Sinclair 1967) and recently this claim has been challenged due to various abiotic factors that exist in areas exhibiting tree dieback as well as the localized nature of the dieback areas (Coyle et al. 2015). Regardless, tree mortality is occurring in southeastern pine stands, and this investigation into the causal factor(s) has invigorated research into these understudied systems.

In the study area for this thesis work, it has been determined that the following three species belonging to the *Leptographium* complex of fungi: *Grosmannia alacris* Duong et al., *G. huntii* Zipfel et al., and *Leptographium profanum* K. Jacobs et al. were commonly associated with the root-feeding beetles (Buland 2019). Species within the *Leptographium* complex can be taxonomically confusing as the *Leptographium* genus refers to asexual forms and the *Grosmannia* genus refers to sexual forms of the same group of organisms. Morphologically distinguishing the two genera can be difficult as teleomorph (sexual) structures are rarely produced in culture (Jacobs and Wingfield 2001). Both genera produce the asexual structures called conidiophores which are dark, single-hyphal stalks bearing conidia (asexual spores) that accumulate in a sticky matrix (Jacobs and Wingfield 2001). *Grosmannia* spp. differ in that they also produce perithecia, flask-shaped, long-necked sexual structures that contain sticky ascospores (Jacobs and Wingfield 2001). While there are some *Leptographium/Grosmannia* species that can cause notable diseases, most are usually saprophytic or weakly pathogenic with undetermined ecological roles (Harrington 1988).

1.3.3 Loblolly Chemical Defense Response

The loblolly defense response to attack follows the general conifer response as previously described in section 1.1. A lot of attention has been given to the terpenoid component of the defense mechanisms (Ro and Bohlmann 2006, Thompson et al. 2006, Harman-Ware et al. 2016). Specific studies, for instance, have shown that the terpene content of heartwood is greater than in sapwood (Thompson et al. 2006), the resin response is supplied by resin ducts (Turner et al. 2019) and wounding to the tree will cause increased resin flow (Knebel et al. 2008). The most prevalent monoterpenes in loblolly pine resin are α- and ß-pinene, while humulene, caryophyllene, and bisabolene constitute the major component of sesquiterpenes (Harman-Ware et al. 2016). Loblolly diterpenes, which are the non-volatile component of resins, are abietic, neoabietic, isopimaric, palustric, dehydroabietic and levopimaric acids (Harman-Ware et al. 2016). It has also been shown that loblolly's hypersensitive reaction to wounding (observed as visible lesions in the phloem) can differ from the response of other southern pines (Cook and Hain 1986). This suggests that different pine species in the same regions have adapted different defense strategies. However, it has also been shown that the resin response of loblolly pine can be influenced by environmental factors (Lombardero et al. 2000). Observed resin responses in loblolly seem to support Berryman's (1972) hypothesis that the primary resin flow acts to prevent insect colonization and the hypersensitive reaction acts to contain associated fungi.

While a lot is known about the terpenoid metabolism in loblolly pine, almost nothing is known about the phenolic metabolism. Investigations into the total phenolic content have been conducted in the past (Jordan et al. 1991, Booker and Maier 2001) and specific phenolic classes have been assessed (Booker et al. 1996), but there have been no investigations as to what

individual compounds are present. Further, much of the methodology and instrumentation of past analyses have improved, highlighting the need for further investigation in the matter.

1.4 The Whitebark System

1.4.1 The Host: *Pinus albicaulis*

Whitebark pine is a five-needled pine that is a foundational and keystone species in high elevation, subalpine forest ecosystems in North America. It is a slow-growing and long-lived species, taking around 50 years to reach cone-bearing age and not reaching a large size until at least 250 years of age (Arno and Hoff 1990, Logan et al. 2010). The oldest recorded specimen was determined to be 1,294 years of age (USDA FS). Due to harsh growing conditions in the highest elevation ecosystems, whitebark pine is a significant percentage of the system's biomass and primary production and has shrub-like growth, referred to as krummholz (Arno and Hoff 1990). Whitebark pine improves the soil conditions and alters snow dynamics, which helps other conifer species grow (Logan et al. 2010). The seeds of whitebark pine are fleshy and highly nutritious and support wildlife species such as Clark's Nutcracker (*Nucifraga columbiana* Wilson), red squirrels (*Tamiasciurus hudsonicusto* Erxleben), and grizzly bears (*Ursus arctos* Linnaeus) (Logan and Powell 2001). In return, whitebark pine relies on these wildlife species to disperse their seeds.

In the last century, whitebark pine has faced considerable challenges to its survival. Regeneration has been altered by changing fire regimes (Tomback et al. 2001); white pine blister rust (*Cronartium ribicola* A. Dietr. [WPBR]), a nonnative pathogen, has caused extensive mortality in whitebark stands (Kendall and Keane 2001); and mountain pine beetle (*D. ponderosae*) outbreaks are causing even more mortality and exacerbating the effects of WPBR

(Tomback and Achuff 2008). Unfortunately, a recent study estimates that at least 50% of the standing whitebark pine in the U.S. is dead (Goeking and Izlar 2018). This mortality is especially concerning when the damage is compared to historic mortality events. According to data recorded in the Greater Yellowstone Ecosystem (GYE), in the 1930s, warmer than average temperatures led to a MPB outbreak that was causing substantial damage and at the time, the outlook for those forests was bleak (Gibson et al. 2008). However, the 1930s outbreak ended up being relatively short-lived and did not cause as much damage as was expected but still left "ghost forests" that are still present today (Logan et al. 2010). In recent land surveys of the GYE, it became clear that ghost forests from the 1930s outbreak paled in comparison to the magnitude of damage from recent outbreaks (Logan et al. 2010). In a 2008 visit to Yellowstone, the Whitebark Pine Ecosystem Foundation director, Diana Tomback, reported finding whitebark pines that were at least a thousand years of age that had been attacked by MPB (Tomback 2008). Given how rapidly these landscapes are being affected by these outbreaks and the slow recovery time of these ecosystems, it is hard to anticipate how these ecosystems may respond, though it is clear that these disturbance events are unprecedented.

1.4.2 The Biotic Agent: *Dendroctonus ponderosae*

Currently, stands of whitebark pine are facing a threat that is historically infrequent, but may now be ever persisting. A member of the large bark beetle family (Coleoptera: Curculionidae), the mountain pine beetle (MPB) is a native, "aggressive" bark beetle that can kill healthy hosts—much like the southern pine beetle in the loblolly pine system. The preferred host species to MPB are ponderosa pine (*Pinus ponderosae* P. & C. Lawson) and lodgepole pine (*Pinus contorta* Douglas ex Loudon) but most western pines can be colonized (Logan and Powell

2001). When the population is at endemic levels, MPBs provide a valuable ecosystem service by removing old or maladaptive trees from the population, helping to regulate the natural fire regime (Logan and Powell 2001) and strengthening the gene pool in the long-term. However, if local populations of MPB increase to reach outbreak level, the beetles can colonize and overwhelm healthier trees. Higher average temperatures caused by climate change have allowed for an unprecedented outbreak of MPB which has led to range expansion and the devastation of many whitebark pine and other subalpine forests in the northwestern U.S. and into Canada (Buotte et al. 2016, Corbett et al. 2016, Six et al. 2018). Whitebark pine is considered a naïve host for MPB (Raffa et al. 2013) as it is historically uncommon for this beetle to establish in such cold climates (Logan and Powell 2001). It is also expected that MPB will remain present in whitebark pine forests as the climate continues to warm (Buotte et al. 2016, Buotte et al. 2017).

Adult MPBs are dark brown to black, ranging from 2.5-7.5 mm, or about the size of a grain of rice (Safranyik 1989). Before reaching maturity, the majority of the MPB life cycle is spent in the larval stage, feeding on the phloem and the inner bark as well as on fungi growing along the walls of their galleries (Safranyik 1989, Logan and Powell 2001). Before emerging to seek new trees, the young adult beetles continue feeding and moving through the galleries, gathering fungal spores in a specialized structure called the mycangium, as well as phoretically along their exoskeletons (Whitney and Farris 1970, Safranyik 1989). The mycangia essentially acts as a fungal culture, ensuring the maintenance of the right species of fungi, and that those fungi are ready to begin colonization when a new host is selected. Several fungal species belonging to the genera *Ophiostoma* and *Leptographium* have been isolated from MPB (Lee et al. 2006). MPB and their associated fungi have developed a symbiotic relationship as the fungi rely on the

beetles for dispersal and the beetles rely on the fungi to aid in overwhelming tree defenses as well as supplementing their diet (Safranyik 1989, Six 2003).

Upon emerging and seeking new hosts, female beetles make selections based on chemical cues produced by the tree (Blomquist et al. 2010) as well as visual cues (Shepherd 1966). Through these cues, beetles are assessing host susceptibility as well as suitability. Once a tree is undergoing colonization, beetles convert some of the tree's terpenes into semiochemicals (pheromones) signaling other beetles to either attack or avoid that tree (Safranyik 1989, Blomquist et al. 2010). After a tree has been successfully colonized, the cycle begins again. Typically, MPB are univoltine, having only one brood per year, but climate conditions can affect development with warmer climates sometimes resulting in two broods, or bivoltine, and colder climates requiring longer than a year to finish development (Safranyik 1985, Safranyik 1989). It appears likely that as the climate continues to warm, MPB will more regularly exhibit a bivoltine life cycle (Mitton and Ferrenberg 2012).

1.4.3 Whitebark Chemical Defense Response

Like loblolly pine, the defense response of whitebark pine to attack follows the general conifer defense described in section 1.1. Much of the specifics of how whitebark pine responds to MPB is detailed in Raffa et al. (2017). In short, monoterpenes, diterpenes, and sesquiterpenes all increase in concentration following attack. Of those, diterpenes were present in the highest concentrations with the three highest compounds being abietic, isopimaric, and levopimaric acids. Conversely, the phenolic classes of vanilloids, flavonoids, hydroxycinnamic acids, phenylpropanoids, and lignans all decrease in concentration following attack. Of those phenolic classes, the individual compounds hydroxypropiovanillone hexoside, taxifolin hexoside,

coumaric acid hexoside, and ferulic acid hexoside were present in the highest concentrations. Stilbenes are the only class of phenolic compounds that increase in concentration once attacked and those compounds are pinosylvin and pinosylvin monomethyl ether.

1.5 Ultra-High-Performance Liquid Chromatography (UHPLC)

Chromatography is a technique used for separating chemicals in a mixture and is a commonly used approach for investigating the chemistry of plants (Pereira et al. 2009, Villari et al. 2012, Villari et al. 2014, Lopez-Goldar et al. 2018). Chromatography is performed in various ways, but even in its most basic form, there is some sort of medium that facilitates the separation, as well as a mobile phase that passes through that medium and separates compounds based on their chemical affinity to the mobile phase, causing compounds to elute at different times. One of the biggest benefits to using high-performance liquid chromatography (HPLC) is that we can run a gradient of solutions for our mobile phase rather than only an isocratic mobile phase. In other words, rather than trying to separate compounds using one liquid (e.g., methanol) at one concentration, we can separate using two liquids (e.g., methanol and water) and vary the concentrations throughout the run. This allows for multiple compounds to elute in a single run of a sample rather than having to run the same sample repeatedly with different mobile phases. Another benefit to HPLC is that this whole process can be automated, allowing for long sequences to be run using the same batch of mobile phase. Automation aids in the consistency of each run because we use organic solvents which must be replaced often to prevent contamination. Ultra-high-performance liquid chromatography (UHPLC) improves on HPLC by running at much higher pressures, allowing us to separate complex mixtures, like the non-

volatile fraction of the secondary metabolites of conifers, rapidly and accurately (Dong and Zhang 2014).

After compounds are separated, they are sent through a diode array detector (DAD) at a predetermined wavelength to report the ultraviolet and visible (UV-vis) absorption spectra of the compounds. The absorption spectra can almost be thought of as the chemical fingerprint of a compound, thus being valuable for identifying unknown compounds. Unfortunately, these fingerprints are not always unique and further identification requires the use of other techniques such as mass spectrometry (MS). Mass spectrometry works by first producing gas-phase ions of the compounds, allowing them to start fragmenting into the different comprising ions to then be separated by their mass-to-charge ratio, resulting in the compound's mass (de Hoffmann and Stroobant 2007). The MS data is often cross-referenced with chemical databases to obtain an identity, but if there is no match in a database, the MS data becomes just another characteristic of the compound as numerous compounds can have the same mass.

1.6 Aims of This Thesis

The goal of this thesis is to investigate the phenolic component of the defense mechanisms of two pine species that are, for different reasons, crucial species in their respective environments. Phenolic compounds are a significant component of the defense mechanisms of pines, but since they are not as abundant as their terpenoid counterparts, they have often been overlooked and are way less understood in their potential role in the host-biotic agent interactions than other pine secondary metabolites (Witzell and Martín 2008). By investigating the different scenarios occurring in the loblolly and whitebark pine systems, we aim to learn more about the role of phenolics, the occurrence and the strength of their induction, as well as the potential for phenolics to predict the resistance phenotype of an individual tree.

The first species is loblolly pine, the most important southeastern U.S. pine in terms of economic and ecological importance in its region (Fox et al. 2004). Since no current literature is available on the phenolic profile of the species, neither constitutive nor induced, the objective of **Chapter 2** was to characterize the phenolic compounds present in the tree phloem, before and after the induction with *Leptographium* species associated with loblolly pine collar and root-feeding beetles commonly found in the Southeast. Results from this study will provide a profile of identified loblolly pine phenolic defense compounds to the scientific community. These results could be the foundation for future studies on loblolly phenolics and defense mechanisms as they will provide a reference for comparison. Results from the study will also provide information on the ecology of phytopathogenic fungal species in the southeastern United States, shedding additional light on the role they might play in overwhelming loblolly pine defenses. The proposed research is an important step to understanding how the *Leptographium* fungal complex reflects the classic paradigm of the bark beetle-fungal association (Six and Wingfield 2011). It is also important to understand how all species within a system, even the less aggressive ones, interact if we are to continue improving management techniques. If we fail to acknowledge and investigate all species present, we run the risk of misdiagnosing problems, which can lead to inefficient management and waste of resources.

In **Chapter 3**, I focus on whitebark pine, a foundational and keystone species in its ecosystems due to soil stabilization and providing nutrition to various wildlife species (Logan and Powell 2001). Whitebark pine is experiencing massive outbreaks of the aggressive MPB, but even in the most devastating aftermath, some trees remain alive, which gives some hope for the possibility of genetic resistance in the population. Recently, an investigation looked at whether the terpenoid profile of survivors can be used as a marker to predict the resistance level of the

trees (Six et al. 2021). The objective of **Chapter 3** of my thesis was similar, but this time comparing the phenolic profile of surviving whitebark pine trees that should have been colonized by MPB to those of trees that escaped the outbreak only because of their age/dimensions. Results from this study will provide an additional reference as to what phenolic compounds are constitutively present in whitebark pine. Results will also serve as the first reference detailing if phenolic compositions can explain whitebark pine resistance to MPB.

1.7 Literature Cited

Arno, S. F., and R. J. Hoff. 1990. *Pinus albicaulis* Engelm. Whitebark Pine. Pages 268-279 *in* R. M. Burns and B. H. Honkala, editors. Silvics of North America: Conifers. USDA Forest Service, Washington, DC.

Aspinwall, M. J., S. E. McKeand, and J. S. King. 2012. Carbon Sequestration from 40 Years of Planting Genetically Improved Loblolly Pine across the Southeast United States. Forest Science **58**:446-456.

Baker, J. B., and G. Langdon. 1990. *Pinus taeda* L. Pages 497-512 *in* R. M. Burns and B. H. Honkala, editors. Silvics of North America: Conifers. U.S. Department of Agriculture, Washington, DC.

Berryman, A. A. 1972. Resistance of Conifers to Invasion by Bark Beetle-Fungus Associations. BioScience **22**:598-602.

Blomquist, G. J., R. Figueroa-Teran, M. Aw, M. Song, A. Gorzalski, N. L. Abbott, E. Chang, and C. Tittiger. 2010. Pheromone production in bark beetles. Insect Biochem Mol Biol **40**:699-712.

