Survey of volatile organic chemicals emitted by four ophiostomatoid fungi

by

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Abstract

Loblolly pine (*Pinus taeda* L.) is an important commercial timber species in the southeastern United States. It contributes to the growth of the economy by serving as raw material for the forest product industry in the region and the entire United States. However, bark beetle vectored root infecting ophiostomatoid fungi: *Grosmannia alacris, G. huntii, Leptographium terebrantis* and *L. procerum* are threats to the growth and productivity of *P. taeda.* The ophiostomatoid fungi have been a major contributing factor of decline disease in loblolly pines. The interaction between beetle vectors and their fungi can be mediated by volatile organic compounds (VOCs) produced by the fungi, acting as communication cues which influences the behavior of plants and animals. Although VOC interactions between many organisms have been studied, ecological roles of VOCs from fungi remain largely unknown.

The study investigated whether: (i) the fungal produced VOC profiles differed between species; (ii) the presence of a resource-sharing fungus affects the VOC production of a given fungal species; (iii) if seedlings inoculated with ophiostomatoid fungi (*G. alacris, G. huntii, L. terebrantis,* and *L. procerum*) produced VOCs that differed from controls.

Volatiles were collected from *G. alacris, G. huntii, L. terebrantis,* and *L. procerum* in the lab and from seedlings inoculated with the four different fungi. Volatiles from seedlings were randomly and destructively sampled from the inoculation area at 4, 8 and 12 weeks after fungi inoculation. Volatiles were identified with gas chromatography – mass spectrometer (GC-MS).

Eight compounds (ethyl acetate, isoamyl acetate, phenylethyl acetate (esters), isobutanol, 2-methyl-1-butanol, ethyl alcohol, phenylethyl alcohol (alcohols), and verbenone were identified and quantified from the ophiostomatoid fungi. Ophiostomatoid fungi can stimulate or inhibit the

production of fungal VOCs in the presence of other fungi occupying the same ecological niche. Fungal VOC profiles were detected in higher concentrations when fungi were grown on the same culture plate, however, lower concentrations of compounds were recorded when fungi were grown on separate plate in the same chamber without touching each other.

The compounds found in loblolly pine seedlings inoculated with ophiostomatoid fungi were identified as α-pinene, camphene, β-pinene, limonene, myrcene, terpineol, p-cymene, bornyl acetate, ocimene, γ-terpinene, *trans*-verbenol, 3-carene, camphene, *cis*-verbenol, and borneol. Loblolly pine seedlings produced monoterpenes after fungal inoculation, and the compounds increased until they peaked at week 8 and then declined during week 12.

The results suggest that there are similarities in different fungal volatile organic compounds of species which occupy the same ecological niche, and the presence of different fungi can stimulate or inhibit the production of volatile organic compounds. The study also demonstrates that fungal volatile organic compounds can drive interactions between bark beetles and fungal symbiont. Also, the study showed that the compounds detected can be used to manage both bark beetle and its fungal symbiont.

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CHAPTER I

Introduction and Literature review

1.1 Forestry in the world

Forestry can be defined as the art and science of protecting, conserving, and managing the various components of the forest for human and environmental benefits. Forestry is practiced both in plantations and natural stands. It is estimated that forests cover 31 percent of the world's land surface which is about 4 billion hectares (FAO, 2010). Out of this, 31 percent is found in Asia including Asian Russian, 21 percent in South America, 17 percent in North and Central America, 17 percent in Africa, 9 percent in Europe and 5 percent in Oceania (FAO 2010). Also, 5 percent of the world's forests are plantations which are generally used for commercial purposes. In 2011, the forestry sector in the world directly employed 13 million people and contributed over $ 600 million into the global economy (FAO 2014).

Pines are made up of 126 species of conifers in the genus *Pinu*s that are distributed across the world but native to the northern hemisphere. They form a major component of the world's forest ecosystem by providing economic, environmental and aesthetic values. They are also grown in temperate and subtropical regions of the world as timber and ornamental plants. According to Mason et al., (2006), loblolly pine is an important conifer that dominates many of the world's temperate and boreal ecosystems. Although loblolly pine is native only to the southern United States, its adaptability and potential in other parts of the world has long been recognized (Lindsay, 1932).

1.2 Forestry in the southeastern United States

The southeastern United States is known for commercial production of timber, forms a major component of the regional economy (Schultz, 1997). The forest establishment in the region covers about 108.1 million hectares and 16% of timber production globally (Prestemon and Abt, 2002). The region also serves as the wood basket of the United States, representing about 58% of timber production in the country (Neale and Wheeler, 2004). A large number of forest plantations in the southeastern United States grow pines (Wear and Gries, 2012), with a few hardwood species including oaks, magnolia, poplar, walnuts, ash, and maple. Loblolly pine (*Pinus taeda* L.) is the most planted tree species for the commercial timber industry which is native to the region (Oswalt et al., 2014). The region is a source of ecosystem services, namely, clean air and water, and recreation and aesthetic values (Anderson and Sassaman, 1996). The forest cover in this region plays a vital role of the entire carbon credit of the United States which accounts for 36% of forest carbon sequestered in the country (Turner et al., 1995).

1.3 *Pinus taeda*

1.3.1 Importance

Loblolly pine is an important commercial timber species in the southeastern United States (Rauscher, 2004) and a coniferous tree belonging to the family Pinacaeae. It covers a land area of approximately 13.4 million hectares throughout the southeastern forests (Shultz, 1997) which forms 80% of commercial pine plantations in the region (Smith et al., 2001). The native range of loblolly pine extends from southern New Jersey south to Florida and west to eastern Texas (Schultz, 1997).

More than 1.3 billion seedlings of loblolly are planted each year (Daine et al., 2020), representing about 75% of loblolly pine seedlings planted in the United States for afforestation

and reforestation programs (McKeand et al., 2003). The tree species is fast-growing and can tolerate stress. Loblolly pine provides food for wildlife as well as providing ecosystem services and recreation (Poudel, 2014; Schultz, 1997). The tree species contributes to the growth of the economy by serving as raw material for the forest product industry in the United States. These reasons make loblolly pine the most economical pine species in the southeastern United States.

1.3.2 Biology

Loblolly pine is dominant on many sites in its range because of its unique biology. The tree is a consistent prolific producer of seed along the coastal plain, becoming a more periodic producer as you move inland (Baker and Langdon, 1990). The seeds go through a dormant stage prior to germination, which lasts longer than any other southern pine. Though dormant seed is susceptible to predation by many pests, when established its growth is rapid and consistent throughout the stand. In the natural stands, different growth rates exist, and individual trees express early dominance when growing under the best microsite conditions (Baker and Langdon, 1990). On good sites, growth differentiation occurs early compared to poor sites, separating trees into different crown classes. Loblolly pine produces a relatively short taproot and extensive shallow lateral roots with grafting capabilities in dense plantations (Baker and Langdon, 1990). In the early stages, it is moderately tolerant to shade but becomes intolerant to shade as tree ages (Schultz, 1997).

The growth and productivity of loblolly pine depends on several factors such as soil properties (physical and chemical), genetics, temperature, light, moisture, wind and flooding, drought, pest and pathogens, carbon dioxide and competition from neighboring flora (Schultz, 1997; Baker and Langdon, 1990). Loblolly pine grows better in sandy-loam, acidic, moist, clay and well-drained soil which are critical for the tree's survival. Though the tree can endure

moderate drought, extended moisture stress can impede tree growth, further changing the physiology (Schultz, 1997). With proper management and fertilization, loblolly pine thrives (Colberx et al., 1990). It also grows well on reclaimed mine land (Priest et al., 2015). Lack of essential nutrients (nitrogen and phosphorus) together with competition from nearby vegetation have adverse effects on loblolly in the southeastern United States (Smethurst and Nambiar, 1989; Fox et al., 2007).

1.3.3 Insect and Pest Tolerance

Loblolly pine can be susceptible to a number of abiotic and biotic stresses. Abiotic factors such as wind, flooding, and high and freezing temperatures can result in decreased vigor and tree growth. Abiotic factors may predispose pine trees to biotic stressors such as insects and fungi (Connor and Wilkinson, 1983; Baker, 1972). *Pinus taeda* is susceptible to pest attack that can cause devastating damage to the tree. A major pest associated with the tree in the southeastern United States is *Dendroctonus frontalis* Zimmermann (southern pine beetle) which cause destruction to loblolly pine (Baker and Langdon, 1990). The beetle caused an estimated 900 million dollars' in damage between 1960 and 1990 (Price et al., 1992). Other insect pests that affect or damage loblolly pines are *Ips* species, *Hylastes salebrosus* Eichoff and *H. tenuis* Eichoff beetles which are associated with pine decline (Klepzig et al., 1995; Jacobs and Wingfield, 2001; Eckhardt et al., 2007). These insects attack dying, unhealthy and recently felled trees (Connor and Wilkinson, 1983). Regeneration weevils such as *Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) and their ophiostomatoid fungal symbionts are associated with *P. taeda* decline in the northeastern and the southern United States (Erbilgin and Raffa, 2000; Eckhardt et al., 2007), respectively. During initial growth stages (under 5 years), loblolly pines are attacked by pine tip moth (*Rhyacionia frustrana* (Comstock)) (Fettig et al., 2000). The tree is

also host or susceptible to damping-off diseases in nurseries, fusiform rust *Cronartium quercuum* (Berkeley) Miyabe ex Shirai f.sp. *fusiforme* Burdsall and Snow (Phelps and Czabator, 1978), the root disease pathogen *Heterobasidion irregulare* (Underw.) (formerly *H. annosum* (Fr.) Bref.) (Gonthier et al., 2012; Robbins 1984), heart rot (*Phellinus pini* Tho. Ex. Fr.) (Baker and Balmer, 1983) and pitch canker (*Fusarium circinatum* Nirenberg and O'Donnell). The lateral roots of loblolly pines are infected by ophiostomatoid fungi, that can be associated with pine decline (Harrington and Cobb, 1988; Eckhardt et al., 2004; Eckhardt et al., 2007).