Bolton, M. D. 2009. Primary Metabolism and Plant Defense--Fuel for the Fire. MPMI **22**:487-497.

Bonello, P., and J. T. Blodgett. 2003. Pinus nigra–Sphaeropsis sapinea as a model pathosystem to investigate local and systemic effects of fungal infection of pines. Physiological and Molecular Plant Pathology **63**:249-261.

Bonello, P., W. Heller, and H. Sandermann. 1993. Ozone effects on root-disease susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedlings of Scots pine (*Pinus sylvestris* L.). New Phytol **124**:653-663.

Booker, F. L., S. Anttonen, and A. S. Heagle. 1996. Catechin, proanthocyanidin and lignin contents of loblolly pine (*Pinus taeda*) needles after chronic exposure to ozone. New Phytol **132**:483-492.

Booker, F. L., and C. A. Maier. 2001. Atmospheric carbon dioxide, irrigation, and fertilization effects on phenolic and nitrogen concentrations in loblolly pine (*Pinus taeda*) needles. Tree Physiol **21**:609-616.

Buland, M. A. 2019. Root-feeding Curculionid Beetles in Unmanaged and Managed Loblolly Pine Stands of the Georgia Piedmont and Detection of Their Associated Phoretic Ophiostomatoid Fungal Communities. University of Georgia.

Buotte, P. C., J. A. Hicke, H. K. Preisler, J. T. Abatzoglou, K. F. Raffa, and J. A. Logan. 2016. Climate influences on whitebark pine mortality from mountain pine beetle in the Greater Yellowstone Ecosystem. Ecological Applications **26**:2507-2524.

Buotte, P. C., J. A. Hicke, H. K. Preisler, J. T. Abatzoglou, K. F. Raffa, and J. A. Logan. 2017. Recent and future climate suitability for whitebark pine mortality from mountain pine beetles varies across the western US. Forest Ecology and Management **399**:132-142.

Cook, S., and F. Hain. 1986. Defense Mechanisms of Loblolly and Shortleaf Pine Against Attack by Southern Pine Beetle, *Dendroctonus frontalis* Zimmermann, and its Fungal Associate, *Ceratocystis minor* (Hedgecock) Hunt. Journal of Chemical Ecology **12**:10.

Corbett, L. J., P. Withey, V. A. Lantz, and T. O. Ochuodho. 2016. The economic impact of the mountain pine beetle infestation in British Columbia: provincial estimates from a CGE analysis. Forestry: An International Journal of Forest Research **89**:100-105.

Coyle, D. R., K. D. Klepzig, F. H. Koch, L. A. Morris, J. T. Nowak, S. W. Oak, W. J. Otrosina, W. D. Smith, and K. J. K. Gandhi. 2015. A review of southern pine decline in North America. Forest Ecology and Management **349**:134-148.

Cubbage, F. W., J. M. Pye, T. P. Holmes, and J. E. Wagner. 2000. An Economic Evaluation of Fusiform Rust Protection Research. Southern Journal of Applied Forestry **24**:77-85.

Cvikrová, M., J. Malá, M. Hrubcová, and J. Eder. 2006. Soluble and cell wall-bound phenolics and lignin in Ascocalyx abietina infected Norway spruces. Plant Science **170**:563-570.

de Hoffmann, E., and V. Stroobant. 2007. Mass Spectrometry: Principles and Applications. John Wiley & Sons, Chichester, West Sussex, England.

Dong, M. W., and K. Zhang. 2014. Ultra-high-pressure liquid chromatography (UHPLC) in method development. TrAC Trends in Analytical Chemistry **63**:21-30.

Dorman, K. W. 1976. The Genetics and Breeding of Southern Pines. U.S. Department of Agriculture, Forest Service, Washington, DC.

Eckhardt, L. G., A. M. Weber, R. D. Menard, J. P. Jones, and N. J. Hess. 2007. Insect-Fungal Complex Associated with Loblolly Pine Decline in Central Alabama. Forest Science **53**:9.

Evensen, P. C., H. Solheim, K. Høiland, and J. Stenersen. 2000. Induced resistance of Norway spruce, variation of phenolic compounds and their effects on fungal pathogens. Forest Pathology **30**:97-108.

Farmer, E. E., R. R. Johnson, and C. A. Ryan. 1992. Regulation of Expression of Proteinase Inhibitor Genes by Methyl Jasmonate and Jasmonic Acid. Plant Physiol **98**:995-1002.

Fox, T. R., E. J. Jokela, and H. L. Allen. 2004. The Evolution of Pine Plantation Silviculture in the Southern United States. Pages 63-82 General Technical Report. U.S. Department of Agriculture, Forest Service, Southern Research Station, Asheville, NC.

Franceschi, V. R., P. Krokene, T. Krekling, and E. Christiansen. 2000. Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). American Journal of Botany **87**:314-326.

Gibson, K., K. Skov, S. Kegley, C. Jorgensen, S. Smith, and J. Witcosky. 2008. Mountain pine beetle impacts in high-elevation five-needle pines: current trends and challenges.*in* F. S. USDA, Forest Health Protection, editor., Missoula, Montana, USA.

Goeking, S., and D. Izlar. 2018. Pinus albicaulis Engelm. (Whitebark Pine) in Mixed-Species Stands throughout Its US Range: Broad-Scale Indicators of Extent and Recent Decline. Forests **9**.

Hammerschmidt, R. 2005. Phenols and plant–pathogen interactions: The saga continues. Physiological and Molecular Plant Pathology **66**:77-78.

Harborne, J. B. 1989. Methods in Plant Biochemistry, Vol. 1, Plant Phenolics. Academic Press Ltd, London, UK.

Harman-Ware, A. E., R. Sykes, G. F. Peter, and M. Davis. 2016. Determination of Terpenoid Content in Pine by Organic Solvent Extraction and Fast-GC Analysis. Frontiers in Energy Research **4**.

Harrington, T. C. 1988. *Leptographium* species, their distributions, hosts and insect vectors. Pages 1-39 *in* T. C. Harrington and j. Cobb, F. W., editors. *Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minnesota, U.S.A.

Harrington, T. C. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. Pages 257-291 Ecological and Evolutionary Advances in Insect-Fungal Associations.

Herms, D. A., and W. J. Mattson. 1992. The Dilemma of Plants: To Grow or Defend. The Quarterly Review of Biology **67**:283-335.

Hofstetter, R. W., J. B. Mahfouz, K. D. Klepzig, and M. P. Ayres. 2005. Effects Of Tree Phytochemistry On The Interactions Among Endophloedic Fungi Associated With The Southern Pine Beetle. Journal of Chemical Ecology **31**:539-560.

Jacobs, K., and M. J. Wingfield. 2001. *Leptographium* Species: Tree Pathogens, Insect Associates, and Agents of Blue-Stain. The American Phytopathological Society, St. Paul, Minnesota.

Jordan, D. N., T. H. Green, A. H. Chappelka, B. G. Lockaby, R. S. Meldahl, and D. H. Gjerstad. 1991. Response of total tannins and phenolics in loblolly pine foliage exposed to ozone and acid rain. Journal of Chemical Ecology **17**:505-513.

Kaku, H., Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami, and N. Shibuya. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. PNAS **103**:11086-11091.

Keeling, C. I., and J. Bohlmann. 2006. Diterpene resin acids in conifers. Phytochemistry **67**:2415-2423.

Kendall, K. C., and R. E. Keane. 2001. Whitebark pine decline: Infection, mortality and population trends. Pages 221-242 *in* D. F. Tomback, S. F. Arno, and R. E. Keane, editors. Whitebark pine communities: ecology and restoration. Island Press, Washington, DC.

Knebel, L., D. J. Robison, T. R. Wentworth, and K. D. Klepzig. 2008. Resin flow responses to fertilization, wounding and fungal inoculation in loblolly pine (*Pinus taeda*) in North Carolina. Tree Physiol **28**:7.

Koricheva, J., H. Nykänen, and E. Gianoli. 2004. Meta-analysis of Trade-offs among Plant Antiherbivore Defenses: Are Plants Jacks-of-All-Trades, Masters of All? The American Naturalist **163**.

Lattanzio, V., V. M. T. Lattanzio, and A. Cardinali. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry: Advances in Research:23-67.

Lee, S., J.-J. Kim, and C. Breuil. 2006. Diversity of fungi associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. Fungal Diversity **22**:91-105.

Lieutier, F., D. Sauvard, F. Brignolas, V. Picron, A. Yart, C. Bastien, and C. Jay-Allemand. 1996. Changes in phenolic metabolites of Scots-pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. Eur. J. For. Path. **26**:145-158.

Loehle, C., and G. Namkoong. 1987. Constraints on tree breeding: growth tradeoffs, growth strategies, and defensive investments. Forest Science **33**:1089-1097.

Logan, J. A., W. W. Macfarlane, and L. Willcox. 2010. Whitebark pine vulnerability to climate-driven mountain pine beetle disturbance in the Greater Yellowstone Ecosystem. Ecological Applications **20**:895-902.

Logan, J. A., and J. A. Powell. 2001. Ghost Forests, Global Warming, and the Mountain Pine Beetle (Coleoptera: Scolytidae). American Entomologist **47**:160-172.

Lombardero, M. J., M. P. Ayres, P. L. J. Lorio, and J. J. Ruel. 2000. Environmental effects on constitutive and inducible resin defenses of *Pinus taeda*. Ecology Letters **3**:329-339.

Lopez-Goldar, X., C. Villari, P. Bonello, A. K. Borg-Karlson, D. Grivet, R. Zas, and L. Sampedro. 2018. Inducibility of Plant Secondary Metabolites in the Stem Predicts Genetic Variation in Resistance Against a Key Insect Herbivore in Maritime Pine. Front Plant Sci **9**:1651.

Martin, D., D. Tholl, J. Gershenzon, and J. Bohlmann. 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol **129**:1003-1018.

Matusick, G., R. D. Menard, Y. Zeng, and L. G. Eckhardt. 2013. Root-Inhabiting Bark Beetles (Coleoptera: Curculionidae) and their Fungal Associates Breeding in Dying Loblolly Pine in Alabama. Florida Entomologist **96**:238-241.

McKeand, S. E. 2019. The Evolution of a Seedling Market for Genetically Improved Loblolly Pine in the Southern United States. Journal of Forestry **117**:293-301.

Mitton, J. B., and S. M. Ferrenberg. 2012. Mountain pine beetle develops an unprecedented summer generation in response to climate warming. Am Nat **179**:E163-171.

Moore, B. D., R. L. Andrew, C. Kulheim, and W. J. Foley. 2014. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytol **201**:733-750.

Nagy, N. E., V. R. Franceschi, H. Solheim, T. Krekling, and E. Christiansen. 2000. Wound-Induced Traumatic Resin Duct Development in Stems of Norway Spruce (Pinaceae): Anatomy and Cytochemical Traits. American Journal of Botany **87**:302-313.

Pereira, D., P. Valentão, J. Pereira, and P. Andrade. 2009. Phenolics: From Chemistry to Biology. Molecules **14**:2202-2211.

Raffa, K. F. 2014. Terpenes Tell Different Tales at Different Scales: Glimpses into the Chemical Ecology of Conifer - Bark Beetle - Microbial Interactions. J Chem Ecol **40**:1-20.

Raffa, K. F., C. J. Mason, P. Bonello, S. Cook, N. Erbilgin, K. Keefover-Ring, J. G. Klutsch, C. Villari, and P. A. Townsend. 2017. Defence syndromes in lodgepole - whitebark pine ecosystems relate to degree of historical exposure to mountain pine beetles. Plant Cell Environ **40**:1791-1806.

Raffa, K. F., E. N. Powell, and P. A. Townsend. 2013. Temperature-driven range expansion of an irruptive insect heightened by weakly coevolved plant defenses. Proc Natl Acad Sci U S A **110**:2193-2198.

Ro, D. K., and J. Bohlmann. 2006. Diterpene resin acid biosynthesis in loblolly pine (Pinus taeda): functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1). Phytochemistry **67**:1572-1578.

Safranyik. 1989. Mountain Pine Beetle: Biology Overview. Pages 9-13 *in* Symposium on the Management of Lodgepole Pine to Minimize Losses to the Mountain Pine Beetle. USDA Forest Service, Intermountain Forest and Range Experiment Station.

Safranyik, L. 1985. Effect of climatic factors on development, survival, and life cycle of the mountain pine beetle. Pages 14-24 *in* Mountain Pine Beetle Symposium. British Columbia Ministry of Forests, Smithers, BC.

Schultz, R. P. 1997. Loblolly Pine. U.S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, New Orleans, LA.

Seabra, R. M., P. B. Andrade, P. Valentão, E. Fernandes, F. Carvalho, and M. L. Bastos. 2006. Antioxidant Compounds Extracted from Several Plant Materials. Pages 115-174 *in* M. Fingerman and R. Nagabhushanam, editors. Biomaterials from Aquatic and Terrestrial Organisms. Science Publishers, Enfield, NH.

Shepherd, R. F. 1966. Factors Influencing the Orientation and Rates of Activity of *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). The Canadian Entomologist **98**:507-518.

Sinclair, W. A. 1967. Decline of hardwoods: possible causes. Proc. Int. Shade Tree Conference **42**:17-32.

Six, D. L. 2003. Bark Beetle-Fungus Symbioses. Pages 97-114 *in* K. Bourtzis and T. A. Miller, editors. Insect Symbiosis. CRC Press, Florida, USA.

Six, D. L., A. Trowbridge, M. Howe, D. Perkins, E. Berglund, P. Brown, J. A. Hicke, and G. Balasubramanian. 2021. Growth, Chemistry, and Genetic Profiles of Whitebark Pine Forests Affected by Climate-Driven Mountain Pine Beetle Outbreaks. Frontiers in Forests and Global Change **4**.

Six, D. L., C. Vergobbi, and M. Cutter. 2018. Are Survivors Different? Genetic-Based Selection of Trees by Mountain Pine Beetle During a Climate Change-Driven Outbreak in a High-Elevation Pine Forest. Front Plant Sci **9**:993.

Six, D. L., and M. J. Wingfield. 2011. The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. Annu Rev Entomol **56**:255-272.

Stephen, F. M. 2011. Southern Pine Beetle Competitors. Pages 183-198 *in* R. N. Coulson and K. D. Klepzig, editors. Southern Pine Beetle II. U.S. Department of Agriculture Forest Service, Southern Research Station, Asheville, NC.

Thompson, A., J. Cooper, and L. L. J. Ingram. 2006. Distribution of terpenes in heartwood and sapwood of loblolly pine. Forest Products Journal **56**:46-48.

Tomback, D. 2008. Director's message. Nutcracker Notes **15**.

Tomback, D. F., and P. L. Achuff. 2008. Blister rust and western forest biodiversity: status, trends, and restoration issues for white pines. J For Pathology.

Tomback, D. F., A. J. Anderies, K. S. Carsey, M. L. Powell, and S. Mellmann-Brown. 2001. Delayed Seed Germination in Whitebark Pine and Regeneration Patterns Following the Yellowstone Fires. Ecology **82**:2587-2600.

Turner, G. W., A. N. Parrish, J. J. Zager, J. T. Fischedick, and B. M. Lange. 2019. Assessment of flux through oleoresin biosynthesis in epithelial cells of loblolly pine resin ducts. J Exp Bot **70**:217-230.

Villari, C., A. Battisti, S. Chakraborty, M. Michelozzi, P. Bonello, and M. Faccoli. 2012. Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine. Tree Physiol **32**:867-879.

Villari, C., M. Faccoli, A. Battisti, P. Bonello, and L. Marini. 2014. Testing phenotypic trade-offs in the chemical defence strategy of Scots pine under growth-limiting field conditions. Tree Physiol **34**:919-930.

Wear, D. N. 2013. Forecasts of land uses. Pages 45-71 *in* D. N. Wear and J. G. Greis, editors. The Southern Forest Futures Project: technical report. USDA-Forest Service, Southern Research Station, Asheville, NC.

Whitney, H. S., and S. H. Farris. 1970. Maxillary Mycangium in the Mountain Pine Beetle. Science **167**:54-55.

Wingfield, M. J., K. A. Seifert, and J. F. Webber. 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. APS Press.