1.4 Insect and fungal association

1.4.1 Bark beetles

Bark beetles are insects from the order Coleoptera belonging to the Curculionidae family and Scolytinae subfamily. The beetles are distributed worldwide with over 6000 species and about 550 species in North America. Bark beetles have variable population and scattered habitats with new generations in search of new reproduction sites. These insects occur on a wide range of host trees species, including commercially important trees such as *Pinus taeda, Pinus sylvestris, Pinus palustris* and *Pinus elliottii.* Bark beetles are important pests of conifers, known to cause significant damage and mortality to forests trees (Klepzig et al., 1991; Eckhardt et al., 2004; Knižek and Beaver, 2007). Bark beetles are described as primary or aggressive pest species when they colonize and kill healthy trees, and as secondary or non-aggressive pests when they attack stressed, dying or dead trees (Raffa, 1991).

Several ophiostomatoid fungi have close and widespread association with bark beetles (Eckhardt et al., 2007, Linnakoski et al., 2012). It is an established fact that some of the bark beetles depend on the fungi as a source of food, or for killing trees through mycelial penetration and toxin release, thus making the habitat more favorable for insect development, while the fungi rely on

the beetle for dispersal to new host trees (Paine et al., 1997; Popa et al., 2012). Insect vectored ophiostomatoid fungi such as *Leptographium truncatum* Wingfield and Marasas, *Leptographium procerum, Leptographium terebrantis, Grosmannia alacris,* and *Grosmannia huntii* have been isolated from roots of loblolly pine trees at various stages of decline, from regeneration weevil species such as *H. pales* and *P. picivorus,* and from root feeding bark beetles *H. tenuis* and *H. salebrosus* which typically attack stressed and declining pines. The fungi colonize roots and lower stumps for loblolly pine in the southeastern United States (Eckhardt et al., 2007).

1.4.2 Ophiostomatoid fungi

Ophiostomatoid fungi (Ascomycetes, Ophiostomataceae) represent a group of morphologically and ecologically similar species (De Beer et al., 2013; Spatafora and Blackwell, 1994). They include the teleomorph genera: *Ophiostoma* Syd. and P. Syd., *Ceratocystiopsis* Upadhyay and Kendr., and *Grosmannia* Goid., together with anamorphs genera: *Leptographium* produced by the genus *Grosmannia* (Wingfield et al., 1993; Zipfel et al., 2006). Apart from their morphological and ecological features, they also have similar chemical composition but different phylogenetically (Kirisitis, 2007).

Members of the Ophiostomataceae producing *Leptographium* anamorphs were previously described as species of *Ophiostoma* (Jacobs and Wingfield, 2001). The form-genus *Leptographium* has been shown to be a monophyletic lineage (Jacobs and Wingfield, 2001; Zipfel et al., 2006), and all *Ophiostoma* species with *Leptographium* anamorphs have been transferred to *Grosmannia,* with *Ophiostoma* sensu stricto and *Ceratocystiopsis* applied to species with *Pesotum, Sporothrix,* or *Hyalorhinocladiella* anamorphs (Zipfel et al., 2006).

The fungi are considered to be pathogens that reduce the quality of coniferous trees by producing dark pigment forming a key part in blue staining of infected wood leading to high value losses (Uzunovic et al., 1999; Zhou et al., 2001). Staining is caused by fungal hyphae usually growing in the ray parenchyma cells and resin ducts (Gibbs, 1993; Seifert, 1993). They have mucilaginous ascospores and conidia. The slimy masses of the spores are produced at the top of long conidiophores that help spores attach to vectors (Wingfield et al.,1993). The fungi lead to superficial discoloration on the wood surface during sporulation.

1.5 Volatile Organic Compounds

Volatile organic compounds (VOCs) are a heterogenous group of carbon-based organic compounds, with low molecular weights and that vaporize or evaporate at normal temperatures and pressures (Rowan, 2011). Volatile organic compounds often have a unique odor and generally have low to medium solubility in water (Herrmann, 2010). Volatile organic compounds include chemical solvents and other industrial compounds such as alkanes, alkenes and alkynes, aromatic compounds and terpenes, as well as oxygenated compounds such as alcohols, esters, aldehydes, ketones and organic acids which are highly volatile (McFee and Zavon, 1988; Demeestere et al., 2007; Talapatra and Srivastava, 2011). These chemicals have different physical properties and molecular sizes (Lundström et al., 2003).

1.5.1 Bark beetle volatiles

Bark beetles are obligate parasites of pine trees which feed on phloem and may take short flights to find hosts. Host colonization occurs when adult (male or female) beetle depending on insect species initially bore into a tree (Tittiger and Blomquist, 2017). Bark beetles locate new hosts using identified chemical compounds (Schlyter and Birgersson, 1999). For instance, *Dendroctonus valens* are attracted to volatiles from ponderosa pine (Kelsey and Westlind, 2020).

Pine trees usually defend themselves against attack, but bark beetles can overwhelm the tree's defenses by using aggregation pheromones which act as kairomones or allomones to coordinate a mass-attack by both sexes of beetles. For example, intraspecific pheromone inhibitors of attraction such as verbenone and *trans*-verbenol cause individuals to avoid colonizing in high attack density patches and thus function in terminating the aggregation (Byers, 1989).

Ipsenol, ipsdienol, and *cis*-verbenol are pheromone components of bark beetles that were the first to be identified. These chemicals are hydroxylated derivatives of common plant monoterpenes found in tree resin: myrcene and a-pinene (Seybold et al., 2000). Most monoterpenoid pheromone components are synthesized *de novo* from the mevalonate pathway. Frontalin, a major monoterpene-derived component of many *Dendroctonus* spp., is produced at the beginning of the mevalonate pathway (Barkawi et al., 2003). Frontalin and *exo*-brevicomin are aggregation pheromone of many *Dendroctonus* spp. *exo*-Brevicomin is modified from fatty acid precursors and a product of endogenous metabolism (Vanderwel et al., 1992). On the other hand, *Dendroctonus jeffreyi* produces 1-heptanol and 2-heptanol (derived from n-heptane) which are metabolites of host resin components (Paine et al., 1999).

1.5.2 Fungal volatiles

Fungal volatiles were first described in 1973 by Hutchinson and was focused on carbon dioxide. During this period, fungal VOCs were usually isolated by steam distillation followed by liquid-liquid extraction and concentration of the organic extract in the laboratory (Kaminski et al., 1972). These approaches have been refined and provided more sophisticated trapping, separation, and identification of varieties of VOCs from fungi. Fungi are known to produce a large number of VOCs and there are approximately 250 fungal VOCs identified that exist as

mixtures of simple hydrocarbons, heterocycles, alcohols, phenols, thioalcohols, thioesters, and their derivatives (Chiron and Michelot, 2005; Korpi et al., 2009).

Many VOCs have distinctive odors but not all can be detected by humans. A huge number of volatile compounds exists in nature. Because of their high vapor pressure and low molecular weight, they can readily diffuse in the gas phase through biological systems. Therefore, they may act as signaling molecules passing information within or between organisms.

Fungal VOCs are used in many applications and fields including agriculture, energy, chemistry, food industry and entomology. In agriculture, fungal VOCs are used as part of biological control methods to prevent pathogen growth in plants. Studies have also shown that fungal VOCs promote the growth of plants (Morath et al., 2012). Fungal VOCs are used as mycofumigation (the use of biological control properties of fungal VOCs to prevent post-harvest fungal growth) in the food industry. Fungal VOCs can be used to convert plant waste into diesel by utilizing biologically based energy sources, a procedure called mycodiesel (Strobel et al., 2011). Sesquiterpenes produced from *Ascocoryne* fungi are potential source of diesel or jet fuel alternatives due to their cyclic and branched nature (Rude and Schirmer, 2009; Griffin et al., 2010).

1.5.3 Collection and detection of fungal VOCs

In the study of fungal VOCs, they must be first be isolated and characterized. Several methodologies are used for sampling, sample preparation, separation, concentration, identification, and quantification of gas phase molecules (Zhang and Li, 2010), the separation, identification and quantification steps are critical. Recently, new analytical techniques have been

employed for the study of biological volatiles. These techniques (headspace and solvent) are used to collect and concentrate volatiles from a sample. Headspace collection is the direct extraction of volatiles from the surrounding air around the sample while solvent extraction is the extraction of volatiles from the sample with further purification of volatiles from non-volatile materials.

Direct extraction is made up of a purge and trap method and solid phase micro-extraction. Solvent extraction on the other hand has four types, which are, steam distillation, liquid-liquid extraction, supercritical fluid extraction and simultaneous distillation extraction. While headspace sampling is simple and environmentally friendly in terms of assessing VOCs, solvent extraction is a complicated process which involves large volumes of solvents and multiple steps (Kimbaris et al., 2006).

Generally, the method used for collecting volatiles from samples is headspace volatiles (Qualley and Dudareva, 2009). The method is used to obtain VOCs from several fungal species grown under standardized laboratory conditions (Mattheis and Roberts, 1992; Börjesson et al., 1993). Charcoal, tenax or super Q is usually used as an absorbent material to trap VOC depending on the binding properties of the compounds. The volatiles trapped in the absorbent material are then dissolved in an organic solvent such as dichloromethane or hexane (Kai et al., 2009). The absorbent material and organic solvent used can affect the VOC profile obtained because different VOC profiles can be achieved with different collection methods (Larsen and Frisvad, 1995). Volatiles are extracted with solvent and then transferred to a gas chromatograph (GC), which separates mixed compounds at high temperature to release the volatile for identification and quantification.