Witzell, J., and J. A. Martín. 2008. Phenolic metabolites in the resistance of northern forest trees to pathogens — past experiences and future prospects. Canadian Journal of Forest Research **38**:2711-2727.

Young, T. P., and B. D. Okello. 1998. Relaxation of an induced defense after exclusion of herbivores: spines on *Acacia drepanolobium*. Oecologia **115**:508-513.

Zanzot, J. W., G. Matusick, and L. G. Eckhardt. 2010. Ecology of root-feeding beetles and their associated fungi on longleaf pine in Georgia. Environ Entomol **39**:415-423.

Zobel, B., and J. Talbert. 1984. Applied Forest Tree Improvement. John Wiley & Sons, New York, USA.

CHAPTER 2

PHENOLIC DEFENSE RESPONSE OF LOBLOLLY PINE TO ROOT-FEEDING BEETLE VECTORED FUNGAL PATHOGENS

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Abstract

Loblolly pine (*Pinus taeda*) is the most economically important timber species in the southeastern United States. Recently, loblolly pine mortality has increased in some counties along the fall line in Alabama and Georgia. Although no causal factor has been determined, root pathogens may be contributing factors. Investigations of the chemical interactions among loblolly pine and its associated pests and pathogens may assist with understanding this mortality better. However, among the numerous defense metabolites, little is known about phenolics in loblolly pines. Furthermore, the general role of phenolics in plant defenses across all systems is poorly understood. Hence, our objectives are to: 1) characterize the constitutive phenolic profile of loblolly pine phloem; and 2) investigate how the profile may change after inoculations with root-infecting fungal pathogens commonly associated with root feeding beetles. Forty mature loblolly pines were selected from a planted stand and assigned to one of five different treatments: (i) inoculation with *Leptographium profanum*, (ii) *Grosmannia alacris*, (iii) *G. huntii*, (iv) sterile wounding, or (v) non-wounded control. Resulting lesions on the phloem were recorded four weeks after the inoculation, and phenolics were analyzed using a combination of ultra-high-performance liquid chromatography—diode array detector (UHPLC-DAD) and mass spectrometry (MS). Inoculation with fungi induced variable lesion lengths in loblolly pine phloem and phenolic concentrations differed from their constitutive amounts after fungal inoculation. Results from this study may contribute to a broader understanding of the defense response of loblolly pine to fungal infections, as well as providing a foundation for future studies by identifying phenolic compounds present in loblolly pine defense mechanisms.

INDEX WORDS: Chromatography, *Grosmannia*, *Leptographium*, Loblolly pine, Phenolics

2.1 Introduction

In the southeastern United States, loblolly pine (*Pinus taeda* L.) is the dominant timber species grown commercially, providing softwood products to much of the world (Fox et al. 2004) and having a significant impact on the economy of the southeast. In the state of Georgia, the forestry industry alone provided 48,444 jobs and its exported output was valued at $19.8 billion in 2018 (GFC). When accounting for other Georgia industries impacted by the forest industry in 2018, the economic activity was valued at $36.3 billion. Because loblolly pine is such an asset, during the last few decades the species has undergone intensive genetic improvement and seen better silvicultural practices (Dorman 1976, Zobel and Talbert 1984, Fox et al. 2004). While growth and resistance to fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*) have improved, loblolly pine is still vulnerable to pests and pathogens, especially because these interactions are an ever-lasting arms race (Mello and Silva-Filho 2002, Anderson et al. 2010). Additionally, the amount of planted pine in the southeast is expected to increase in the future (Wear 2013), meaning losses from pests and pathogens will remain a persistent issue.

Like all conifers, the defense response of loblolly pine consists of an integration of anatomical and chemical strategies that are both constitutive (preformed, always present) and induced (Franceschi et al. 2005). Chemical tree defense is executed by secondary metabolites that are mostly comprised of compounds classified as terpenoids and phenolics (Hofstetter et al. 2005) and defense strategies follow the three-step defense sequence first hypothesized by Berryman (1972): first is wound cleansing, followed by containment of infection, and lastly comes wound healing. Wound cleansing is primarily performed by constitutive oleoresin comprised of several classes of terpenoids (monoterpenoids, sesquiterpenoids, and diterpene acids) that push out or entomb invading insects while cleaning the wound (Keeling and

Bohlmann 2006). Containment of infection is primarily handled by induced terpenoids and phenolics, which have insecticidal, antifungal and antioxidant properties (Hammerschmidt 2005, Bohlmann 2012). Wound healing is more of a physical process through the formation of callus and wound periderm (Berryman 1972).

When only considering the chemical defense response of loblolly pine, a lot of work has been done focusing on the resin response. In addition to characterizing its composition (Harman-Ware et al. 2016) and the different genes involved in resin production (Ro and Bohlmann 2006, Mao et al. 2019), it has been shown that the resin response is supplied by resin ducts (Turner et al. 2019) and that once a tree is wounded, resin flow will increase (Knebel et al. 2008). The distribution of terpenes throughout loblolly's heartwood and sapwood has also been analyzed (Thompson et al. 2006). However, when it comes to phenolics in loblolly, there is little known. Previous investigations into loblolly phenolics focused on total contents rather than individual compounds (Jordan et al. 1991, Booker et al. 1996, Gebauer et al. 1997, Booker and Maier 2001) and used old quantification methods that today are considered inaccurate (Appel et al. 2001). Additionally, while we have a decent understanding of the role induced phenolics play in plant defense, the role constitutive phenolics play is still poorly understood and they may rather act as precursors for larger molecules (Witzell and Martín 2008).

Recently, interactions among root-feeding beetles (*Hylobius*, *Hylastes*, and *Pachylobius* genera), their associated fungi, and loblolly pine have been receiving more attention in the southeastern United States. These beetles are generally secondary invaders, only attempting to colonize a tree after defenses have been already weakened (Eckhardt et al. 2004, Matusick et al. 2013). They are associated with phoretic, ophiostomatoid fungi belonging to the *Leptographium*/*Grosmannia* complex where some species have been found to cause fine root

mortality and root staining on loblolly pine (e.g., *Leptographium procerum* (W.B. Kendr.) M.J. Wingf.) (Eckhardt et al. 2004, Eckhardt et al. 2007). These are Ascomycete fungi that are adapted to colonize wood in live trees and dispersal by insects (Wingfield et al. 1993) and most species are usually saprophytic or weakly pathogenic with unknown ecological roles (Harrington 1988) but likely assist with root turnover.

Some southern pine stands, particularly around the fall line, have recently been exhibiting tree mortality and this mortality has been referenced as a phenomenon called "southern pine decline" (Eckhardt et al. 2007, Zanzot et al. 2010, Matusick et al. 2013). In some Alabama pine forests, this decline has been correlated with the presence of root-feeding beetles and their associated fungi (Eckhardt et al. 2007). While the southern pine decline phenomenon has been challenged (Coyle et al. 2015), tree mortality is occurring and investigation into the causal factor(s) has invigorated research into understudied interactions. Hence, our objectives are: 1) to characterize the constitutive phenolic profile of mature loblolly pine phloem and 2) investigate how the profile may change after inoculation with *Leptographium profanum*, *Grosmannia alacris*, and *G. Huntii*, which are ophiostomatoid fungal species commonly associated with root-feeding beetles in the southeastern United States.

2.2 Materials and Methods

2.2.1 Field Site and Sample Collection

The field component of the experiment was conducted between September and October 2017. A stand of mature, planted loblolly pine trees was selected in Whitehall Forest (Athens, Georgia, USA (33.8848395 N, -83.357658 W). The understory of this stand is periodically subjected to prescribed burns, keeping surrounding vegetation low with the only competing tree

species being sweetgum (*Liquidambar styraciflua* L.). The soil type of Athens-Clarke County is predominantly sandy clay loam (2006). Following a randomized complete block design, 40 trees were randomly selected from those that had no physical defects (crooked form, abnormal growths, wounds, etc.) as four blocks of ten trees each. Within each block, two trees were assigned one of five treatments: (i) inoculation with *Leptographium profanum* (isolate CV20170072), (ii) *Grosmannia alacris* (isolate 0529H3T6), (iii) *G. huntii* (isolate CV20170089), (iv) sterile wounding and (v) non-wounded control, for a total of eight replicates per treatment. Sterile wounding served as a control for any response we may have induced by physically damaging the tree while inoculating. Non-wounded trees served as a control for any environmental changes that may occur throughout the experiment. Using a 7 mm. cork borer, we removed four perpendicular, evenly spaced plugs of phloem from around the root collar of each tree (excluding non-wounded controls). Before taking each plug, we shaved the outer bark slightly to ensure we were reaching the phloem when boring the plugs. We labelled these plugs 'week 0' and used them for the analysis of constitutive defense compounds. To prevent oxidation of secondary metabolites, we immediately flash froze all samples in liquid nitrogen until returning to the lab where we stored them at -80°C until processing. For inoculated trees (treatments i-iii), we substituted the removed phloem plug with another loblolly pine phloem plug that we had previously sterilized by autoclaving twice at 121 °C for 25 minutes and then colonized for 12 days with each respective treatment fungi (Fig. 2.1), following the protocol described in Villari et al. (2012). All fungal isolates used in the experiment had been previously isolated from root-feeding beetles trapped within Whitehall Forest (Buland 2019).

Four weeks after inoculation, we returned to the trees and randomly selected two opposing side inoculation sites for sampling. We carefully removed the outer periderm

surrounding the inoculation sites until the phloem layer was visible and then measured the lesion length (cm.) visible on the phloem using a ruler (Fig. 2.2). At the same time, we also collected phloem samples from the margins of the lesion using sterilized scalpel and tweezers, flash froze them in liquid nitrogen, and stored them at -80°C until processing. We labelled these samples 'week 4' and used them for the analysis of induced profiles. Additional phloem samples were also taken from each tree so that we could re-isolate the fungi and confirm successful inoculation. We also removed phloem plugs from control trees at this time, following the same procedure as described for the collection of samples at the beginning of the experiment.

2.2.2 Extraction of Phenolic Compounds

Phloem plugs from the same tree and same collection date were combined and ground to a fine powder in liquid nitrogen using pestle and mortar. Ground material was weighed into 100 ± 5 mg aliquots and stored in a -80°C freezer until phenolic compounds extraction. To extract phenolic compounds, we used a three-day protocol as described in Lopez-Goldar et al. (2018). On the first day, each aliquot was soaked in 500 µL HPLC-grade methanol containing 0.5 mg/mL resorcinol as an internal standard (IS), vortexed for 5-10 seconds, and stored at 4°C overnight. The following afternoon, aliquots were centrifuged at 16,000 rcf for 8 minutes. The resulting supernatants were transferred to new tubes and stored at -20°C, while the remaining pellets were processed again as in day 1. On the third afternoon, the samples were again centrifuged at 16,000 rcf for 8 minutes and the resulting supernatants were merged with the previous extracts and stored at -20°C.

Since we were only interested in the phenolic compounds, we removed non-polar resins from our samples as they are too viscous and would have interfered with our analysis. For this

purpose, we added 500 µL of HPLC-grade water to 500 µL of each sample, causing the diterpenes to flocculate and fall out of solution. Samples were then briefly vortexed and centrifuged at 16,000 rcf for 15 minutes. The resulting supernatants were transferred to a new tube and the pellets were discarded. The supernatants were then dried down using a vacuum centrifuge, and the resulting pellets were resuspended in 500 µL of HPLC-grade methanol, briefly vortexed, then sonicated for 10 minutes and stored at -20°C until analysis.

2.2.3 Acquisition of the Phenolic Profiles

We acquired phenolic profiles using an ultra-high-performance liquid chromatography—diode array detector (UHPLC-DAD) with an Agilent 1290 Infinity II UHPLC system. All UHPLC-DAD parts and software used were developed by Agilent Technologies (CA, USA). The column was a ZORBAX Eclipse Plus C18 Rapid Resolution HD (2.1x100mm, 1.8-micron) with a ZORBAX Eclipse Plus C18 guard column (2.1x5mm, 1.8-micron). The autosampler temperature was held at 22ºC and the column temperature was held at 50ºC. We used a binary mobile phase of 0.1% acetic acid in HPLC-grade methanol (solvent A) and 0.1% acetic acid in HPLC-grade water (solvent B) with a constant flow rate of 0.5 ml/min. Samples were injected at a volume of 0.5 µL and were run on the following linear gradient: (cumulative run time (min), % solvent A) 0.0, 95.0; 12.0, 0.0; 14.0, 0.0, 14.25, 95.0; 19.25, 95.0 (run time totaled 19.25 minutes). A methanol blank was run every 3 samples to prevent column carryover and sample cross-contamination. A standard check (i.e., trans-ferulic authentic standard at a concentration of 0.2 mg/ml) was run every 6 samples to verify that the instrument calibration was maintained for the duration of the whole run. All samples were run in one single continuous sequence that lasted a total of ~44 hours. DAD spectral data were recorded from 210 to 400 nm and phenolic

compounds were detected at 280 nm (Lopez-Goldar et al. 2018). Compounds eluting after minute 10.5 were not included in analysis to exclude terpenes that remained in the samples after purification.

2.2.4 Identification of the Phenolic Compounds

We used high-performance liquid chromatography—mass spectrometry (HPLC-MS) using a HPLC 1200 (Agilent Technologies, CA, USA) coupled to an Accurate-Mass TOF LC/MS 6220 (Agilent Technologies, CA, USA) to obtain molecular mass (m/z) for the peaks we previously identified on the UHPLC. Mass spectrometry data were acquired in negative ion mode using electrospray ionization and analysis was performed on Agilent's MassHunter software. We used the same solvent system, column, and gradient as we did on the Agilent 1290 Infinity II UHPLC-DAD system. Flow rate had to be lowered to 0.4 ml/min to maintain a stable pressure. Identification of phenolic compounds followed the procedure described by Raffa et al. (2017). Although there was a retention time shift between the resulting chromatograms (UHPLC-DAD vs. HPLC-MS), phenolic compounds could still be matched based on order of elution and congruence of λ_{max}. Compounds were then identified based on negative ion fragmentation patterns, matching retention time and λ_{max} to standards, and comparing order of elution and λ_{max} to literature (see Table 2.1).

2.2.5 Quantifying the Phenolic Compounds

Quantification of phenolic compounds followed the procedure described by Raffa et al. (2017). Using the DAD chromatograms generated by Agilent OpenLab, we calculated the peak areas using the following parameters: Tangent Skim Mode: New Exponential, Tail Peak Height

Ratio: 11.00, Front Peak Skim Height Ratio: 12.00, Skim Valley Ratio: 1.00, Baseline Correction: Advanced, Peak to Valley Ratio: 1.60, Slope Sensitivity: 2.300, Peak Width: 0.010, Area Reject: 1.000, Height Reject: 1.000, Shoulders: DROP, Area Percent Reject: 0.00. All peak areas were then normalized by the internal standard and by mg of fresh weight used in extraction. Peaks were quantified by fitting them to a 5-point calibration curve ($R^2 > 0.99$) prepared from the appropriate analytical pure standards (Extrasynthese, France; Sigma-Aldrich, USA) (Table 2.1). Phenolic compounds of known identity for which a corresponding pure standard was available were fit to those respective calibration curves. If a standard was not available, relative quantification was done using the calibration curves from pure standards of closely related compounds. If compounds were not closely related to pure standards, or did not have a putative identity assigned, they were quantified as internal standard equivalents.

2.2.6 Statistical Analyses

All statistical analyses of loblolly phenolics were performed in RStudio version 1.3.1056 (R Core Team). Using a one-way analysis of variance (ANOVA), we first verified that there was no significant difference in lesion lengths among blocks, although it was marginal ($P = 0.0742$). We also verified that there was no significant difference in lesion lengths among trees with different stem diameters (ANOVA, $P = 0.1867$). After knowing that DBH and blocking had no significant effect on lesion lengths, these factors were removed from subsequent tests on lesion length. A linear mixed-effects model and Tukey's HSD were then run to determine any significant differences among fungal inoculation treatments.