The procedures involved in the collection and extraction of fungal volatiles may introduce errors and inaccuracies in results (Kataoka and Saito, 2011). To address some of these errors, solid phase micro-extraction (SPME) was introduced (Zhang, 2010). A SPME fiber is exposed to the atmosphere and traps compounds to be transferred and analyzed by gas chromatography-mass spectrometer GC-MS. Solid phase micro-extraction provides simpler and shorter times to complete tasks, as well as its high extraction capacity and selectivity of metabolites (Jeleń, 2003). This method is mostly used in the food industry for monitoring fungal contamination of stored products (Stoppacher et al., 2010; Zhang, 2010).

Separation and detection of fungal metabolites is usually done with a GC-MS because of its high efficiency in identification and quantification (Rösecke et al., 2000; Matysik et al., 2009; Roze et al., 2012; Dickschat, 2014). Molecules are absorbed and ionized into fragments by a mass spectrometer. Compounds are identified using a database where the fragments are compared to standards, to predict identity (Stoppacher et al., 2010).

1.6 Volatiles in insect management

Fungi are known to produce a large number of volatile organic compounds. However, such VOCs have received limited attention their possible benefit to pest management (Korpi et al., 2009). Entomologists and chemical ecologists have explained that many fungal VOCs act as semiochemicals or infochemicals and function as attractants and deterrents to insects and other invertebrates (Dicke et al., 2009). Fungal VOCs can also mediate interactions among different organisms in ecosystems. Fungal VOCs may act as pheromones, allomones, or kairomones to affect the behavior of organisms (Rohlfs et al., 2005; Mburu et al., 2011). The close interaction between insects and fungi can be facilitated by VOCs emitted by the fungus (Kandasamy et al., 2016; Cale et al., 2019). During the life cycle of some bark beetles, fungal VOCs may play roles

in the attraction of beetles to hosts trees, aggregation for beetle mass attacks, and repulsion of beetle competitors (Kandasamy et al., 2016). Fungal VOCs which are mostly described as monoterpenes, sesquiterpenes and aromatic compounds have ecological importance by possessing antifungal activity which could represent an antagonistic mechanism during interspecific interactions (Viiri et al., 2001).

Wertheim et al., (2005) noted that a striking number of insects that exhibit aggregation behaviors are strongly associated with specific microbial communities, although few researchers have treated microbial VOCs directly as pheromonal communications. Ophiostomatoid fungi produce different kinds of VOCs (Cale et al., 2016). For instance, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) and *Ophiostoma ips* (Rumbold) Nannfeldt are ophiostomatoid fungi that can emit verbenone, a VOC that has strong semiochemical effect on their vectored bark beetles (Cale et al., 2019). According to Wertheim et al., (2005), different insects exhibit aggregation behaviors that are associated with their symbiotic fungi. Verbenone, an anti-aggregation chemical reduces competition within or outside bark beetle interactions (Lindgren and Miller, 2002). The compound repelled other insects from attacking host trees already colonized by mountain pine beetle (*Dendroctonus ponderosae* Hopkins) and its associated fungi (Cale et al., 2017). According to Cale et al., (2016), fungal VOCs from the ophiostomatoid fungi; *Grosmannia clavigera, Ophiostoma montium* Rumbold and *Leptographium longiclavatum* Lee, Kim and Breuil differ between species and can influence the growth and spore production of other fungal species.

1.7 Objectives

The specific objectives of the study were to:

1. Identify and quantify volatile organic compounds emitted by ophiostomatoid fungi associated with loblolly pine-infesting root bark beetles: *G. alacris, G. huntii, L. terebrantis,* and *L. procerum.*
2. Determine how the presence of a resource-sharing fungus affect the VOC produced by another fungal species.
3. Determine if seedlings inoculated with ophiostomatoid fungi: *G. alacris, G. huntii, L. terebrantis,* and *L. procerum* produce allelochemicals that differed.

CHAPTER II

Production of volatile organic compounds from ophiostomatoid fungi: single and combination

2.1 Abstract

Southern pine decline is a complex disease syndrome that slowly and progressively weakens a tree's ability to grow. *Pinus taeda* (loblolly pine), the predominant and most economically important tree crop in the southeastern United States, can suffer from southern pine decline. Root-feeding bark beetles and their associated ophiostomatoid fungi contribute to southern pine decline. Volatile organic compounds emitted by fungi can be used as semiochemical in managing both insect and associated fungi. Headspace volatiles were collected, identified, and quantified fungi beetle-associated fungi to determine their species-specific volatile profiles. Eight compounds: ethyl acetate, isoamyl acetate and phenylethyl acetate (esters), isobutanol, ethyl alcohol, 2-methyl-1-butanol and phenylethyl alcohol (alcohols) and verbenone were identified by GC analysis. The results suggests that there are similarities in the different fungal volatile organic compounds produced by fungal species that occupy the same ecological niche. The study also demonstrates that fungal volatile organic compounds can drive interactions between bark beetles and fungal symbiont.

2.2 Introduction

Volatile organic compounds (VOCs) are a heterogenous group of carbon-based organic compounds, with low molecular weights that vaporize or evaporate at normal temperatures and pressures (Rowan, 2011). Volatile organic compounds include chemical solvents and other industrial compounds such as alkanes, alkenes and alkynes, aromatic compounds, and terpenes, as well as oxygenated compounds such as alcohols, esters, aldehydes, ketones and organic acids

which are highly volatile (McFee and Zavon, 1988; Demeestere et al., 2007; Talapatra and Srivastava, 2011). Fungi emit diverse types and sizes of moleculars during metabolism and there are approximately 300 fungal VOCs identified. These can exist as mixtures of simple hydrocarbons, heterocycles, alcohols, phenols, thioalcohols, thioesters, and their derivatives (Chiron and Michelot, 2005; Korpi et al., 2009; Morath et al., 2012).

Fungal VOCs mediate the interaction between fungi and their host by acting as communication cues which influences the behavior of plants and animals (Cale et al., 2016; Davis et al., 2013; Davis and Landolt, 2013; Schulz-Bohm et al., 2017; Schenkel et al., 2018). Fungal VOCs can also regulate antagonistic and beneficial interactions among fungi (Nishino et al., 2013), acting specifically as infochemicals within and between kingdoms. For instance, FVOCs from some fungal species can stimulate or inhibit the reproduction and growth of cultures from other fungal species (Cale et al., 2016). Alternatively, organisms found in the same ecological niche can be influenced positively by fungal VOCs (Schmidt et al., 2015). Cale et al., (2016), demonstrated that fungal VOCs emitted by phytopathogenic fungi can be used as carbon source to support fungal growth. Also, VOCs emitted by *Rhizoctonia solani* Kühn (teleomorph: *Thanathephorus cucumeris* Frank Donk), a root pathogen can also increase the shoot and root biomass in *Arabidopsis* plants (Cordovez et al., 2017). At the moment, several studies have been conducted into VOC interactions in many origins (Schulz and Dickschat, 2007; Junker and Tholl, 2013), however, the ecological role of VOCs in fungi remains largely unknown.

Blue stain ophiostomatoid fungi (Ascomycetes, Ophiostomataceae), which is associated with and vectored by bark beetles (Curculionidae, Coleoptera), infect coniferous trees such as loblolly pines (Schultz, 1999; Eckhardt et al., 2007). *Leptographium procerum*, *Leptographium*

terebrantis, *Grosmannia alacris*, and *Grosmannia huntii* are ophiostomatoid fungi associated with root-feeding bark beetles and are implicated as contributing factors of pine decline disease (Eckhardt et al., 2007; Mensah et al., 2021). The beetles *Hylastes tenuis, Hylastes salebrosus*, *Hylobius pales* and *Pachylobius picivorus* have been collected from declining loblolly pines (Eckhardt, 2004; Matusick, et al., 2013). Generally, there is a mutualistic association between bark beetles and at least some of their associated ophiostomatoid fungi. The beetles depend on the fungi as a source of food, or for killing host trees through mycelial penetration and toxins released during infection, thus making the habitat more favorable for insect development (Six et al., 2007; Cale et al., 2017; Wang et al., 2020). The fungi also provide protection for the beetles against predation, while the fungi rely on the beetle for dispersal to new host trees (Paine et al., 1997).

In the present study, root feeding bark beetle symbionts *G. alacris*, *G. huntii*, *L. procerum*, and *L. terebrantis* were used in a laboratory experiment to investigate the differences in VOC profiles among ophiostomatoid fungi. Headspace fungal volatiles were collected from emitting cultures of *G. alacris*, *G. huntii*, *L. procerum*, and *L. terebrantis* either individually or in the presence of another. Determination was made on whether the fungi qualitatively and quantitatively differed from each other.

2.3 Materials and methods

2.3.1 Fungal volatile collection and quantification *in situ*

A push-pull system was used to collect headspace fungal volatile (as described by (Cale et al., 2016)) from cultures of four fungal species alone or in combination. Emitted fungal VOCs from *G. alacris* (Ga), *G. huntii* (Gh), *L. procerum* (Lp) and *L. terebrantis* (Lt), as well as control without fungal cultures and combined (Ga + Gh, Ga + Lp, Ga + Lt, Gh + Lp, Gh + Lt, and Lp +

Lt) grown on the same plate. The fungi were grown on potato dextrose media (PDA), malt extract agar (MEA) and pine twig agar (PTA) to provide diverse compounds. One fungal isolate was used in the experiment and replicates 10 times. Fungal cultures were obtained from pathogenic species isolated and provided by the Forest Health Dynamics Laboratory in Auburn University. The fungi were collected from *Hylastes* galleries in loblolly pine roots. Cultures used for the experiment were subcultured using 5 mm diameter plugs from 10-day old, actively growing margins of cultures. Fungal cultures were incubated in permanent darkness at 22°C covered 80% of the plate. The cultures were placed in the volatile collection chamber and the petri dish opened to expedite the diffusion of volatiles.

The volatile collection chamber is made up of a glass jar (473 mL) covered with a metal cap having two holes. Fitted to each hole is a Teflon tube (6.35 mm) moving into the glass chamber. The first tube was 15 cm long was filled with activated carbon (800 mg) and glass wool fixed at the ends. The purpose of this tube is to purify incoming ambient as it enters the chamber. The second tube was 8 cm long and was attached to a volatile trap contained 150 mg of activated carbon with glass wool fitted at the ends. The activated carbon serves as absorbent of volatiles emitting from the fungal cultures. A gang-valve is connected to a jointed inlet spigot of a bellows vacuum pump, all joined to another trap. Each gang-valve manifold was connected to five other identical collection system. A flowmeter was used to set a constant flow of 450 mL min^{-1} for all chambers.

Fungal cultures were placed in the collection chamber for 24 hours to build up volatiles. The pump is switched on for another 24 hours for volatiles trapping. Headspace volatiles were extracted after 48 hours. The activated carbon was removed and transferred into a microtube containing 1 mL of dichloromethane with tridecane as internal standard (0.002%). This mixture

was vortexed for 30 seconds, sonicated for 10 minutes and centrifuged (at 30,000 rpm) for 30 minutes. The extract was transferred to a gas chromatograph (GC) vial. The same procedure was repeated a second time before chromatographic separation. All samples were analyzed with GC system (GC: 7890A; Agilent Tech., Santa Clara, CA, USA) fitted with a mass spectrometer (MS: 5062C, Agilent Tech., Santa Clara, CA, USA) with a DB-5MS UI column (30 m x 0.25 mm ID x 0.25 μm film). Helium was used as a carrier gas flowing at 1 mL min^{-1} with a temperature programbeginning at 50°C (held for 1 min) then increased by 5°C min^{-1} to 200°C, followed by an increase of 30°C min^{-1} to 325°C (held for 2 min). It was maintained at 250 °C, a 1 μl sample injection volume was used, and samples were run in splitless mode. Peaks present in chromatograms of control treatment were ignored from those of fungal cultures to determine peaks unique to the media. Library matches using NIST/EPA/NIH Mass Spectral library version 2.0f for all detected fungal volatiles were verified and quantified using the following standards: acetoin (≥ 96%), ethyl acetate (≥ 99%), *cis*-grandisol (≥ 96%), isobutanol (≥ 99%), 2-methyl-1-butanol (≥ 99% pure), isoamyl alcohol (≥98%), phenylethyl acetate (≥ 98%), and phenylethyl alcohol (≥ 99%). All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analyte concentrations was standardized by the culture area prior to data analysis.

2.3.2 Data analysis

The quantity of each compound detected in fungal treatments were calculated as amount (ng) of compound per unit (mm^2) of fungal culture area per day ($ng/mm^2/day$). Data were analyzed using SAS (PROC GLM, SAS Inc., Cary, NC, USA) statistical software. A multivariate analysis of variance (MANOVA) was conducted to determine if there were differences between the quantity of compounds produced across fungi treatments. Data were first checked for normality and equal variance using Wilks' lambda. Pair-wise comparisons were

conducted at using the post-hoc Bonferroni test on the fungal treatments at $\alpha = 0.05$. Graphs were drawn with Microsoft Excel.

2.4 Results

Eight fungal VOCs, representing three chemical classes were detected in extraction of headspace volatiles of *Grosmannia alacris, Grosmannia huntii, Leptographium terebrantis,* and *Leptographium procerum* as well as fungi combination during the 48-hour sampling period. The compounds detected were ethyl acetate, isoamyl acetate and phenylethyl acetate (esters), isobutanol, 2-methyl-1-butanol, ethyl alcohol and phenylethyl alcohol (alcohols) and verbenone (monoterpene) (Fig 2.1 and 2.2).

Fungal VOC profiles significantly differed among treatments ($p < 0.001$) (Table 2.1). Phenylethyl alcohol, phenylethyl acetate and verbenone were detected in all fungal treatments. *Grosmannia alacris, G. huntii, Leptographium terebrantis, Leptographium procerum* and the combination treatments produced different VOCs. More individual compounds were detected in *G. huntii* (phenylethyl alcohol, phenylethyl acetate, 2-methyl-1-butanol, isobutanol, verbenone and isoamyl acetate). Fifty eight percent of the compound detected was phenylethyl alcohol. *Grosmannia alacris* produced five compounds with phenylethyl acetate (58%) constituting the greater part. The other compounds are phenylethyl alcohol, 2-methyl-1-butanol, isobutanol and verbenone. Five compounds (phenylethyl acetate, phenylethyl alcohol, verbenone, ethyl acetate and ethyl alcohol) were detected in *Leptographium terebrantis* with ethyl alcohol, ethyl acetate and phenylethyl alcohol constituting 39%, 33% and 25% respectively. Four compounds were detected in *Leptographium procerum:* phenylethyl alcohol (49%), verbenone (30%), ethyl acetate (12%) and phenylethyl acetate (9%).

Verbenone was the dominant compound when fungal treatments were combined. Isoamyl acetate was not detected in the combined treatments. Fungal VOCs generally observed higher quantities in combined treatments than the individual fungi. Fungal VOC profiles significantly differed among treatments ($p = 0.0037$) (Table 2.2). The combination of *G. alacris* + *L. procerum*, *G. huntii* + *L. procerum*, + *L. procerum* + *L. terebrantis* recorded a higher number (6) of compounds. Combined *G. alacris* + *L. terebrantis* detected five compounds. Four compounds were detected each in *G. alacris* + *G. huntii*, and *G. huntii* + *L. terebrantis.*

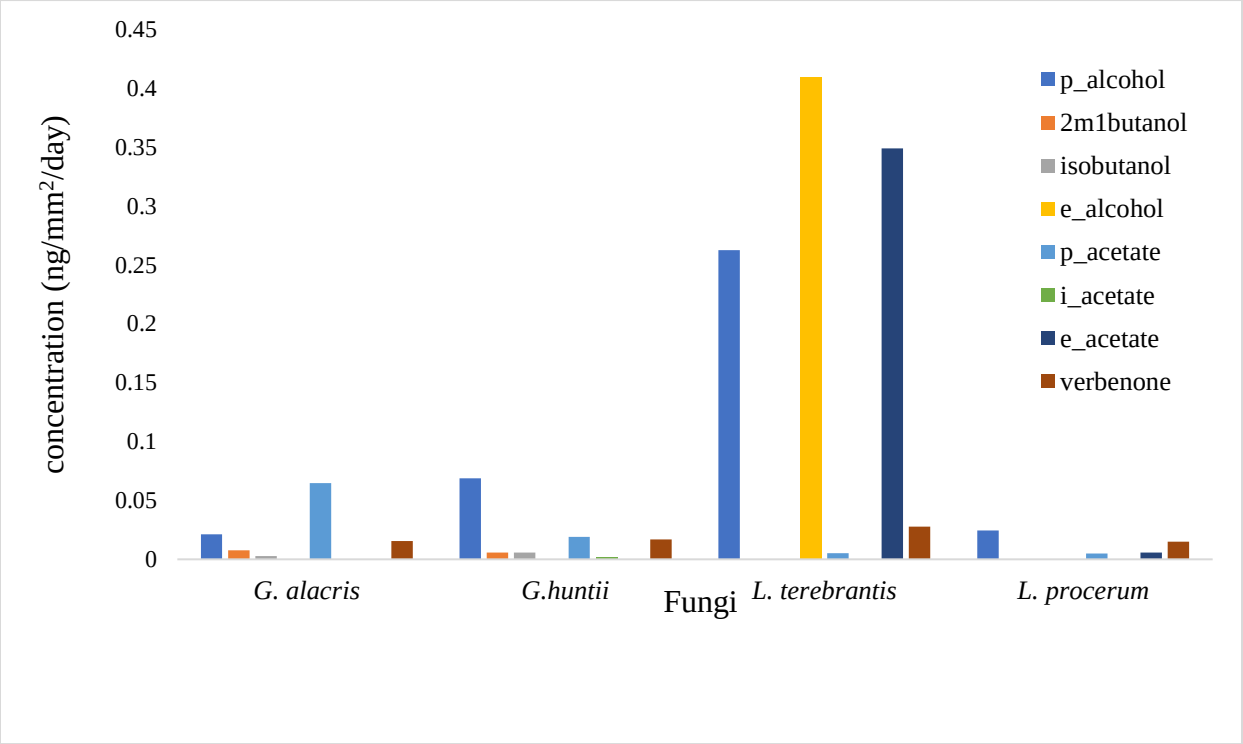

Figure 2.1 Graph of compounds detected in *G. alacris*, *G. huntii*, *L. terebrantis*, and *L. procerum.*