An ANOVA was run on the change in concentration between the constitutive and induced (week 4 – week 0) phenolic compounds present in at least 70% of samples within each

treatment combination. Compounds not detected in individual samples were assigned a concentration of '0'. Exceptions were made for the compounds putatively identified as pinoresinol, pinosylvin, pinocembrin, and pinosylvin monomethyl ether, because they have been recognized to be important compounds in conifer defense responses (Witzell and Martín 2008). Before running the ANOVA, each compound was tested for normality and those that failed the normality test were log-transformed. If a compound returned as significantly different ($P < 0.05$) across treatments, Tukey's HSD was run to compare the different treatments. To determine if the change in concentration between weeks was only due to the treatment and not to the elapsed time between measurements, an ANOVA was run comparing the week 0 constitutive amounts of each compound to the amounts found in the week 4 unwounded control trees. This experimental design, modified from Lombardero et al. (2000), was chosen to control for the high variability expected for constitutive metabolite profiles.

2.3 Results

2.3.1 Lesion Lengths

We found a significant difference in lesion lengths among the treatments (ANOVA, $F_{3,28} = 22.43$, $P = <0.001$) (Figure 2.3). *Grosmannia huntii* caused significantly larger lesions than both *G. alacris* ($P = 0.005$) and *L. profanum* ($P = <0.001$). Lesions caused by *G. alacris* and *L. profanum* did not significantly differ from each other ($P = 0.669$). All fungal induced lesions were significantly bigger than the control of sterile wounding ($P = < 0.013$).

2.3.2 Re-isolation of Fungi

Re-isolation of fungi was successful for 100% of the *L. profanum* inoculated trees, for 87% of the *G. alacris* inoculated trees, and for 75% of the *G. huntii* inoculated trees.

2.3.3 Compound Identities and Quantities

We detected a total of 61 possible phenolic compounds in loblolly pine phloem (Fig 2.4). Of those, 25 were found to be in at least 70% of samples within a treatment combination. We were able to putatively assign an identity to 17 phenolic compounds with 10 being flavonoids, 3 being hydroxycinnamic acids, 2 being stilbenes, and 2 being lignans (Table 2.1).

2.3.4 Treatment Effects on Phenolic composition

Constitutive concentrations and inducible variation are reported in Table 2.2. For most identified compounds, we saw a change between the constitutive level at week zero and the level at week 4, however, the type of induction treatment (sterile wounding vs fungal inoculation with different species) had no significant effect on the inducible change in concentration. PK3F, a ferulic acid derivative was the only compound showing a significant difference in the fungal induced changes (irrespective of the fungal species) compared to sterile wounding. In the few other compounds where a significant effect was observed, it was almost always due to *G. alacris* inducing a significant change in the profile compared to all other treatments (Table 2.3). Comparisons of the week 0 constitutive amounts to the week 4 unwounded control trees determined that 12 different compounds were significantly different between the two groups (Table 2.4).

2.4 Discussion

In this study, we sought to: 1) characterize the constitutive phenolic profile of mature loblolly pine phloem; and 2) investigate how the profile may change after inoculation with *L. profanum*, *G. alacris*, and *G. huntii*. We assigned putative identities to 17 phenolic compounds present in loblolly pine phloem. Of those, 12 belong to the phenolic classes of flavonoids and stilbenes, which are most associated with the defense response of conifers to pathogens (Witzell and Martín 2008). All flavonoid compounds were detected in both constitutive and induced profiles whereas the stilbenes, pinosylvin and pinosylvin monomethyl ether, were only detected in induced profiles. This finding is similar to the phenolic composition that was found in the reaction zone to fungal infection in Scots pine (*P. sylvestris* L.) where the same stilbenes were detected (Lieutier et al. 1996, Villari et al. 2012). In another experiment on Scots pine, these same compounds were shown to have antifungal properties (Bonello et al. 1993). Though our experiment did not test antifungal properties, it is not surprising that these compounds were only detected after fungal inoculation.

Most of the compounds, except for stilbenes, decreased in concentration after fungal inoculation, but not at significantly different amounts. One possible explanation for this could be that some compounds decrease in concentration so that the tree can allocate more energy in the production of compounds better suited to defense (e.g., stilbenes) (Witzell and Martín 2008). Another possible explanation for decreased concentrations could be that they are being catabolized by the inoculated fungi (Zhao et al. 2019). Unfortunately, our results might also be confounded by the fact that we found an unexpected difference between the constitutive levels at week 0 and the unwounded controls at week 4 for many of the analyzed compounds, indicating a possible effect of time or other disturbance events that could have occurred between our

sampling periods on the metabolites of sampled trees. A way to obviate to this analytical issue would be to compare all week 4 profiles, including the unwounded control ones, instead of analyzing the difference between week 4 and week 0 of every single plant. We chose this approach because of the known high intra-specific variability of secondary metabolites in pines (Lombardero et al. 2000), but this variation might be negligible compared to the time effect we found.

Interestingly, the trend of the measured lesion lengths and that of the comparison of the change in compound concentrations from week 0 to week 4 differ. While inoculation with *G. huntii* produced the largest lesions, *G. alacris* was attributed with causing the largest changes in phenolic compound concentration. Differences in lesion lengths among fungal species supports the findings of a previous study on loblolly pine, longleaf pine (*P. palustris* Mill.), and slash pine (*P. elliotii* Engelm.) seedlings conducted by Matusick and Eckhardt (2010). Additionally, all fungal inoculations produced significantly longer lesions than sterile wounding but when comparing the phenolic compound concentrations, there was rarely a difference in the concentration changes among all treatments, similarly to what previously observed by Villari and coauthors in Scots pine (Villari et al. 2012). A possible explanation for this is that the produced lesion, a hypersensitive reaction, is comprised mostly of terpenes in order to seal and clean the wound created when inoculating the trees (Keeling and Bohlmann 2006), or that for pine trees, a generic induced response rather than a specific one might be more functional, as by activating multiple generic mechanisms, there are higher chances that at least some may be effective (Katagiri 2004, Villari et al. 2012).

The constitutive phenolic profile of loblolly pine has now been categorized which should serve as a springboard into further studies aiming at better understanding the complex host-

pathogen chemical interactions. Moving forward, for instance, it would be pertinent to isolate and collect individual phenolic compounds found in loblolly pine and investigate their antifungal properties, if any, using bioassays.

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2.6 Literature Cited

2006. Community Assessment Chapter Four: Natural and Cultural Resources. ACC Gov.

Anderson, J. P., C. A. Gleason, R. C. Foley, P. H. Thrall, J. B. Burdon, and K. B. Singh. 2010. Plants versus pathogens: an evolutionary arms race. Functional Plant Biology **37**:499-512.

Appel, H. M., H. L. Govenor, M. D'Ascenzo, E. Siska, and J. C. Schultz. 2001. Limitations of folin assays of foliar phenolics in ecological studies. Journal of Chemical Ecology **27**:761-778.

Berryman, A. A. 1972. Resistance of Conifers to Invasion by Bark Beetle-Fungus Associations. BioScience **22**:598-602.

Bohlmann, J. 2012. Pine terpenoid defences in the mountain pine beetle epidemic and in other conifer pest interactions: specialized enemies are eating holes into a diverse, dynamic and durable defence system. Tree Physiol **32**:943-945.

Bonello, P., W. Heller, and H. Sandermann. 1993. Ozone effects on root-disease susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedlings of Scots pine (*Pinus sylvestris* L.). New Phytol **124**:653-663.

Booker, F. L., S. Anttonen, and A. S. Heagle. 1996. Catechin, proanthocyanidin and lignin contents of loblolly pine (*Pinus taeda*) needles after chronic exposure to ozone. New Phytol **132**:483-492.

Booker, F. L., and C. A. Maier. 2001. Atmospheric carbon dioxide, irrigation, and fertilization effects on phenolic and nitrogen concentrations in loblolly pine (*Pinus taeda*) needles. Tree Physiol **21**:609-616.

Buland, M. A. 2019. Root-feeding Curculionid Beetles in Unmanaged and Managed Loblolly Pine Stands of the Georgia Piedmont and Detection of Their Associated Phoretic Ophiostomatoid Fungal Communities. University of Georgia.

Coyle, D. R., K. D. Klepzig, F. H. Koch, L. A. Morris, J. T. Nowak, S. W. Oak, W. J. Otrosina, W. D. Smith, and K. J. K. Gandhi. 2015. A review of southern pine decline in North America. Forest Ecology and Management **349**:134-148.

Dorman, K. W. 1976. The Genetics and Breeding of Southern Pines. U.S. Department of Agriculture, Forest Service, Washington, DC.

Eckhardt, L. G., J. P. Jones, and K. D. Klepzig. 2004. Pathogenicity of *Leptographium* Species Associated with Loblolly Pine Decline. Plant Disease **88**:5.

Eckhardt, L. G., A. M. Weber, R. D. Menard, J. P. Jones, and N. J. Hess. 2007. Insect-Fungal Complex Associated with Loblolly Pine Decline in Central Alabama. Forest Science **53**:9.

Fox, T. R., E. J. Jokela, and H. L. Allen. 2004. The Evolution of Pine Plantation Silviculture in the Southern United States. Pages 63-82 General Technical Report. U.S. Department of Agriculture, Forest Service, Southern Research Station, Asheville, NC.

Franceschi, V. R., P. Krokene, E. Christiansen, and T. Krekling. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytol **167**:353-375.

Gebauer, R. L. E., B. R. Strain, and J. F. Reynolds. 1997. The effect of elevated CO2 and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). Oecologia **113**:29-36.

GFC. 2018. Economic Benefits of the Forest Industry in Georgia: 2018. Enterprise Innovation Institute, Georgia Institute of Technology.

Hammerschmidt, R. 2005. Phenols and plant–pathogen interactions: The saga continues. Physiological and Molecular Plant Pathology **66**:77-78.

Harman-Ware, A. E., R. Sykes, G. F. Peter, and M. Davis. 2016. Determination of Terpenoid Content in Pine by Organic Solvent Extraction and Fast-GC Analysis. Frontiers in Energy Research **4**.

Harrington, T. C. 1988. *Leptographium* species, their distributions, hosts and insect vectors. Pages 1-39 *in* T. C. Harrington and j. Cobb, F. W., editors. *Leptographium* root diseases on conifers. American Phytopathological Society, St. Paul, Minnesota, U.S.A.

Hofstetter, R. W., J. B. Mahfouz, K. D. Klepzig, and M. P. Ayres. 2005. Effects Of Tree Phytochemistry On The Interactions Among Endophloedic Fungi Associated With The Southern Pine Beetle. Journal of Chemical Ecology **31**:539-560.

Jordan, D. N., T. H. Green, A. H. Chappelka, B. G. Lockaby, R. S. Meldahl, and D. H. Gjerstad. 1991. Response of total tannins and phenolics in loblolly pine foliage exposed to ozone and acid rain. Journal of Chemical Ecology **17**:505-513.

Katagiri, F. 2004. A global view of defense gene expression regulation--a highly interconnected signaling network. Curr Opin Plant Biol **7**:506-511.

Keeling, C. I., and J. Bohlmann. 2006. Diterpene resin acids in conifers. Phytochemistry **67**:2415-2423.

Knebel, L., D. J. Robison, T. R. Wentworth, and K. D. Klepzig. 2008. Resin flow responses to fertilization, wounding and fungal inoculation in loblolly pine (*Pinus taeda*) in North Carolina. Tree Physiol **28**:7.

Lieutier, F., D. Sauvard, F. Brignolas, V. Picron, A. Yart, C. Bastien, and C. Jay-Allemand. 1996. Changes in phenolic metabolites of Scots-pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. Eur. J. For. Path. **26**:145-158.

Lombardero, M. J., M. P. Ayres, P. L. J. Lorio, and J. J. Ruel. 2000. Environmental effects on constitutive and inducible resin defenses of *Pinus taeda*. Ecology Letters **3**:329-339.

Lopez-Goldar, X., C. Villari, P. Bonello, A. K. Borg-Karlson, D. Grivet, R. Zas, and L. Sampedro. 2018. Inducibility of Plant Secondary Metabolites in the Stem Predicts Genetic Variation in Resistance Against a Key Insect Herbivore in Maritime Pine. Front Plant Sci **9**:1651.

Mao, J., Z. He, J. Hao, T. Liu, J. Chen, and S. Huang. 2019. Identification, expression, and phylogenetic analyses of terpenoid biosynthesis-related genes in secondary xylem of loblolly pine (*Pinus taeda* L.) based on transcriptome analyses. PeerJ.

Matusick, G., and L. G. Eckhardt. 2010. Variation in virulence among four root-inhabiting Ophiostomatoid fungi onPinus taedaL.,P. palustrisMill, andP. elliottiiEngelm. seedlings. Canadian Journal of Plant Pathology **32**:361-367.

Matusick, G., R. D. Menard, Y. Zeng, and L. G. Eckhardt. 2013. Root-Inhabiting Bark Beetles (Coleoptera: Curculionidae) and their Fungal Associates Breeding in Dying Loblolly Pine in Alabama. Florida Entomologist **96**:238-241.

Mello, M. O., and M. C. Silva-Filho. 2002. Plant-insect interactions: an evolutionary arms race between two distinct defense mechanisms. Braz. J. Plant Physiol. **14**:71-81.

Raffa, K. F., C. J. Mason, P. Bonello, S. Cook, N. Erbilgin, K. Keefover-Ring, J. G. Klutsch, C. Villari, and P. A. Townsend. 2017. Defence syndromes in lodgepole - whitebark pine

ecosystems relate to degree of historical exposure to mountain pine beetles. Plant Cell Environ **40**:1791-1806.

Ro, D. K., and J. Bohlmann. 2006. Diterpene resin acid biosynthesis in loblolly pine (Pinus taeda): functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1). Phytochemistry **67**:1572-1578.

Thompson, A., J. Cooper, and L. L. J. Ingram. 2006. Distribution of terpenes in heartwood and sapwood of loblolly pine. Forest Products Journal **56**:46-48.

Turner, G. W., A. N. Parrish, J. J. Zager, J. T. Fischedick, and B. M. Lange. 2019. Assessment of flux through oleoresin biosynthesis in epithelial cells of loblolly pine resin ducts. J Exp Bot **70**:217-230.

Villari, C., A. Battisti, S. Chakraborty, M. Michelozzi, P. Bonello, and M. Faccoli. 2012. Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine. Tree Physiol **32**:867-879.

Wear, D. N. 2013. Forecasts of land uses. Pages 45-71 *in* D. N. Wear and J. G. Greis, editors. The Southern Forest Futures Project: technical report. USDA-Forest Service, Southern Research Station, Asheville, NC.

Wingfield, M. J., K. A. Seifert, and J. F. Webber. 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. APS Press.

Witzell, J., and J. A. Martín. 2008. Phenolic metabolites in the resistance of northern forest trees to pathogens — past experiences and future prospects. Canadian Journal of Forest Research **38**:2711-2727.

Zanzot, J. W., G. Matusick, and L. G. Eckhardt. 2010. Ecology of root-feeding beetles and their associated fungi on longleaf pine in Georgia. Environ Entomol **39**:415-423.

Zhao, T., D. Kandasamy, P. Krokene, J. Chen, J. Gershenzon, and A. Hammerbacher. 2019. Fungal associates of the tree-killing bark beetle, Ips typographus, vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior. Fungal Ecology **38**:71-79.

Zobel, B., and J. Talbert. 1984. Applied Forest Tree Improvement. John Wiley & Sons, New York, USA.

Table 2.1. Chromatographic, UV, mass-spectral data, and assigned identities of phenolic compounds isolated from outer bark and phloem of *Pinus taeda*.

Code	PDA RT	$[M-Z]^-$	Main fragments by ESI-MS	λ_{max} (nm)	Assigned identity	Standard equivalent	References
PK2F	2.61	329	155	254, 286	UNK	-	
PK2I	2.81	577	373	280	Procyanidin dimer	Procyanidin B2	Raffa et al. 2017
PK2J	2.90	865	577, 432, 359	280	Procyanidin trimer	Procyanidin B2	Raffa et al. 2017
PK3A	3.00	865	720, 577, 432	280	Procyanidin trimer	Procyanidin B2	Raffa et al. 2017
PK3B	3.04	325	163	224, 294	Coumaric acid derivative[a]	pCoumaric acid	Raffa et al. 2017
PK3C	3.17	865	720, 577, 432	280	Procyanidin trimer	Procyanidin B2	Raffa et al. 2017
PK3D	3.23	289	-	230, 280	Catechin	Catechin	Verified by standard
PK3E	3.46	327	-	226, 280	UNK 5	-	Raffa et al. 2017
PK3F	3.53	711	577, 415, 391, 355, 193	216, 230, 290, 315	Ferulic acid derivative	Ferulic acid	Raffa et al. 2017
PK3F2	3.61	481	-	230, 286, 320	UNK	-	
PK3G	3.80	319	-	226, 290, 330	UNK	-	
PK3G2	3.87	405	319	230, 276, 306	UNK	-	
PK4C	4.28	495	445, 421	226, 280	Lignan xyloside	Pinoresinol	Verified by standard
PK4D	4.52	ND	ND	216, 238, 302, 318	Ferulic acid derivative	Ferulic acid	Raffa et al. 2017
PK4F	4.67	525	491, 401	224, 278	UNK 14	-	Raffa et al. 2017
PK4H	4.92	465	303, 162	290	Taxifolin hexoside	Taxifolin	Raffa et al. 2017
PK5A	4.97	303	-	290	Taxifolin	Taxifolin	Verified by standard
PK5B	5.18	243	155	226, 282	UNK	-	
PK5C	5.38	303	-	230, 282	UNK	-	
PK5D	5.46	327	287	230, 290	UNK	-	
PK5G	5.83	603	301	252, 300, 372	Quercetin derivative[a]		
PK5H	5.94	449	391	230, 284	UNK	-	
PK6A	6.29	317	315	252, 300, 372	UNK	-	
PK6C	6.54	287	-	228, 288	UNK	-	
PK7A	7.04	357	317	230, 280	Pinoresinol	Pinoresinol	Verified by standard
PK7B	7.16	603	301	254, 300, 372	Quercetin	Quercetin	Verified by standard
PK8A	8.15	211	-	230, 300, 308	Pinosylvin	Pinosylvin	Verified by standard
PK8C	8.88	255	235, 163	230, 290, 336	Pinocembrin	Pinocembrin	Verified by standard
PK9D	9.67	ND	ND	228, 300, 308	Pinosylvin monomethyl ether	Pinosylvin monomethyl ether	Verified by standard

PDA RT = Retention time at 280 nm

ND = Not detected

[a] = Coeluted with an unknown compound

Table 2.2. Assigned identities and mean concentrations of constitutive phenolic compounds and their inducible variation isolated from outer bark and phloem of *Pinus taeda*.

Code	Assigned identity	Constitutive composition Wk_0 (ng g^{-1} FW)*		Inducible variation $Wk_4 - Wk_0$ (ng g^{-1} FW)		Quantification Method
		Mean and $CI_{95\%}$		Mean and $CI_{95\%}$		
PK2F	UNK	4.27E-04	(3.92E-04, 4.61E-04)	3.43E-06	(-5.14E-05, 5.82E-05)	Peak area equivalent
PK2I	Procyanidin dimer	0.119	(0.076, 0.162)	0.443	(0.343, 0.544)	Analytical standard
PK2J	Procyanidin trimer	0.954	(0.841, 1.066)	-0.012	(-0.136, 0.114)	Analytical standard
PK3A	Procyanidin trimer	0.222	(0.185, 0.258)	0.275	(0.186, 0.364)	Analytical standard
PK3B	Coumaric acid derivative	0.027	(0.014, 0.041)	-0.017	(-0.028, -0.005)	Analytical standard
PK3C	Procyanidin trimer	0.156	(0.116, 0.196)	0.063	(0.023, 0.103)	Analytical standard
PK3D	Catechin	1.079	(0.931, 1.227)	1.065	(0.773, 1.356)	Analytical standard
PK3E	UNK 5	1.06E-04	(5.52E-05, 1.56E-04)	8.31E-05	(3.63E-05, 1.29E-04)	Peak area equivalent
PK3F	Ferulic acid derivative	0.374	(0.301, 0.447)	-0.084	(-0.151, -0.018)	Analytical standard
PK3F2	UNK	6.46E-04	(5.19E-04, 7.73E-04)	-2.53E-04	(-3.79E-04, -1.28E-04)	Peak area equivalent
PK3G	UNK	0.013	(0.011, 0.016)	-0.006	(-0.008, -0.004)	Peak area equivalent
PK3G2	UNK	5.74E-04	(2.67E-04, 8.81E-04)	-2.42E-04	(-5.52E-04, 6.71E-05)	Peak area equivalent
PK4C	Lignan xyloside	0.078	(0.051, 0.104)	-2.02E-04	(-0.022, 0.022)	Analytical standard
PK4D	Ferulic acid derivative	0.074	(0.061, 0.087)	-0.024	(-0.036, -0.012)	Analytical standard
PK4F	UNK 14	8.01E-04	(6.54E-04, 9.48E-04)	-8.78E-05	(-2.29E-04, 5.33E-05)	Peak area equivalent
PK4H	Taxifolin hexoside	0.102	(0.082, 0.122)	-0.005	(-0.022, 0.012)	Analytical standard
PK5A	Taxifolin	1.830	(1.458, 2.203)	-0.759	(-1.009, -0.509)	Analytical standard
PK5B	UNK	0.001	(8.56E-04, 1.18E-03)	1.14E-04	(-2.91E-05, 2.57E-04)	Peak area equivalent
PK5C	UNK	1.51E-04	(1.3E-04, 1.73E-04)	-2.17E-05	(-5.32E-05, 9.86E-06)	Peak area equivalent
PK5D	UNK	6.57E-04	(5.0E-04, 8.14E-04)	-3.12E-04	(-4.09E-04, -2.14E-04)	Peak area equivalent
PK5G	Quercetin derivative	0.119	(0.101, 0.139)	-0.047	(-0.062, -0.033)	Analytical standard
PK5H	UNK	1.53E-04	(1.2E-04, 1.86E-04)	5.79E-05	(1.14E-05, 1.04E-04)	Peak area equivalent
PK6A	UNK	0.003	(0.003, 0.004)	-0.001	(-0.002, -7.61E-04)	Peak area equivalent
PK6C	UNK	7.28E-04	(5.4E-04, 9.15E-04)	-3.01E-04	(-3.98E-04, -2.02E-04)	Peak area equivalent
PK7A	Pinoresinol	0.084	(0, 0.199)	-0.059	(-0.184, 0.065)	Analytical standard
PK7B	Quercetin	0.481	(0.333, 0.629)	-0.182	(-0.287, -0.077)	Analytical standard
PK8A	Pinosylvin	0	Not detected constitutively	0.033	(0.022, 0.044)	Analytical standard
PK8C	Pinocembrin	0	Not detected constitutively	0.049	(0.025, 0.072)	Analytical standard
PK9D	Pinosylvin monomethyl ether	0	Not detected constitutively	0.022	(0.013, 0.031)	Analytical standard

*Unknown (UNK) compounds are reported as Internal Standard equivalent peak areas per g^{-1} FW

Table 2.3. Treatment effects of phenolic compounds isolated from outer bark and phloem of *Pinus taeda*.

Code	Assigned identity	Treatment effects $Wk_4 - Wk_0$			Significant treatments (*P* value)
		df	*F* value	*P* value	
PK2F	UNK	3	3.811	0.022	G.a. – St.w. (0.014)
PK2I	Procyanidin dimer	3	0.128	0.942	
PK2J	Procyanidin trimer	3	3.57	0.028	G.a. – St.w. (0.027)
PK3A	Procyanidin trimer	3	0.812	0.499	
PK3B	Coumaric acid derivative	3	2.092	0.127	
PK3C	Procyanidin trimer	3	1.013	0.404	
PK3D	Catechin	3	2.144	0.120	
PK3E	UNK 5	3	0.518	0.674	
PK3F	Ferulic acid derivative	3	6.463	0.002	All to St.w. (G.a. 0.005)(G.h. 0.004) (L.p. 0.041)
PK3F2	UNK	3	0.536	0.662	
PK3G	UNK	3	0.024	0.995	
PK3G2	UNK	3	0.766	0.524	
PK4C	Lignan xyloside	3	7.032	0.001	G.a. – L.p. (0.037) G.a. – St.w. (0.0009) G.h. – St.w. (0.049)
PK4D	Ferulic acid derivative	3	0.532	0.664	
PK4F	UNK 14	3	3.896	0.021	G.a. – St.w. (0.012)
PK4H	Taxifolin hexoside	3	2.527	0.080	
PK5A	Taxifolin	3	0.922	0.445	
PK5B	UNK	3	1.807	0.172	
PK5C	UNK	3	2.152	0.119	
PK5D	UNK	3	0.072	0.974	
PK5G	Quercetin derivative	3	0.212	0.887	
PK5H	UNK	3	0.491	0.692	
PK6A	UNK	3	0.635	0.600	
PK6C	UNK	3	0.269	0.847	
PK7A	Pinoresinol	3	1.20	0.330	
PK7B	Quercetin	3	1.432	0.257	
PK8A	Pinosylvin	3	4.175	0.016	G.h. – St.w. (0.009)
PK8C	Pinocembrin	3	2.751	0.064	
PK9D	Pinosylvin monomethyl ether	3	2.012	0.138	

G.a. = *G. alacris* G.h. = *G. huntii* L.p. = *L. profanum* St.w. = Sterile wounding

Table 2.4. Assigned identities and constitutive profiles vs. Week 4 controls of phenolic compounds isolated from outer bark and phloem of *Pinus taeda*.

Code	Assigned identity	Constitutive composition Wk_0 vs. *Unwounded Controls*				Direction of Change
		df	*F* value	*P* value		
PK2F	UNK	1	27.64	5.92E-06	***	+
PK2I	Procyanidin dimer	1	8.636	0.006	**	+
PK2J	Procyanidin trimer	1.	20.51	5.71E-05	***	-
PK3A	Procyanidin trimer	1	18.71	1.06E-04	***	+
PK3B	Coumaric acid derivative	1	2.024	0.163		-
PK3C	Procyanidin trimer	1	14.41	5.15E-04	***	+
PK3D	Catechin	1	12.96	9.07E-04	***	+
PK3E	UNK 5	1	10.07	0.003	**	+
PK3F	Ferulic acid derivative	1	3.809	0.058		-
PK3F2	UNK	1	1.162	0.288		-
PK3G	UNK	1	3.097	0.087		-
PK3G2	UNK	1	1.644	0.207		-
PK4C	Lignan xyloside	1	15.56	3.32E-04	***	-
PK4D	Ferulic acid derivative	1	1.822	0.185		-
PK4F	UNK 14	1	18.23	1.26E-04	***	-
PK4H	Taxifolin hexoside	1	6.915	0.012	*	-
PK5A	Taxifolin	1	1.966	0.169		-
PK5B	UNK	1	0.836	0.366		+
PK5C	UNK	1	7.726	0.008	**	-
PK5D	UNK	1	3.686	0.062		-
PK5G	Quercetin derivative	1	4.647	0.038	*	-
PK5H	UNK	1	1.261	0.269		+
PK6A	UNK	1	0.3	0.587		-
PK6C	UNK	1	2.958	0.094		-
PK7A	Pinoresinol	1	2.795	0.103		-
PK7B	Quercetin	1	0.082	0.777		-
PK8A	Pinosylvin	Not detected constitutively				+
PK8C	Pinocembrin	Not detected constitutively				+
PK9D	Pinosylvin monomethyl ether	Not detected constitutively				+

Figure 2.1. Phloem plugs used for inoculation of the loblolly pine (*Pinus taeda*) trees. Plugs were previously collected from a neighboring stand, sterilized, and colonized by treatment fungi.

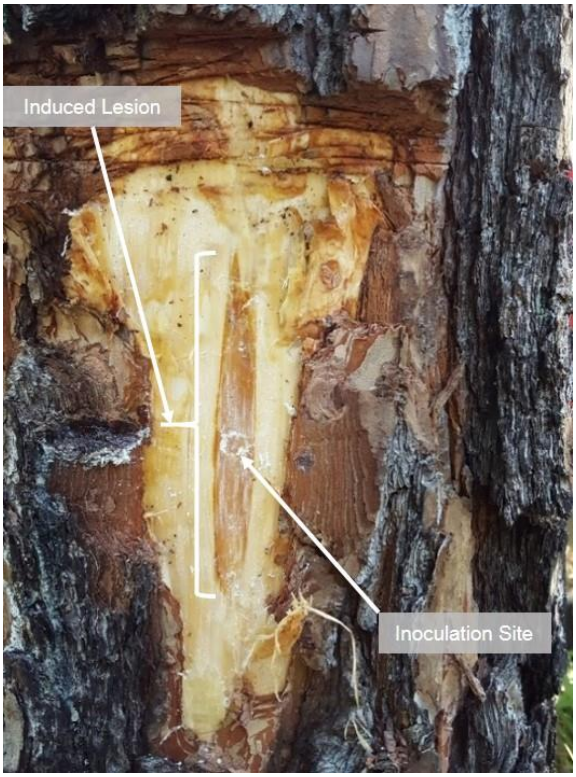

Figure 2.2. An exposed lesion showing the hypersensitive reaction after fungal inoculation

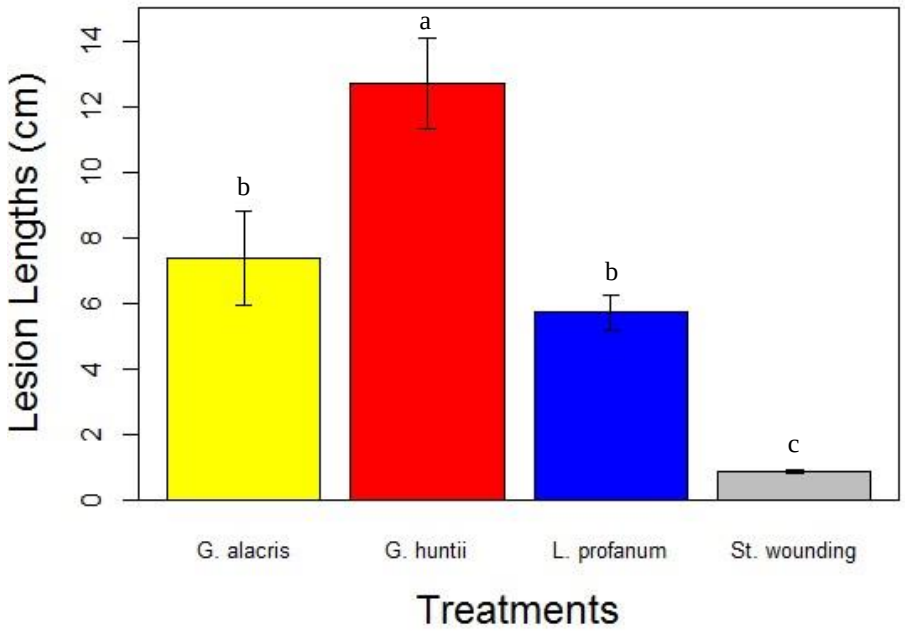

Figure 2.3. Measured lesion lengths in loblolly pine (*Pinus taeda*) induced by fungal inoculation. Error bars shown are the standard errors of the measurements respective to each treatment. Different letters denote significant differences among treatments.