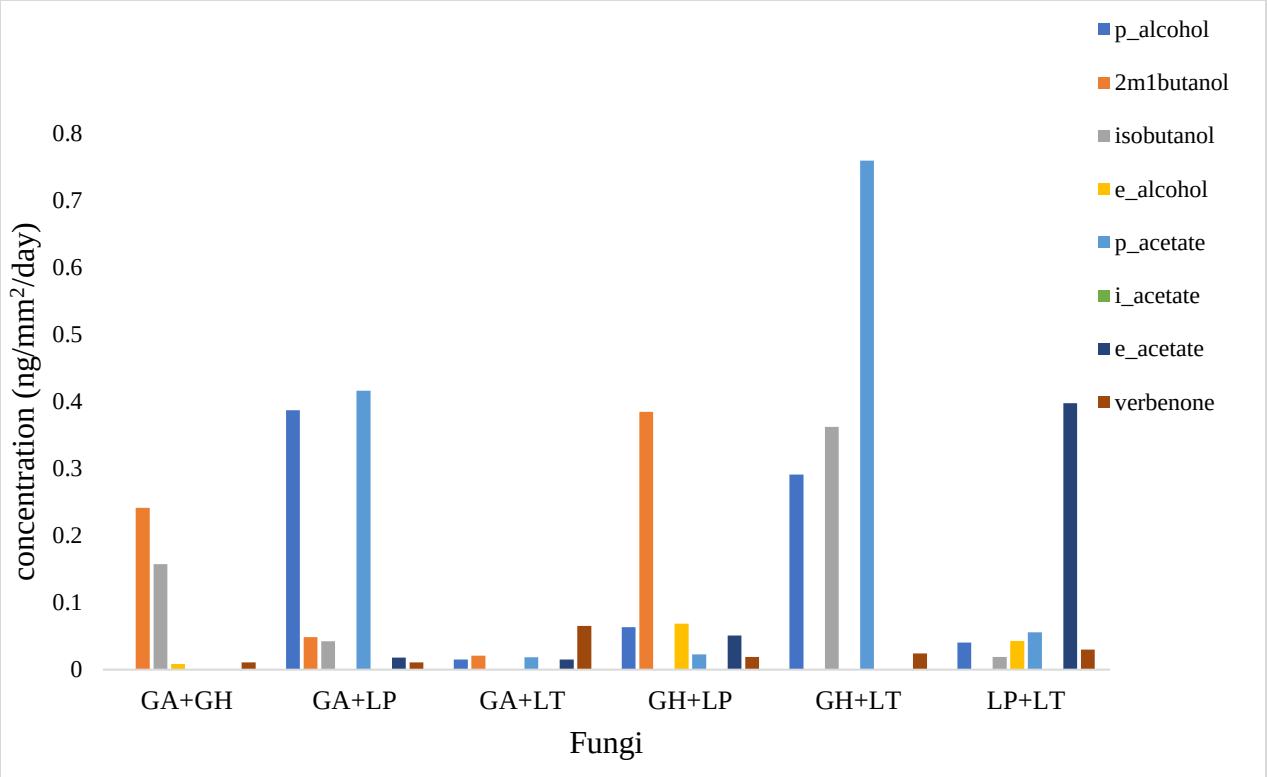

Figure 2.2 Graph of compounds detected in combined fungal treatments.

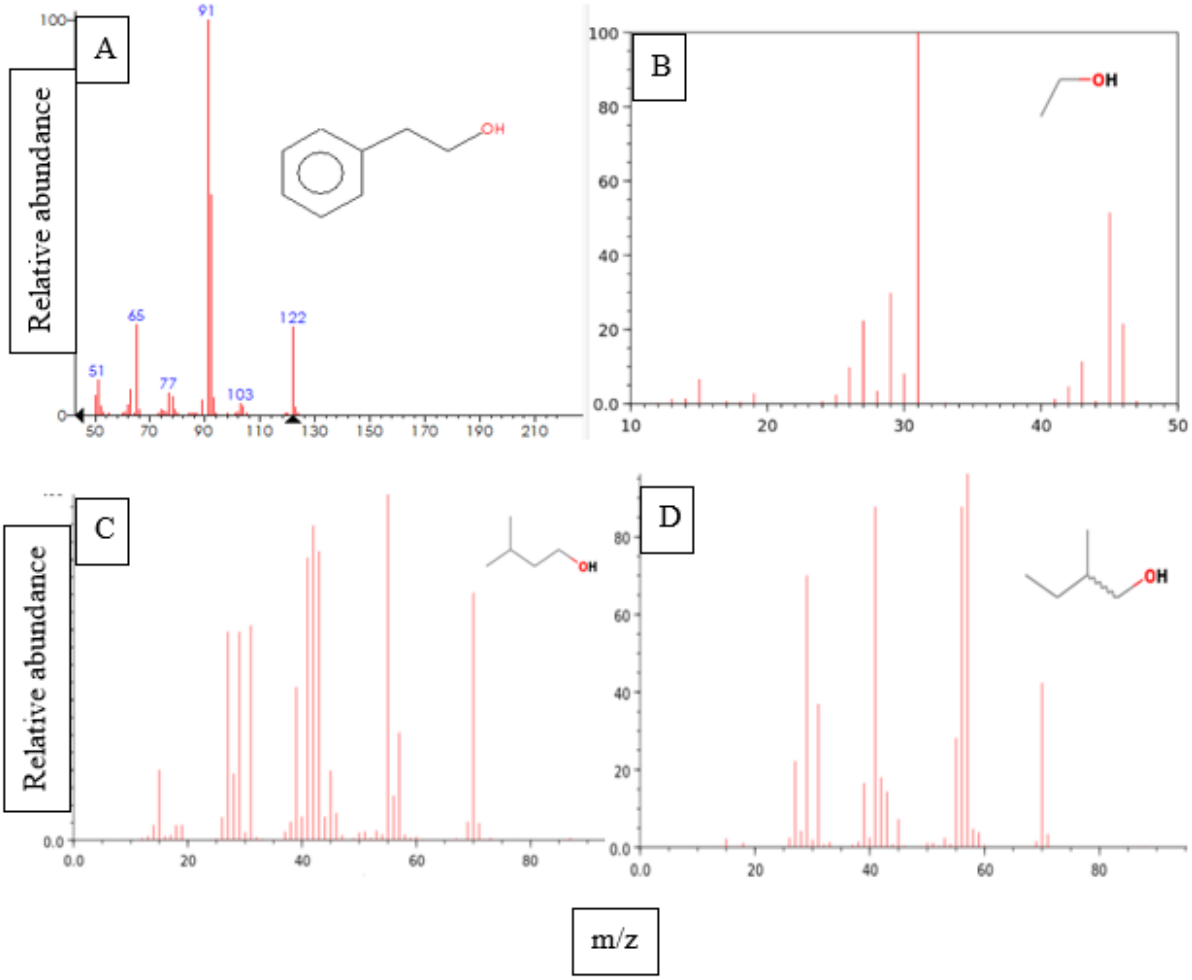

Figure 2.3 Spectral diagrams of abundant monoterpenes phenylethyl alcohol (A), ethyl alcohol (B), isobutanol (C) and 2-methyl-1-butanol (D).

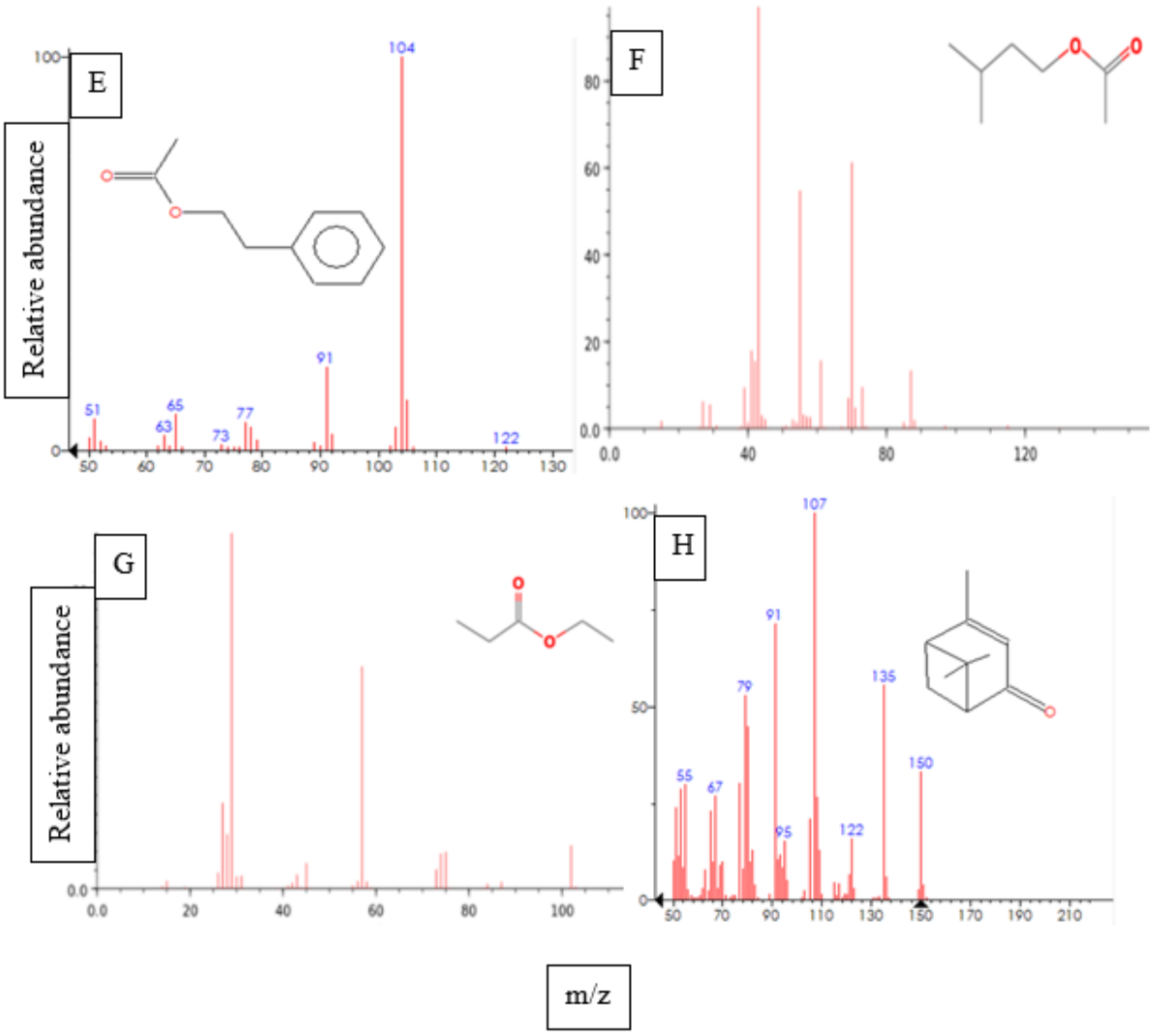

Figure 2.4 Spectral diagrams of abundant phenylethyl acetate (E), isoamyl acetate (F), ethyl acetate (G) and verbenone (H).