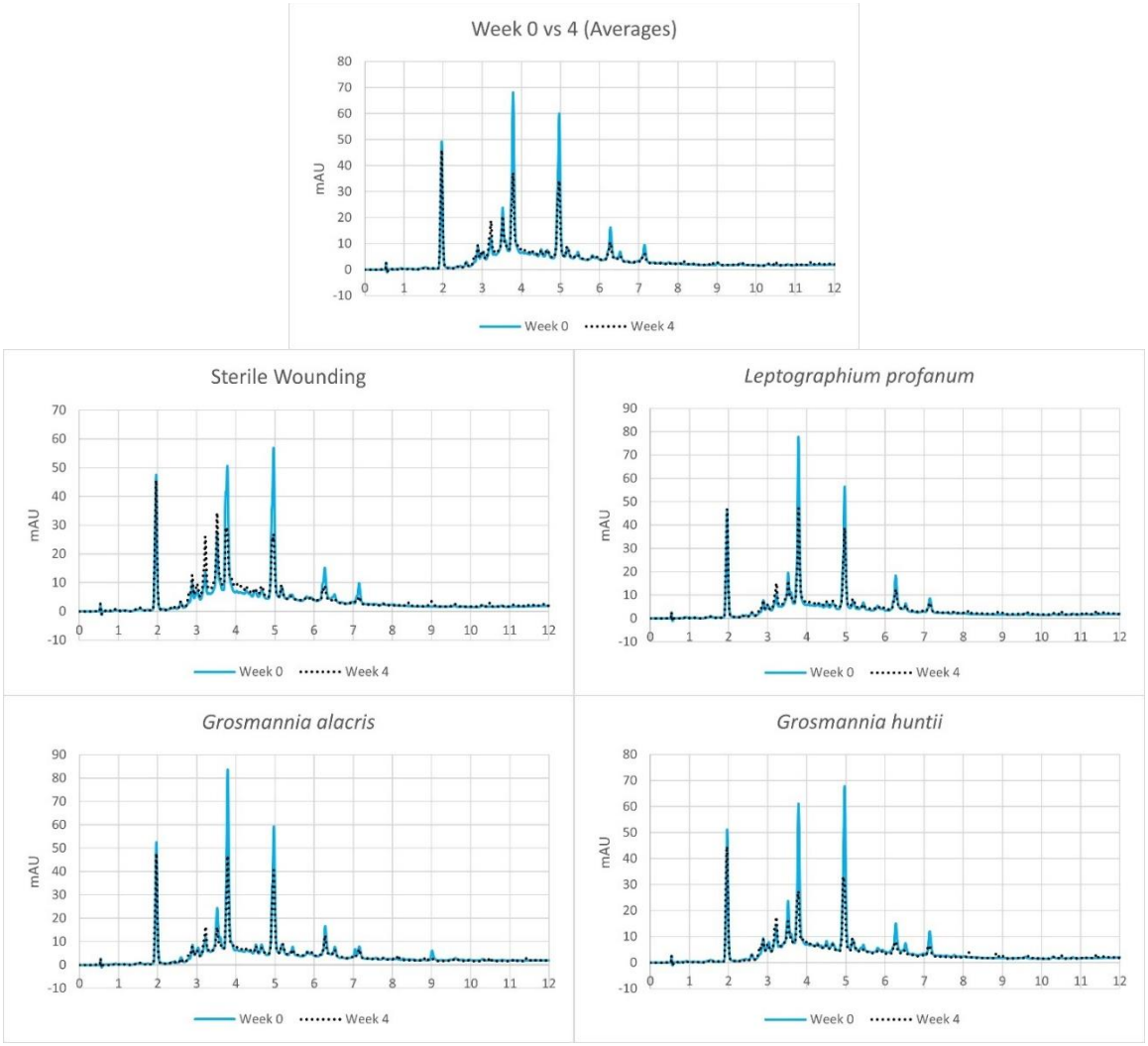

Figure 2.4. Average chromatograms comparing the constitutive profile to the induced profile of phenolic compounds in loblolly pine (*Pinus taeda*) phloem produced by each treatment. Y-axis is mAU recorded by UHPLC-DAD at 280 nm, X-axis is retention time (minutes).

CHAPTER 3

PHENOLIC PROFILES OF SURVIVING WHITEBARK PINES AS A FUNCTION OF THEIR RESISTANCE TO MOUNTAIN PINE BEETLES

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Abstract

Whitebark pine (*Pinus albicaulis*) is a foundational and keystone species in high elevation forests of the northwestern United States currently facing the threat of extinction. A warming climate has led to extensive and severe outbreaks of mountain pine beetle (MPB; *Dendroctonus ponderosae*) that are causing devastating levels of mortality to this naïve host. Outbreaks of MPB are a regular occurrence and forests in this region typically rely on them as regular disturbance agents. Historically, however, outbreaks were relatively short-lived and confined to lower elevation forests where whitebark pine is not found. Only rarely would conditions exist for outbreaks to reach up into the range of whitebark pine, but this has unfortunately changed. To help preserve this critical species, recent studies have been conducted investigating if any whitebark pine present in beetle-killed forests have any chemical markers that could predict resistance to MPB attack. It has been concluded that there exists a genetic basis for survivorship, but how those genetics are phenotypically expressed is still a primary concern. Thus, our objective for this study was to investigate whether the phenolic profile of remaining trees in the aftermath of a MPB outbreak can differentiate between potentially resistant or susceptible trees and help identify resistance mechanisms of whitebark pine toward bark beetles. Whitebark pine phloem was analyzed using ultra-high-performance liquid-chromatography—coupled with either diode array detection (UHPLC-DAD) or with mass spectrometry (UHPLC -MS). Though the overall phenolic profile was a weak predictor for potentially resistant trees, significant differences in individual compound concentrations were found between the two groups of sampled whitebark pine.

INDEX WORDS: Chromatography, Mountain pine beetle, Phenolics, Whitebark pine

3.1 Introduction

In forested ecosystems, disturbance (either natural and anthropogenic) plays an important role in succession and community structure (Doyle 1981). Forest succession is described as either being primary or secondary. Primary succession occurs when pioneer species begin to inhabit an area with little to no existing life and as these pioneer species continue to colonize the area, the area becomes more inhabitable to other species (Finegan 1985). Once this happens, the community has reached secondary succession, where new life either outcompetes old life, or the old life is removed in some manner (disturbance), allowing for new life to take over. Succession is how forest communities change over time—the most adaptable responses to disturbance persist on the landscape. In many ecosystems, forest succession follows a predictable pattern, and this helps dictate how we manage our forests (e.g., prescribed burns, thinning regimes) (Taylor et al. 2009).

In all forests around the world, there are numerous biological agents such as bark beetles and fungal pathogens, that act as catalysts for forest succession and drive disturbance events. In most cases, this is beneficial for the forest as unhealthy or maladapted trees are targeted, resulting in a healthier forest and ecosystem (Logan and Powell 2001). There are very few species capable of large-scale disturbance events and the mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) is one of those species (Raffa et al. 2013). MPB is native to North America and adult beetles are dark brown to black, ranging from 2.5-7.5 mm (Safranyik 1989). Much of the MPB life cycle is spent as larvae in the inner bark of host trees, feeding on phloem and fungi growing on the walls of their galleries (Safranyik 1989, Logan and Powell 2001). MPBs are known vectors of several fungal species belong to the *Ophiostoma* and *Leptographium* genera (Lee et al. 2006). Outbreaks of MPB are not unusual, typically short-

lived, and have occurred throughout history, at least since the Holocene (Gibson et al. 2008). Unfortunately, the most recent outbreaks of MPB have arguably reached unprecedented levels in all aspects. They have persisted longer than historical MPB outbreaks (Logan et al. 2010), spread to forests previously thought to be unsuitable for MPB colonization (Carroll et al. 2003), and caused billions (USD) in damage (Corbett et al. 2016). Outbreak persistence is partly due to a warming climate. Longer periods of warmer weather and milder winters are conducive to outbreak events and it has been found that the life cycle of some MPB broods has increased to two generations rather than one (Mitton and Ferrenberg 2012). The outlook is grim for high elevation forests if the range expansion of MPB does not slow down, as trees in these ecosystems are naïve hosts and do not stand a fighting chance against colonizing MPB (Raffa et al. 2013).

One species that is threatened by the presence of MPB is whitebark pine (*Pinus albicaulis* Engelm.). Whitebark pine is a five-needled pine that is slow-growing and long-lived, not reaching sexual maturity until around 50 years (Arno and Hoff 1990). It lives in high elevation ecosystems, comprising a significant percentage of its ecosystem's biomass and can exhibit krummholz growth (shrub-like) (Arno and Hoff 1990). In this extreme climate, whitebark pine is considered a foundational and keystone species because of its function of improving soil conditions, altering snow dynamics, and providing nutrition to wildlife with its fleshy seeds (Logan and Powell 2001, Logan et al. 2010). Because of the relative importance of whitebark pine and the need to maximize conservation and mitigation efforts, many different investigations were/are being conducted on the capabilities of this species at defending itself against MPB attacks. One experiment provided evidence that whitebark pine may have a genetic basis for survivorship (Six et al. 2018). Trees from Vipond Park (Montana, United States), a stand that experienced 95% mortality of whitebark pine, were sampled and divided into "survivor" and

"general population" groups. Using diameter at breast height (DBH), trees were determined to be survivors if their DBH fell within the DBH range of beetle-killed trees. Those with a smaller DBH were assumed too small to be attacked by beetles and were chosen to represent the general population of whitebark. When comparing the genetic profiles of survivor trees to the general population, Six et al. (2018) found a low percentage of "survivor" genotypes within the general population, roughly mirroring the proportion of surviving trees within the sampled stand. This finding was encouraging for whitebark pine preservation efforts, but their results could not provide an answer as to how these genetic differences are phenotypically expressed, prompting further investigation into aspects like the chemical phenotypes of surviving trees.

The chemical defense response of conifers is a complex mixture comprised of secondary metabolites, primarily being phenolics and terpenoids. Phenolic compounds derive from the shikimate-phenylpropanoids-flavonoids pathways (Pereira et al. 2009) whereas terpenes derive from the mevalonic acid pathway (Zhou et al. 2012). Previous studies have shown that terpenoids are generally better suited to defend against herbivores and that phenolics are generally better suited to defend against pathogens (Hammerschmidt 2005, Keeling and Bohlmann 2006, Raffa et al. 2017). Compounds in both classes represent adaptive characteristics, and this has resulted in a large diversity of compounds depending on the plant that derives it (Lattanzio et al. 2006, Tholl 2006). While the general role of induced defense phenolics in pine species is understood to be antifungal (Witzell and Martín 2008), these compounds are known to be important component of the response to herbivory in many herbaceous species or hardwoods (Hayes and Strom 1994, Leitner et al. 2005, Villari et al. 2016). In pine species, phenolic compounds belonging to the stilbenes and flavonoids classes increased their concentrations after simulated attacks by MPB (Raffa et al. 2017).

The large-scale mortality of whitebark pine in Vipond Park depicts a grim outlook for the future of the species. To understand how the genetic variation of whitebark pine is phenotypically expressed, a recent investigation into the composition of terpenes was conducted but found no evidence of terpenes being representative of survivorship (Six et al. 2021). In a similar vein, we thought it pertinent to investigate the phenolic composition of the "survivor" and "general population" trees within the Vipond Park whitebark pine population. Our objective for this study was to investigate whether the phenolic profile of remaining trees in the aftermath of a MPB outbreak can differentiate between potentially resistant or susceptible trees and help identify resistance mechanisms of whitebark pine toward bark beetles.

3.2 Materials and Methods

3.2.1 Field Site and Sample Collection

Sampling occurred in Vipond Park, located in the Beaverhead National Forest, Montana, United States (2,501 m elevation, 45.6974ºN, 112.9106ºW). [See Six et al. (2018) for a more detailed description of site and tree selection]. Within Vipond Park, *P. albicaulis* makes up 90% of the tree species and of those, approximately 95% were killed during a *D. ponderosae* outbreak lasting from 2009-2013 (Six et al. 2018, Six et al. 2021). Transects were established in 2017 and 2018 and along them beetle-killed trees had their diameters measured at breast height (DBH, 1.4 m above ground) to determine a diameter distribution of targeted trees. Using that distribution, living trees along sampling transects were divided into two groups. Trees that had a similar diameter as beetle-killed trees were assigned to the 'survivor' group, assuming that they had not been killed because they were resistant, and trees with diameters slightly smaller were assigned to the 'general' group, assuming that they had not been killed because they were too small to be

colonized. The general group served to represent the diversity of the population before the beetle outbreak and because of the way it had been selected, included both potentially susceptible and potentially resistant trees. A total of 30 survivor and 29 general trees were selected for the chemical categorization of their bark and phloem. Using a 2.5 cm arch punch, two disks of bark and phloem were removed from each tree and placed on dry ice until they were returned from the field, immediately stored at -80ºC, and later shipped to the University of Georgia for the analysis of phenolic compounds.

3.2.2 Extraction of Phenolic Compounds

Samples were comprised of outer bark and phloem and were processed following the three-day protocol described in Lopez-Goldar et al. (2018). Bark and phloem plugs were ground in liquid nitrogen using pestle and mortar and the ground material was weighed into 100 ± 5 mg aliquots. To begin extraction, each aliquot was soaked in 500 µL HPLC-grade methanol containing 0.5 mg/mL resorcinol as an internal standard (IS), vortexed for 5-10 seconds, and stored at 4°C overnight. After soaking overnight, aliquots were centrifuged at 16,000 rcf for 8 minutes. The resulting supernatants were removed and stored in new tubes at -20°C. The remaining pellets were again soaked in methanol containing IS as in day one. On the final day of extraction, samples were again centrifuged at 16,000 rcf for 8 minutes and the resulting supernatants were merged with the previous supernatants and stored at -20°C.

Because terpenes have already been analyzed in this study system (Six et al. 2021), non-polar resins were removed so as to not interfere with our analysis. Following the purification methods used in Lopez-Goldar et al. (2018), 500 µL of HPLC-grade water was added to 500 µL of each sample. This caused the diterpene acids to flocculate out of solution. Samples were then

vortexed and centrifuged at 16,000 rcf for 15 minutes. Supernatants were transferred to new tubes and then dried down using a vacuum centrifuge. Once dried, the remaining contents were resuspended in 500 µL of HPLC-grade methanol, mixed by sonication, and stored at -20°C until further analysis.

3.2.3 Acquisition and Quantification of the Phenolic Compounds

Phenolic profiles were acquired using an ultra-high-performance liquid chromatography—diode array detector (UHPLC-DAD) with an Agilent 1290 Infinity II UHPLC system. All UHPLC-DAD parts and software used were developed by Agilent Technologies (CA, USA). The column used for separation was a ZORBAX Eclipse Plus C18 Rapid Resolution HD (2.1x100mm, 1.8-micron) coupled with a ZORBAX Eclipse Plus C18 guard column (2.1x5mm, 1.8-micron). Autosampler temperature was held at 22ºC and the column temperature was held at 50ºC. We used a binary mobile phase of 0.1% acetic acid in HPLC-grade methanol (solvent A) and 0.1% acetic acid in HPLC-grade water (solvent B) with a constant flow rate of 0.5 ml/min. Samples were injected at a volume of 0.5 µL and were run on the following linear gradient: (cumulative run time (min), % solvent A) 0.0, 95.0; 12.0, 0.0; 14.0, 0.0, 14.25, 95.0; 19.25, 95.0 (run time totaled 19.25 minutes). A methanol blank was run every 5 samples to periodically clean the column and channels from any potential carryover. All samples were run in a single, continuous sequence that lasted approximately 24 hours. Spectral data from the DAD were recorded from 210 to 400 nm and detected phenolic compounds at 280 nm (Lopez-Goldar et al. 2018).

With the generated chromatograms (Agilent OpenLab), we compared peak arrangement, retention times, and absorption units (AU) of each peak detected using the following integration

events: Tangent Skim Mode: New Exponential, Tail Peak Skim Height Ratio: 5.00, Front Peak Skim Height Ratio: 5.00, Skim Valley Ratio: 20.00, Baseline Correction: Advanced, Peak to Valley Ratio: 500.00, Slope Sensitivity: 5.000, Peak Width: 0.010, Area Reject: 5.000, Height Reject: 1.450, Shoulders: TAN, Area Percent Reject: 0.000. A manual event was placed at minute 12 to remove peaks of non-interest from the integration results. The baseline for each chromatogram was then visually verified for accuracy and consistency of the automatic integration performed on each peak, making manual adjustments when necessary. Peak areas were exported and normalized by the internal standard and by the fresh weight used for extraction.

3.2.4 Identification of the Phenolic Compounds

Identification of phenolic compounds followed the procedure described by Raffa et al. (2017). We used high-performance liquid chromatography—mass spectrometry (HPLC-MS) using a HPLC 1200 (Agilent Technologies, CA, USA) coupled to an Accurate-Mass TOF LC/MS 6220 (Agilent Technologies, CA, USA) to obtain molecular mass (m/z) for the peaks we previously identified on the UHPLC. Mass spectrometry data were acquired in negative ion mode using electrospray ionization and analysis was performed on Agilent's MassHunter software. Although an imperfect match (UHPLC-DAD vs. HPLC-MS), the same solvent system, column, and mobile phase gradient was used on the HPLC-MS. To maintain a stable pressure, flow rate had to be reduced to 0.4 ml/min. Phenolic compounds were matched based on order of elution and congruence of λ_{max}. Compounds were then identified based on negative ion fragmentation patterns, matching retention time and λ_{max} to standards, and comparing order of elution and λ_{max} to literature (see Table 3.1).

3.2.6 Statistical Analyses

Statistical tests were performed in RStudio version 1.3.1056 (R Core Team) using the following packages: tidyverse, caret, pls, and factoextra. Compounds selected for analysis were those that were found in 70% or more of the samples of either group. Compounds not detected in individual samples were assigned a concentration of '0'. A one-way analysis of variance (ANOVA) was run on each compound to test if compound amounts were significantly different between the groups. Each compound was tested for normality and those that failed the normality test were log-transformed. To determine whether the chemical profile as a whole could be used to discriminate among the trees, a principal component analysis (PCA) and principal component regression (PCR) (Kassambara 2018) were performed on the standardized dataset, after compound concentrations were divided by their standard deviation to reduce the effect of the abundant compounds on the ordination.

3.3 Results

3.3.1 Phenolic Profiles and Compound Identities

We found a total of 47 possible compounds present in whitebark pine phloem (Fig 3.1). Of those, a total of 16 compounds were found in 70% or more of the samples of either group and were further analyzed in terms of identity and IS-relative quantity (Tables 3.1 and 3.2). We were able to putatively assign an identity to 10 phenolic compounds in the following classes: 5 flavonoids, 2 hydroxycinnamic acids, 1 vanilloid, 1 phenylpropanoid, and 1 lignan.

3.3.2 Comparison of Phenolic Profiles

When comparing the concentrations of each compound between the two groups, 12 of the 16 analyzed compounds were found to be significantly different (see Table 3.2 for compound differences). In the PCA, 50.9% of the variation associated with the two groups was explained by the first two principal components. While most trees were closely clustered, there were a handful of survivor trees that did not overlap with the rest (Fig 3.2). In the PCR, the first component alone explained most of the variation associated with the two groups (Fig 3.3). We performed a regression analysis (or analysis of deviance) on this component and results showed that only 26.7% of the deviance was explained, suggesting little difference in the phenolic profiles between the groups. Loading values of most compounds were similar (Table 3.3), suggesting compounds affected the comparison equally.

3.4 Discussion

Whitebark pine, a foundational species at high elevation, is experiencing severe outbreaks of MPB, and is now on the verge of extinction (Goeking and Izlar 2018). While there is some evidence for the presence of a low level of genetic resistance in the natural populations (Six et al. 2018), the chemical phenotype of potentially resistant trees has not been identified yet (Six et al. 2021). In this study, we investigated if the phenolic profile of remaining trees in the aftermath of a MPB outbreak can differentiate between potentially resistant or susceptible trees and help identify resistance mechanisms of whitebark pine towards bark beetles. Phenolic compounds found in our samples match compounds known to be present in whitebark pine (Raffa et al. 2017). While we found significant differences in the individual concentrations of each phenolic compound, the overall phenolic profile was a poor predictor of whitebark pine

resistance to MPB attack. Moreover, it is worth noting that differences in chemical concentrations could be due to the different age classes of the trees, especially considering how the general and survivor populations were sorted by diameter, a characteristic that likely correlates with age in such a long-lived species. Of the compounds showing a significant difference between the two groups, of note is taxifolin, as an investigation on the role of flavonoids in the defense response of Norway spruce (*Picea abies* (L.) Karst) has shown it to be toxic to *Ips typographus* Linnaeus and its associated fungus, *Endoconidiophora polonica* (Siemaszko) de Beer et al. (Hammerbacher et al. 2019).

When analyzing total phenolic content, we found no significant difference in the phenolic profiles between each group, which could indicate that phenolics do not have a predominant role in the defense mechanisms of conifers against bark beetles. This is supported by what is currently known about conifer phenolic defense compounds predominantly having antifungal properties (Witzell and Martín 2008), despite phenolic compounds being well established as defense compounds against bark and wood borers in hardwoods (e.g., (Schultz 1989, Morewood et al. 2004, Villari et al. 2016). Alternatively, this could also be due to the intrinsic limitations of our experimental design, in which the general population was a natural mix of potentially resistant and susceptible trees, and hence a distinct clustering of survivors versus general was not expected, unless the differentiation was very strong. Moreover, it could be because whitebark pine is a naïve host to MPB (Raffa et al. 2013) and survivorship to MPB may not be related to the chemical phenotype. This is supported by the recent findings of Six et al. (2021) where they investigated the terpene content of trees from the same stand as our experiment and determined that the terpene content could not predict differences between the general population and surviving trees.

While our results suggest phenolics might not be important in the defense response of whitebark against MPB, it is still important that we explore all the components of the chemical defense strategy of trees to disturbance, especially for species as ecologically important as whitebark pine. Even though the results from our comparisons were not strong enough to be used as a predictive measure of resistance, the clustering that occurred in our PCA and the different chemical concentrations determined by ANOVA suggest that there is still differing chemical activity occurring and that is enough to grant further investigation into the matter.

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3.6 Literature Cited

Arno, S. F., and R. J. Hoff. 1990. *Pinus albicaulis* Engelm. Whitebark Pine. Pages 268-279 *in* R. M. Burns and B. H. Honkala, editors. Silvics of North America: Conifers. USDA Forest Service, Washington, DC.

Carroll, A. L., S. W. Taylor, J. Regniere, and L. Safranyik. 2003. Effects of climate change on range expansion by the mountain pine beetle in British Columbia.*in* Mountain Pine Beetle Symposium: Challenges and Solutions. Canadian Forest Service, Kelowna, British Columbia.

Corbett, L. J., P. Withey, V. A. Lantz, and T. O. Ochuodho. 2016. The economic impact of the mountain pine beetle infestation in British Columbia: provincial estimates from a CGE analysis. Forestry: An International Journal of Forest Research **89**:100-105.

Doyle, T. W. 1981. The Role of Disturbance in the Gap Dynamics of a Montane Rain Forest: An Application of a Tropical Forest Succession Model.*in* D. C. West, H. H. Shugart, and D. B. Botkin, editors. Forest Succession. Springer, New York, NY.

Finegan, B. 1985. Forest Succession. Nature **315**:692-692.

Gibson, K., K. Skov, S. Kegley, C. Jorgensen, S. Smith, and J. Witcosky. 2008. Mountain pine beetle impacts in high-elevation five-needle pines: current trends and challenges.*in* F. S. USDA, Forest Health Protection, editor., Missoula, Montana, USA.

Goeking, S., and D. Izlar. 2018. Pinus albicaulis Engelm. (Whitebark Pine) in Mixed-Species Stands throughout Its US Range: Broad-Scale Indicators of Extent and Recent Decline. Forests **9**.

Hammerbacher, A., D. Kandasamy, C. Ullah, A. Schmidt, L. P. Wright, and J. Gershenzon. 2019. Flavanone-3-Hydroxylase Plays an Important Role in the Biosynthesis of Spruce

Phenolic Defenses Against Bark Beetles and Their Fungal Associates. Front Plant Sci **10**:208.

Hammerschmidt, R. 2005. Phenols and plant–pathogen interactions: The saga continues. Physiological and Molecular Plant Pathology **66**:77-78.

Hayes, J. L., and B. L. Strom. 1994. 4-Allylanisole as an Inhibitor of Bark Beetle (Coleoptera: Scolytidae) Aggregation. J. Econ. Entomol. **87**:1586-1594.

Kassambara, A. 2018. Machine Learning Essentials: Practical Guide in R. 1 edition. STHDA.

Keeling, C. I., and J. Bohlmann. 2006. Diterpene resin acids in conifers. Phytochemistry **67**:2415-2423.

Lattanzio, V., V. M. T. Lattanzio, and A. Cardinali. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry: Advances in Research:23-67.

Lee, S., J.-J. Kim, and C. Breuil. 2006. Diversity of fungi associated with the mountain pine beetle, *Dendroctonus ponderosae* and infested lodgepole pines in British Columbia. Fungal Diversity **22**:91-105.

Leitner, M., W. Boland, and A. Mithofer. 2005. Direct and indirect defences induced by piercing-sucking and chewing herbivores in Medicago truncatula. New Phytol **167**:597-606.

Logan, J. A., W. W. Macfarlane, and L. Willcox. 2010. Whitebark pine vulnerability to climate-driven mountain pine beetle disturbance in the Greater Yellowstone Ecosystem. Ecological Applications **20**:895-902.

Logan, J. A., and J. A. Powell. 2001. Ghost Forests, Global Warming, and the Mountain Pine Beetle (Coleoptera: Scolytidae). American Entomologist **47**:160-172.

Lopez-Goldar, X., C. Villari, P. Bonello, A. K. Borg-Karlson, D. Grivet, R. Zas, and L. Sampedro. 2018. Inducibility of Plant Secondary Metabolites in the Stem Predicts Genetic Variation in Resistance Against a Key Insect Herbivore in Maritime Pine. Front Plant Sci **9**:1651.

Mitton, J. B., and S. M. Ferrenberg. 2012. Mountain pine beetle develops an unprecedented summer generation in response to climate warming. Am Nat **179**:E163-171.

Morewood, W. D., K. Hoover, P. R. Neiner, J. R. McNeil, and J. C. Sellmer. 2004. Host tree resistance against the polyphagous wood-boring beetle Anoplophora glabripennis. Entomologia Experimentalis et Applicata **110**:79-86.

Pereira, D., P. Valentão, J. Pereira, and P. Andrade. 2009. Phenolics: From Chemistry to Biology. Molecules **14**:2202-2211.

Raffa, K. F., C. J. Mason, P. Bonello, S. Cook, N. Erbilgin, K. Keefover-Ring, J. G. Klutsch, C. Villari, and P. A. Townsend. 2017. Defence syndromes in lodgepole - whitebark pine ecosystems relate to degree of historical exposure to mountain pine beetles. Plant Cell Environ **40**:1791-1806.

Raffa, K. F., E. N. Powell, and P. A. Townsend. 2013. Temperature-driven range expansion of an irruptive insect heightened by weakly coevolved plant defenses. Proc Natl Acad Sci U S A **110**:2193-2198.

Safranyik. 1989. Mountain Pine Beetle: Biology Overview. Pages 9-13 *in* Symposium on the Management of Lodgepole Pine to Minimize Losses to the Mountain Pine Beetle. USDA Forest Service, Intermountain Forest and Range Experiment Station.

Schultz, J. C. 1989. Tannin-insect interactions. Pages 417-433 *in* R. W. Hemingway, J. J. Karchesy, and S. J. Branham, editors. Chemistry and Significance of Condensed Tannins. Springer, New York, NY, USA.

Six, D. L., A. Trowbridge, M. Howe, D. Perkins, E. Berglund, P. Brown, J. A. Hicke, and G. Balasubramanian. 2021. Growth, Chemistry, and Genetic Profiles of Whitebark Pine Forests Affected by Climate-Driven Mountain Pine Beetle Outbreaks. Frontiers in Forests and Global Change **4**.

Six, D. L., C. Vergobbi, and M. Cutter. 2018. Are Survivors Different? Genetic-Based Selection of Trees by Mountain Pine Beetle During a Climate Change-Driven Outbreak in a High-Elevation Pine Forest. Front Plant Sci **9**:993.

Taylor, A. R., H. Y. H. Chen, and L. VanDamme. 2009. A Review of Forest Succession Models and Their Suitability for Forest Management Planning. Forest Science **55**:23-36.

Tholl, D. 2006. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr Opin Plant Biol **9**:297-304.

Villari, C., D. A. Herms, J. G. Whitehill, D. Cipollini, and P. Bonello. 2016. Progress and gaps in understanding mechanisms of ash tree resistance to emerald ash borer, a model for wood-boring insects that kill angiosperms. New Phytol **209**:63-79.

Witzell, J., and J. A. Martín. 2008. Phenolic metabolites in the resistance of northern forest trees to pathogens — past experiences and future prospects. Canadian Journal of Forest Research **38**:2711-2727.

Zhou, Y. J., W. Gao, Q. Rong, G. Jin, H. Chu, W. Liu, W. Yang, Z. Zhu, G. Li, G. Zhu, L. Huang, and Z. K. Zhao. 2012. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc **134**:3234-3241.

Table 3.1. Chromatographic, UV, mass-spectral data, and assigned identities of phenolic compounds isolated from outer bark and phloem of *Pinus albicaulis*.

Code	PDA RT	$[M-Z]^-$	Main fragments by ESI-MS	λ_{max} (nm)	Assigned identity	Standard equivalent	References
PK2B	2.34	343	-	220, 280	Dihydroconiferin	Coniferyl alcohol	Verified by standard
PK2H	2.76	865	577, 373	230, 280, 308	Procyanidin trimer	Procyanidin B2	Raffa et al. 2017
PK2I2	2.85	577	-	230, 280, 310	Procyanidin dimer	Procyanidin B2	Raffa et al. 2017
PK2J	2.93	865	577, 432, 359	280	Procyanidin trimer	Procyanidin B2	Raffa et al. 2017
PK3B	3.09	325	163	224, 294	Coumaric acid derivative	Coumaric acid	Raffa et al. 2017
PK3D	3.28	289	-	230, 280	Catechin	Catechin	Verified by standard
PK3E2	3.52	ND	ND	278	UNK	-	
PK3F	3.58	711	577, 415, 391, 355, 193	216, 230, 290, 315	Ferulic acid derivative	Ferulic acid	Raffa et al. 2017
PK3H	3.93	357	-	230, 276, 304	Hydroxypropiovanillone hexoside	Vanillic acid	Raffa et al. 2017
PK4A	4.08	577	382, 357	228, 278, 308	UNK	-	
PK4C	4.32	495	445, 421	226, 280	Lignan xyloside	Pinoresinol	Raffa et al. 2017
PK4F	4.70	525	491, 401	224, 278	UNK 14	-	Raffa et al. 2017
PK5A	5.03	303	-	290	Taxifolin	Taxifolin	Verified by standard
PK5B	5.21	243	155	226, 282	UNK	-	
PK5E	5.65	441	-	262	UNK 22	-	Raffa et al. 2017
PK9B	9.09	ND	ND	214, 268, 302, 314	UNK	-	

PDA RT = Retention time at 280 nm ND = Not detected

Table 3.2. Assigned identities and comparison of phenolic compound concentrations between the Survivor and General groups isolated from outer bark and phloem of *Pinus albicaulis*. Chemical concentrations were measured by DAD absorbance and are reported as internal standard normalized peak area.