Table 2.1 Mean and standard error concentrations (ng/mm^2/day) of compounds detected in *G. alacris*, *G. huntii*, *L. terebrantis,* and *L. procerum.* Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Fungal treatments**				**df**	**F**	**P-value**
	Grosmannia alacris	*Grosmannia huntii*	*Leptographium terebrantis*	*Leptographium procerum*			
Phenylethyl alcohol	0.02±0.01	0.07±0.03	0.02±0.001	0.26±0.11	3	3.86	0.011
Phenylethyl acetate	0.06±0.03	0.02±0.01	0.005±0.001	0.005±0.003	3	2.34	0.077
2-methyl-1-butanol	0.008±0.002	0.005±0.001	ND	ND	1	8.43	<0.001
Isobutanol	0.003±0.001	0.005±0.002	ND	ND	1	8.19	<0.001
Verbenone	0.02±0.004	0.01±0.006	0.03±0.01	0.02±0.005	3	0.78	0.511
Isoamyl acetate	ND	0.002±0.0005	ND	ND	-	-	-
Ethyl acetate	ND	ND	0.35± 0.16	0.006± 0.003	1	4.75	0.0037
Ethyl alcohol	ND	ND	0.4± 0.17	ND	-	-	-

Table 2.2 Mean and standard error concentrations (ng/mm^2/day) of compounds detected in combined fungi. Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Fungal treatments**						**df**	**F**	**P-value**
	GAGH	GALP	GALT	GHLP	GHLT	LPLT			
Phenylethyl alcohol	ND	0.39±0.26	0.02±0.003	0.06±0.01	0.29±0.27	0.04±0.01	4	1.15	0.346
Phenylethyl acetate	ND	0.41±0.26	0.02±0.005	0.02±0.008	0.76±0.47	0.06±0.03	4	1.98	0.096
2-methyl-1-butanol	0.24±0.19	0.05±0.03	0.02±0.004	0.38±0.36	ND	ND	3	0.92	0.476
Isobutanol	0.16±0.14	0.04±0.02	ND	ND	0.36±0.34	0.02±0.003	3	0.88	0.499
Verbenone	0.01±0.003	0.01±0.0007	0.07±0.04	0.01±0.007	0.02±0.01	0.03±0.008	5	1.49	0.21
Ethyl acetate	ND	0.02±0.005	0.02±0.007	0.05±0.01	ND	0.04±0.029	3	1.69	0.154
Ethyl alcohol	0.008±0.002	ND	ND	0.07±0.03	ND	0.04±0.008	2	6.5	<.001

2.5 Discussion

The profiles of VOCs emitted by ophiostomatoid fungi are similar between species that share the same ecological niche. Bark beetle's symbiotic fungi can emit VOC profiles that qualitatively and quantitatively differ. Three compounds (phenylethyl alcohol, phenylethyl acetate and verbenone) were common between species. Eight compounds were detected in the study and were distributed among *G. alacris, G. huntii, L. terebrantis, L. procerum* and their combination. The study was consistent with previous studies as ethyl acetate, isoamyl acetate, phenylethyl acetate, isobutanol, 2-methyl-1-butanol and phenylethyl alcohol were compounds identified (Cale et al., 2016; Wang et al., 2020; Strobel et al., 2001). Cale et al., (2019) demonstrated that the compounds are byproducts of primary metabolism during vegetative growth. Additionally, similarities in fungal profiles may suggest that phytopathogenic fungi which share the same ecological niche and perform the same function emit similar VOCs (Müller et al., 2013).

Fungal volatiles emitted by bark beetle vectored fungi may affect other organisms (bark beetles and fungi) occupying the same habitat (Kandasamy et al., 2016; Cale et al., 2019). Phenethyl alcohol and 2-methyl-1-butanol are compounds known to attract many insect species (Davis et al., 2013). Specifically, phenethyl alcohol is attractive to southern pine beetle, mountain pine beetle and pine engraver beetle (Pureswaran and Borden, 2004). Several bark beetles are attracted to mixtures containing the detected esters. For example, *D. frontalis* is more attractive to a pheromone blend (frontalin, *trans*-verbenol, turpentine) containing phenethyl acetate, 3-methyl-1-butyl acetate and isoamyl acetate than the blend itself (Cale et al., 2016; Kandasamy et al., 2019). Isoamyl acetate is known to be toxic to some fungi and bacteria (Strobel et al., 2001). Ethyl acetate can be used as insect deterrent or repellent in the field

because of its strong smell or vapor. It is used as a killing agent during insect collection in the field.

Verbenone was an unknown chemical identified during the study. The compound is produced by southern pine beetle and its symbiotic fungi and can serve as a repellent. Also, the compound repelled *Ips pini* from host tree already colonized by mountain pine beetle and the associated fungi. Verbenone can be used to interrupt the attraction of bark beetles to their aggregation pheromones (Lindgren and Miller, 2002). Both the beetle and the fungus appear to produce verbenone from chemicals already present in the tree. *G. clavigera* and *Ophiostoma ips* produced verbenone in the presence of the precursor *trans*-verbenol which is produced by pine trees (Cale et al., 2019).

2.6 Conclusion

The impact of root feeding bark beetles in the forests of the southeastern United States is highly dependent on beetle-associated symbiotic fungi such as *G. alacris, G. huntii, L. terebrantis*, and *L. procerum*. The fungal VOCs (alone and in combination) identified needs to be tested using gas chromatography - electroantennographic etection (GC-EAD) to determine whether the compounds stimulate the olfactory sensilla of root-feeding bark beetle before field experiments.

CHAPTER III

The influence of different fungal interaction on the production of fungal metabolites

3.1 Abstract

Southern pine decline is a disease of loblolly pine and contributed by the activities of a root-feeding bark beetle and its fungal symbiont. Volatile organic compounds emitting from the fungi can mediate interactions between the beetle and the fungi and may be used as infochemicals in managing both insects and their associated fungi. Headspace volatiles were collected, identified, and quantified to determine the species-specific volatile profiles. Eight compounds: ethyl acetate, isoamyl acetate and phenylethyl acetate (esters), isobutanol, 2-methyl-1-butanol, ethyl alcohol and phenylethyl alcohol (alcohols) and verbenone were identified by GC-MS analysis. The results demonstrate that fungal volatile organic compounds profiles can inhibit the production of volatiles emitted by other fungi occupying the same ecological niche.

3.2 Introduction

Southern pine decline (SPD) is a disease of loblolly pine resulting from the complex interaction between abiotic and biotic factors. Stressed trees induced by abiotic factors attract root-feeding and lower stem-feeding bark beetles (Eckhardt et al., 2007). Root-feeding bark beetles and their symbiotic fungi are biotic factors associated with SPD. The insect bores into the bark of a host creating exposure to the associated ophiostomatoid fungi, thus allowing the fungi to colonize the tree phloem. The fungi serve as a source of food for the beetle by providing nitrogen to developing larvae (Six, 2013; Ojeda-Alayon et al., 2017). In return for sustenance, the beetle vectors the fungi to a new host.

The ecological interaction between the beetle and its associated fungi can be mediated by the VOCs produced by the fungi (Kandasamy et al., 2016; Schulz-Bohm et al., 2017). Fungal VOCs are compounds that vaporize and enter a gas phase at normal atmospheric temperature and pressure. These compounds represent several classes of chemicals such as acids, alcohols, aldehydes, esters, ketones, terpenes, and thiols (Morath et al., 2012). The compounds are produced by fungi occupying the same ecological niche and can affect the way fungi interact with plants, animals, and other fungi (Cale et al., 2016; Hulcr et al., 2011; Hung et al., 2013; Davis and Landlot, 2013). Fungi are important in interspecific and intraspecific communication by functioning as semiochemicals or infochemicals during interactions between organisms (Schenkel et al., 2018). Also, fungal VOCs can regulate antagonistic and beneficial interactions with other organisms (Macias-Rubalcava et al., 2010; Briard et al., 2016; Schmidt et al., 2015). For example, fungal VOCs emitted by phytopathogenic fungi can be used as carbon resources to support fungal growth (Cale et al., 2016). On the other hand, volatiles of *Penicillium paneum* Frisvad inhibit spore germination of the same and different species of fungi, representing various genera (Chitarra et al., 2004). Volatile organic compounds emitted by older fungal cultures can facilitate or inhibit the growth and reproduction of other fungi cultures (Hofstetter et al., 2005; Cale et al., 2016; Kandasamy et al., 2019). Studies have shown ophiostomatoid fungi produce a wide range of VOCs known to function as infochemicals or semiochemicals (Cale et al., 2016; Schmidt et al., 2015; Schulz-Bohm et al., 2017). What remains unknown is how fungal interactions adversely affect the fungal VOC production.

In this study, *Grosmannia alacris*, *G. huntii*, *Leptographium terebrantis*, and *L. procerum* were used in a laboratory experiment to determine that the chemical profile of fungal VOCs can be influenced by the presence of another ophiostomatoid fungal species. In particular, the

experiment wanted to determine (i) Can fungal VOCs emit differ qualitatively and qualitatively from each other? (ii) Can fungal VOCs emitted by ophiostomatoid fungi in the same habitat be affected? To answer these questions, headspace volatiles were collected from fungal cultures.

3.3 Materials and methods

3.3.1 Fungal volatile collection and quantification *in situ*

A push-pull system was used to collect headspace fungal volatile (as described by (Cale et al., 2016)) from cultures of four fungal species alone double (two separate plates). Emitting fungal VOCs from *G. alacris* (Ga), *G. huntii* (Gh), *L. procerum* (Lp) and *L. terebrantis* (Lt), as well as control without fungal cultures and double cultures (Ga/Gh, Ga/Lp, Ga/Lt, Gh/Ga, Gh/Lp, Gh/Lt, Lt/Ga, Lt/Gh, Lt/Lp, Lp/Ga, Lp/Gh and Lp/Lt) without physical contact with each other. The fungi were grown on potato dextrose media (PDA), malt extract agar (MEA) and pine twig agar (PTA) to provide diverse compounds. One fungal isolate was used in the experiment with 10 replicates. Fungal cultures were obtained from pathogenic species isolated and provided by the Forest Health Dynamics Laboratory at Auburn University. The cultures were collected from the roots of infected loblolly pine. Cultures used for the experiment were subcultured using 5 mm diameter plugs from 10-day old actively growing margins of cultures. Fungal cultures were incubated in permanent darkness at 22°C until covered 80% of the plate. The cultures were placed in the volatile collection chamber and the petri dish opened to expedite the diffusion of volatiles. For the combination treatment, a metal wire was coiled and bent horizontally, and placed in the glass jar to hold fungal isolates. Full grown (10 days old) cultures known as the source was placed at the bottom and a new inoculum (3 days old) called the resource was placed on top of the coiled wire.

The volatile collection chamber is a glass jar (473 mL) covered with a metal cap having two holes. Fitted in each hole was a Teflon tube (6.35 mm) moving into the glass chamber. The first tube was 15 cm long and was filled with activated carbon (800 mg) and glass wool fixed at the ends. The purpose filter incoming ambient into the chamber. The second tube was 8 cm long and was attached a volatile trap contained 150 mg of activated carbon with glass wool fitted at the ends. The activated carbon absorbs volatiles emitted from the fungal cultures. A gang-valve is connected to the jointed inlet spigot of a bellows vacuum pump, all joined to another trap. Each gang-valve manifold was connected to five other identical collection systems. A flowmeter was used to set a constant flow of 450 mL min^{-1} for all chambers.

Fungal cultures were placed in the collection chamber for 24 hours to build up volatiles. The pump is switched on for another 24 hours for volatiles trapping. Headspace volatiles were extracted after 48 hours. The activated carbon was removed and transferred into a microtube containing 1 mL of dichloromethane with tridecane as internal standard (0.002%). This mixture was vortexed for 30 seconds, sonicated for 10 minutes and centrifuged (at 30,000 rpm) for 30 minutes. The extract was transferred to a gas chromatograph (GC) vial. The same procedure was repeated a second time before chromatographic separation. All samples were analyzed with GC system (GC: 7890A; Agilent Tech., Santa Clara, CA, USA) fitted with a mass spectrometer (MS: 5062C, Agilent Tech., Santa Clara, CA, USA) with a DB-5MS UI column (30 m x 0.25 mm ID x 0.25 μm film). Helium was used as a carrier gas flowing at 1 mL min^{-1} with a temperature programbeginning at 50°C (held for 1 min) then increased by 5°C min^{-1} to 200°C, followed by an increase of 30°C min^{-1} to 325°C (held for 2 min). It was maintained at 250 °C, a 1 μl sample injection volume was used, and samples were run in splitless mode. Peaks present in chromatograms of control treatment were ignored from those of fungal cultures to determine

peaks unique to the media. Library matches using NIST/EPA/NIH Mass Spectral library version 2.0f for all detected fungal volatiles were verified and quantified using the following standards: ethyl acetate (≥ 99%), isobutanol (≥ 99%), 2-methyl-1-butanol (≥ 99% pure), isoamyl alcohol (≥ 98%), isoamyl acetate (≥97%), ethyl alcohol (≥98%), phenylethyl acetate (≥ 98%), and phenylethyl alcohol (≥ 99%). All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analyte concentrations were standardized by the culture area prior to data analysis.

3.3.2 Data analysis

The quantity of each compound detected in fungal treatments were calculated as amount (ng) of compound per unit (mm^2) of fungal culture area per day ($ng/mm^2/day$). Data were analyzed using SAS (PROC GLM, SAS Inc., Cary, NC, USA) statistical software. A multivariate analysis of variance (MANOVA) was conducted to determine if there were differences between the quantity of compounds produced among fungi treatments. Data were first checked for normality and equal variance using Wilks' lambda. Pair-wise comparisons were conducted at undertaken using the post-hoc Bonferroni test on the fungal treatments at $\alpha = 0.05$. Graphs were created in excel.

3.4 Results

After a 48-hour sampling period, eight fungal VOCs were detected in the extraction of headspace volatiles of *G. alacris, G. huntii, L. terebrantis* and *L. procerum* isolates growing alone and double treatments. The compounds represented two chemical classes: ethyl acetate, isoamyl acetate and phenylethyl acetate (esters), isobutanol, 2-methyl-1-butanol, ethyl alcohol and phenylethyl alcohol (alcohols) and verbenone (Figure 3).

The fungal VOC profiles among *G. alacris, G. huntii, L. terebrantis* and *L. procerum,* and double treatment significantly differed ($P < 0.0001$). Volatile organic compound profile

composition among treatments varied and the profiles were influenced by the fungal species. Double treatments had profiles similar to *G. alacris, G. huntii, L. terebrantis* and *L. procerum* (Table 3.2). All compounds detected from the double treatment had lower concentrations compared to the fungal isolates growing alone, indicating that the presence of other fungus did influence the production of compounds.

For fungal isolates growing alone, *G. huntii* produced six compounds: isoamyl acetate, phenylethyl acetate, isobutanol, 2-methyl-1-butanol, phenylethyl alcohol and verbenone. Isoamyl acetate and ethyl alcohol were exclusive to *G. huntii* and *L. procerum* respectively (Table 3.1), however, the compounds were also detected in several fungi within the combination treatment. Quantity of compounds detected significantly differed (P <0.001) among individual treatment (*G. alacris, G. huntii, L. terebrantis* and *L. procerum*). For the combination treatments, seven compounds were detected each in *G. alacris / L. procerum, G. huntii / L. procerum, L. procerum / G. alacris, L. terebrantis / L. procerum, and L. terebrantis / L. procerum.*

Verbenone was a compound detected in all treatments (growing alone and double). Verbenone was not an expected compound for the study. However, the compound did not show significant difference among isolates growing alone (P = 0.702) or double treatment (P = 0.974).

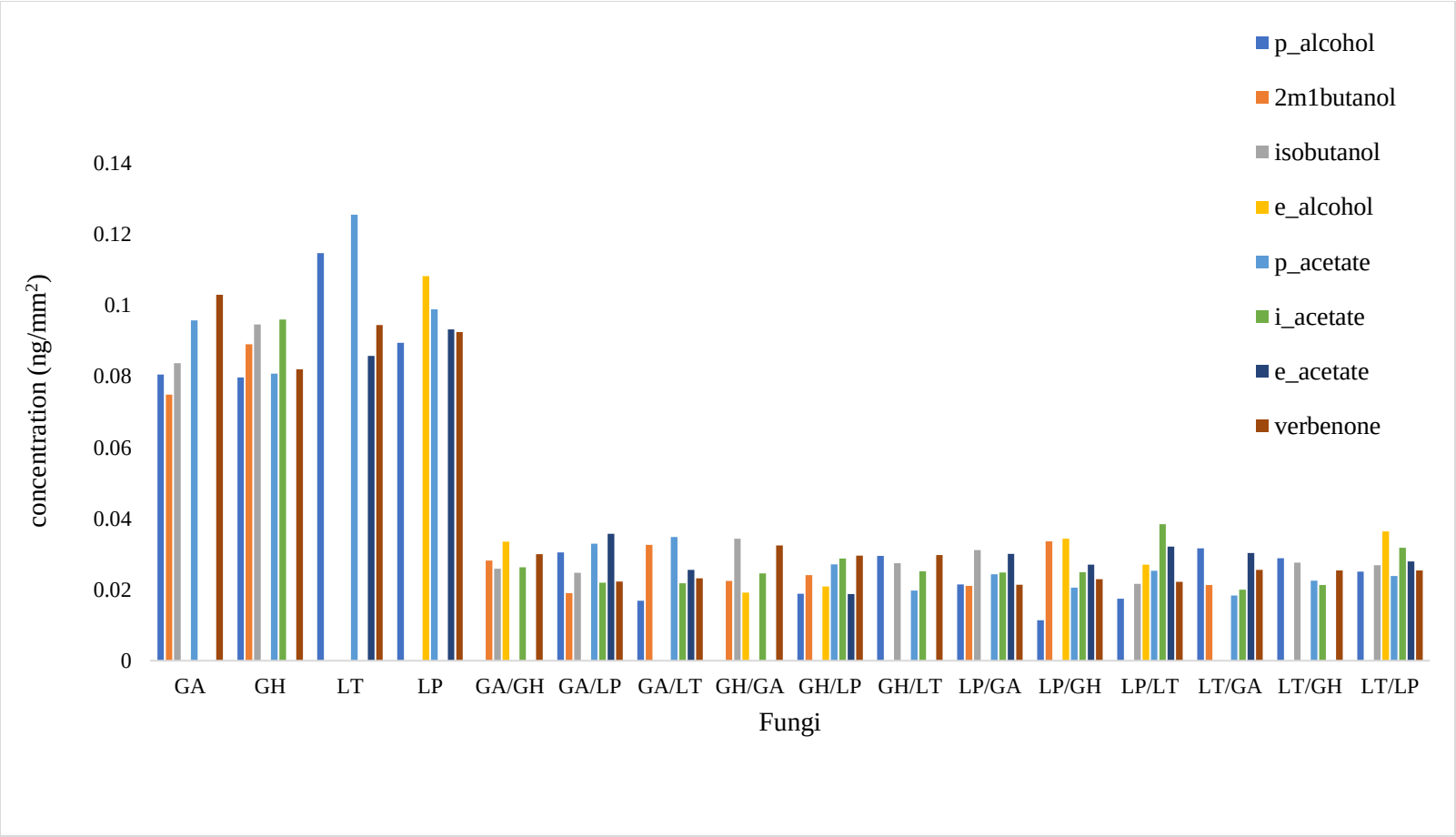

Figure 3.1 Graph of compounds detected in *G. alacris, G. huntii, L. terebrantis,* and *L. procerum* and their combination treatments.