Code	Assigned identity	Survivor Composition		General Composition		Survivor vs. General		
		Mean and $CI_{95\%}$		Mean and $CI_{95\%}$		*F* value	df, *P* value	
PK2B	Dihydroconiferin	1.18E-03	(9.13E-04, 1.45E-03)	4.53E-04	(2.66E-04, 6.41E-04)	18.27	1, 7.36E-05	***
PK2H	Procyanidin trimer	1.49E-03	(1.31E-03, 1.66E-03)	1.23E-03	(1.05E-03, 1.41E-03)	4.823	1, 0.032	*
PK2I2	Procyanidin dimer	1.01E-03	(8.92E-04, 1.14E-03)	7.64E-04	(6.89E-04, 8.40E-04)	11.98	1, 0.001	**
PK2J	Procyanidin trimer	1.44E-03	(1.19E-03, 1.69E-03)	1.17E-03	(9.59E-04, 1.38E-03)	2.349	1, 0.131	
PK3B	Coumaric acid derivative	3.78E-03	(2.87E-03, 4.69E-03)	2.52E-03	(1.74E-03, 2.29E-03)	4.139	1, 0.047	*
PK3D	Catechin	3.50E-03	(2.89E-03, 4.12E-03)	3.17E-03	(2.55E-03, 3.79E-03)	0.831	1, 0.366	
PK3E2	UNK	4.80E-04	(3.28E-04, 6.32E-04)	7.80E-05	(4.00E-06, 1.52E-04)	20.63	1, 2.94E-05	***
PK3F	Ferulic acid derivative	4.59E-03	(3.55E-03, 5.62E-03)	4.44E-03	(3.36E-03, 5.51E-03)	0.101	1, 0.752	
PK3H	Hydroxypropiovanillone hexoside	1.30E-02	(1.18E-02, 1.42E-02)	1.12E-02	(1.06E-02, 1.19E-02)	5.505	1, 0.023	*
PK4A	UNK	1.75E-03	(1.42E-03, 2.07E-03)	9.05E-04	(7.12E-04, 1.09E-03)	17.85	1, 8.72E-07	***
PK4C	Lignan xyloside	1.69E-03	(1.52E-03, 1.86E-03)	1.32E-03	(1.19E-03, 1.43E-03)	12.08	1, 9.82E-04	***
PK4F	UNK 14	1.06E-03	(9.44E-04, 1.17E-03)	8.94E-04	(7.83E-04, 1.00E-03)	3.951	1, 0.0517	
PK5A	Taxifolin	1.46E-02	(1.19E-02, 1.72E-02)	6.02E-03	(4.44E-03, 7.59E-03)	30.64	1, 8.17E-07	***
PK5B	UNK	1.65E-03	(1.48E-03, 1.81E-03)	1.38E-03	(1.23E-03, 1.54E-03)	5.314	1, 0.025	*
PK5E	UNK 22	8.43E-04	(6.83E-04, 1.00E-03)	9.82E-04	(8.52E-04, 1.11E-03)	1.691	1, 0.199	
PK9B	UNK	4.47E-04	(1.81E-04, 7.13E-04)	9.06E-04	(5.77E-04, 1.24E-03)	4.398	1, 0.040	*

Table 3.3. Assigned identities and PCA loading values for comparison 1, determined by PCR to explain most of the variation among the concentrations of detected phenolic compounds in *Pinus albicaulis*.

Code	Assigned identity	PCA Loading Value (Comp 1)
PK2B	Dihydroconiferin	0.322
PK2H	Procyanidin trimer	0.305
PK2I2	Procyanidin dimer	0.324
PK2J	Procyanidin trimer	0.200
PK3B	Coumaric acid derivative	0.119
PK3D	Catechin	0.183
PK3E2	UNK	0.308
PK3F	Ferulic acid derivative	0.082
PK3H	Hydroxypropiovanillone hexoside	0.300
PK4A	UNK	0.308
PK4C	Lignan xyloside	0.300
PK4F	UNK 14	0.210
PK5A	Taxifolin	0.313
PK5B	UNK	0.274
PK5E	UNK 22	-0.054
PK9B	UNK	-0.120

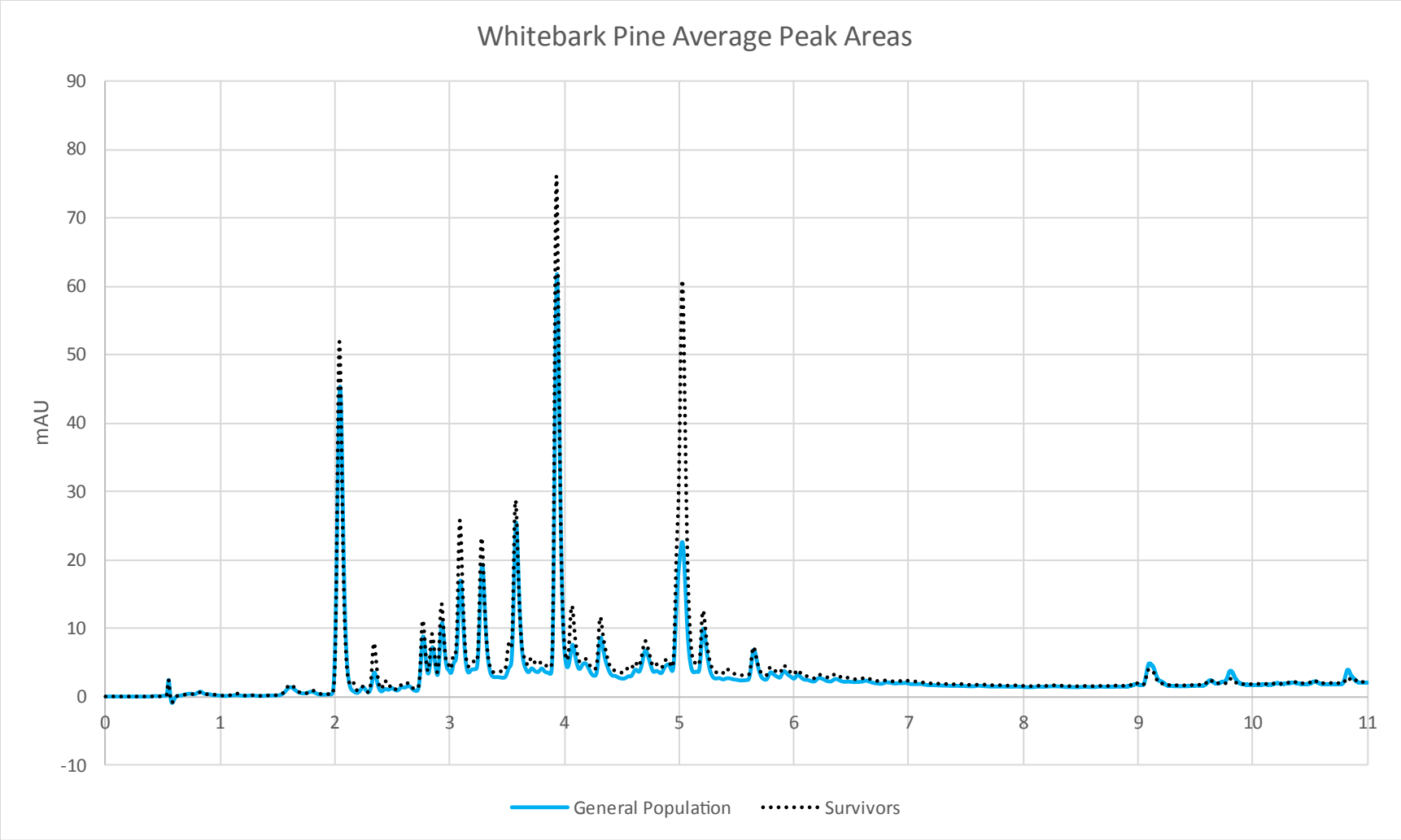

Figure 3.1. Average chromatograms of phenolic compounds detected in both the General and Survivor groups of whitebark pine (*Pinus albicaulis*) phloem. Y-axis is mAU recorded by UHPLC-DAD at 280 nm, X-axis is retention time (minutes).

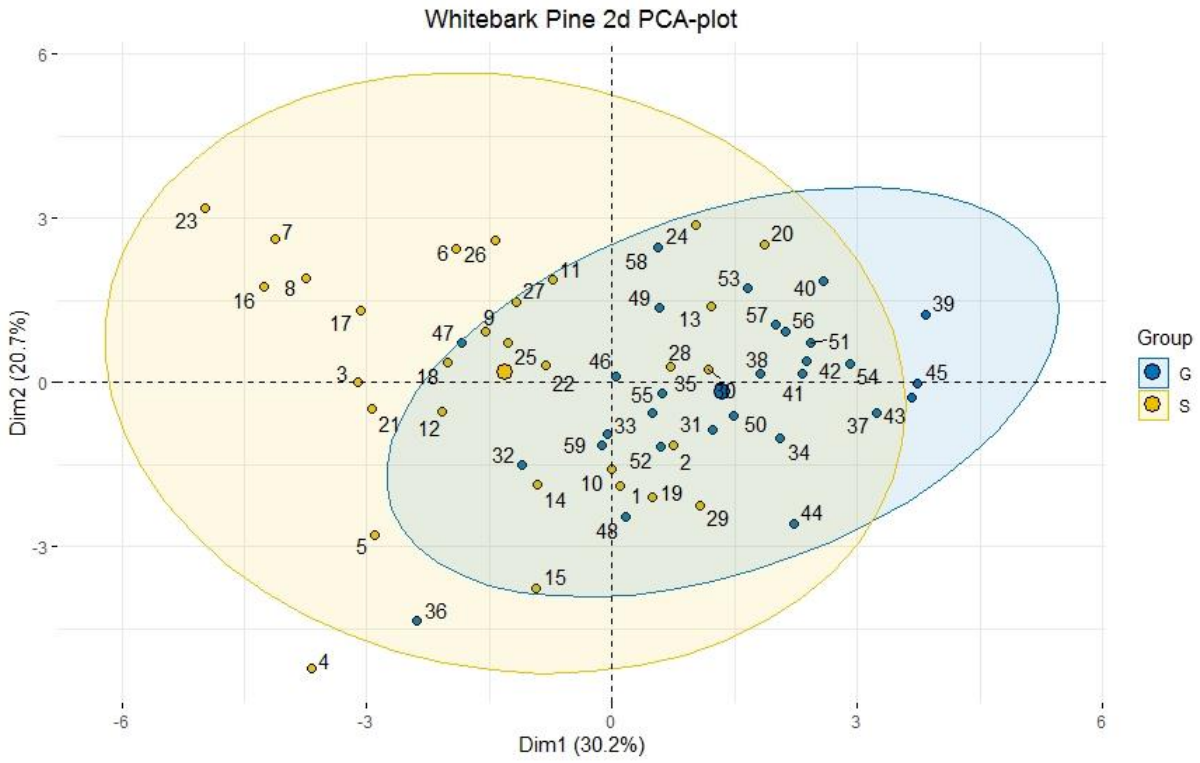

Figure 3.2. Grouping of phenolic compounds detected in whitebark pine (*Pinus albicaulis*) determined by a principal component analysis (PCA). Ellipses highlight the normal distribution of the data for both the General population (G) and Survivors (S), and the graph was created using the factoextra package in R (R Core Team).

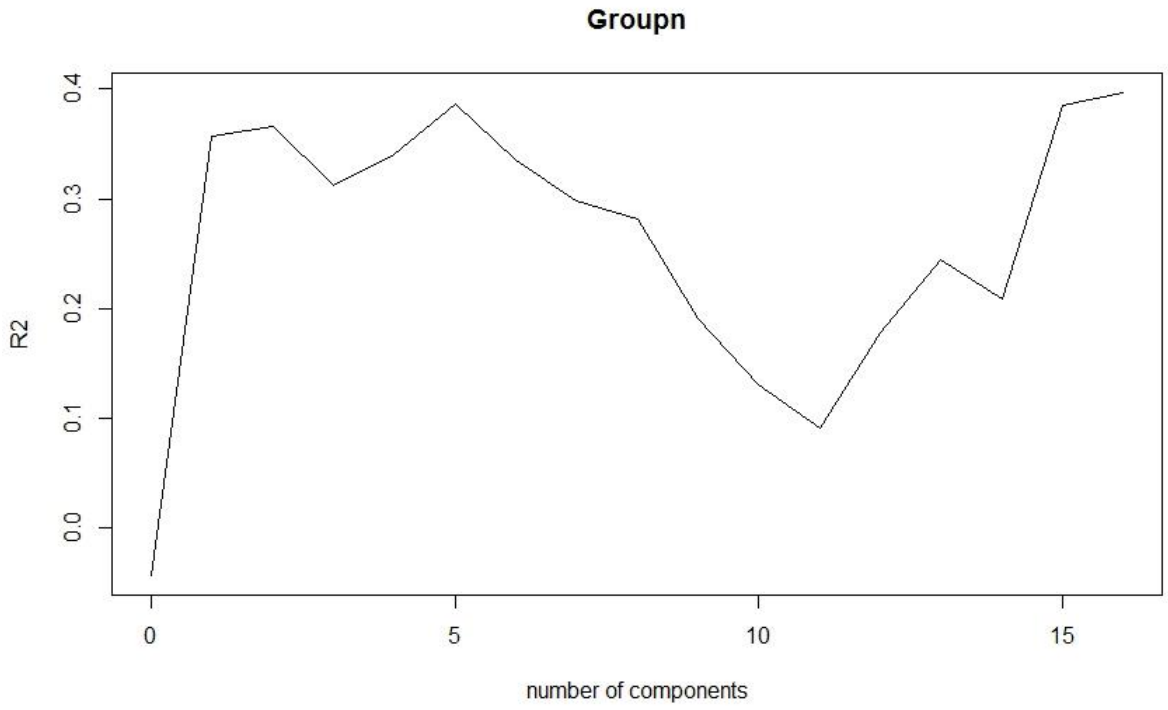

Figure 3.3. Resulting R^2 showing explanation of variance per component. After component 1, no new information was gained regarding the variance.

CHAPTER 4

THESIS CONCLUSIONS

4.1 Conclusions

This thesis sought to investigate the phenolic compounds associated with conifer defense mechanisms in two native North American pine species (*Pinus taeda* and *Pinus albicaulis*), both of which have significant importance to the regions where they are found. In the case of loblolly pine (*P. taeda*), our main objective was to categorize the phenolic compounds present in the phloem of the tree as this information is currently not found in the literature. The phenolic compounds that we identified match the identities of compounds found in investigations of other conifer species, namely those described in Raffa et al. (2017). Additionally, upon investigating the compositional changes to the phenolic profile that occurred after fungal inoculation, we discovered that, like other conifer species, flavonoids and stilbenes were most associated with our treatments. In fact, the stilbenes that we detected were only detected in induced profiles and although we expected stilbenes to be associated with fungal induction, it was interesting to discover that in loblolly pine, they do not appear to be present at all in the phloem until the tree is wounded. Other compounds, however, decreased after the induction, which might suggest an energy tradeoff to enable loblolly pine to deploy the stilbenes. Further research would be needed to effectively investigate this possibility.

In the case of whitebark pine (*P. albicaulis*), many of the phenolic compounds present in this species have already been identified (Raffa et al. 2017) and while we were unable to

elucidate the identities of any of the compounds that remain unknown, our results support what is in current literature. Our main objective with whitebark was to investigate if the phenolic profile could be used to predict resistance of surviving whitebark pine to MPB attack. With an ever-changing climate (that tends to continue warming), the threat that MPB poses to whitebark pine, and other North American conifers will continue to increase. While we determined the total phenolic profile of whitebark pine cannot effectively predict tree resistance to MPB, perhaps future work expanding on our results may discover that compounds we found to be significantly different between the general population and survivor trees act as signaling molecules (Hammerschmidt 2005) for more discreet responses.

4.2 Future Research Directions

Given recent advances in methodology and instrumentation used in chemical analyses of plants, the scientific community is always discovering brand new interactions occurring. Now that there is a phenolic profile categorized for loblolly pine, it would be pertinent to investigate the effects individual compounds may have on fungal growth and insect colonization. Investigations into the individual effects of compounds will also help answer questions about the complicated host-beetle-fungi interactions and the role the fungi have in these systems. It would also be helpful to conduct similar experiments as to what was done in this thesis as methods such as sampling can always be improved. The possibility exists, for instance, that by sampling four weeks after induction, we could have missed the optimal window to observe the most changes in secondary metabolites. An experiment that samples just days after the inoculation, or at multiple time intervals, could reveal interesting and surprising results.

As for the chemical defenses in the whitebark system, future work investigating metabolomics using nuclear magnetic resonance spectroscopy (NMR) (Leiss et al. 2011) could provide more insight into the potential role secondary metabolites play in tree resistance to MPB. Future investigations of whitebark pine secondary metabolites should focus on entire metabolic pathways to try and identify what compounds serve as precursors for others. Unfortunately for the whitebark system, mortality is occurring more rapidly than ever, and time is of the essence if there is any hope in saving this species from extinction.

4.3 Literature Cited

Hammerschmidt, R. 2005. Phenols and plant–pathogen interactions: The saga continues. Physiological and Molecular Plant Pathology **66**:77-78.

Leiss, K. A., Y. H. Choi, R. Verpoorte, and P. G. Klinkhamer. 2011. An overview of NMR-based metabolomics to identify secondary plant compounds involved in host plant resistance. Phytochem Rev **10**:205-216.

Raffa, K. F., C. J. Mason, P. Bonello, S. Cook, N. Erbilgin, K. Keefover-Ring, J. G. Klutsch, C. Villari, and P. A. Townsend. 2017. Defence syndromes in lodgepole - whitebark pine ecosystems relate to degree of historical exposure to mountain pine beetles. Plant Cell Environ **40**:1791-1806.