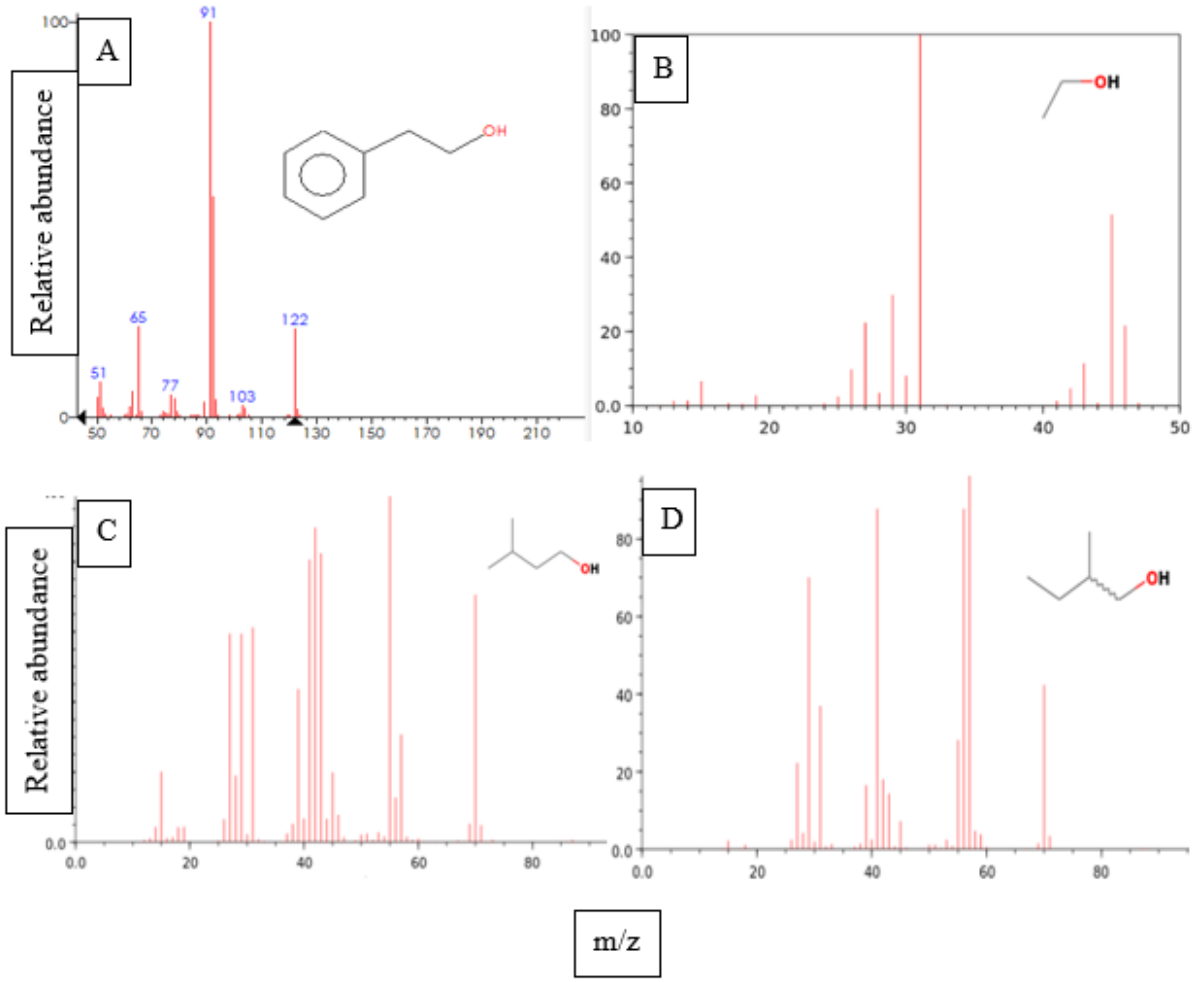

Figure 3.2 Spectral diagrams of abundant monoterpenes phenylethyl alcohol (A), ethyl alcohol (B), isobutanol (C) and 2-methyl-1-butanol (D).

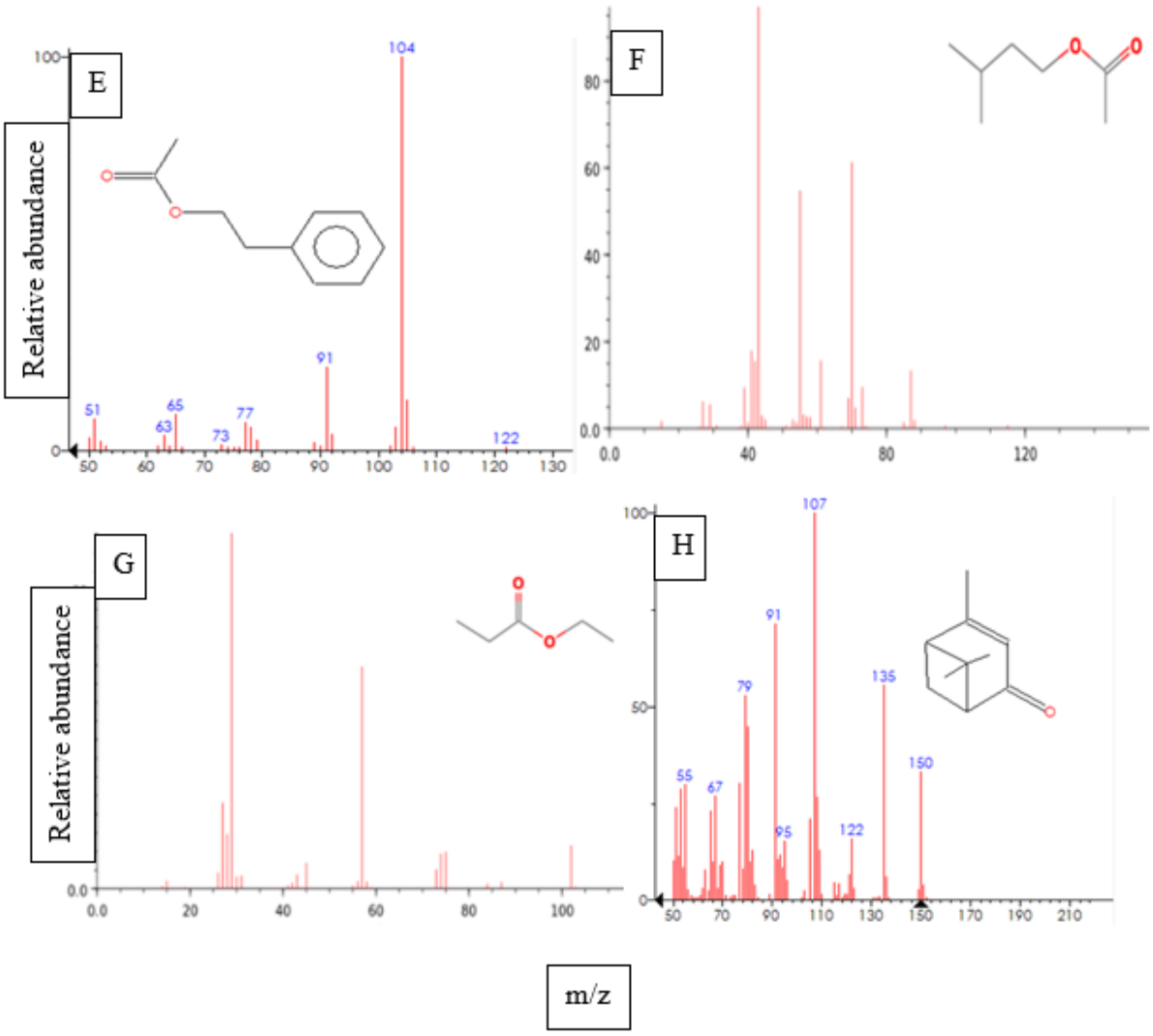

Figure 3.3 Spectral diagrams of abundant phenylethyl acetate (E), isoamyl acetate (F), ethyl acetate (G) and verbenone (H).

Table 3.1 Mean and standard error concentrations (ng/mm^2/day) of compounds detected in *G. alacris*, *G. huntii*, *L. terebrantis*, and *L. procerum.* Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Fungal treatments**				**df**	**F**	**P-value**
	Grosmannia alacris	*Grosmannia huntii*	*Leptographium terebrantis*	*Leptographium procerum*			
Phenylethyl alcohol	0.08±0.006	0.08±0.005	0.11±0.021	0.09±0.01	3	1.63	0.201
Phenylethyl acetate	0.1±0.011	0.08±0.008	0.13±0.02	0.7±0.02	3	1.45	0.245
2-methyl-1-butanol	0.07±0.009	0.09±0.011	ND	ND	1	40.17	<0.0001
Isobutanol	0.08±0.006	0.09±0.006	ND	ND	1	150.56	<0.0001
Verbenone	0.1±0.018	0.08±0.008	0.1±0.013	0.09±0.009	3	0.47	0.702
Isoamyl acetate	ND	0.1±0.013	ND	ND	-	-	-
Ethyl acetate	ND	ND	0.09±0.01	0.09±0.011	1	42.33	<0.0001
Ethyl alcohol	ND	ND	ND	0.1±0.011	-	-	-

Table 3.2 Mean and standard error concentrations (ng/mm^2/day) of compounds detected in combined fungi. Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Fungal treatments**												**df**	**F**	**P-value**
	GAGH	GALP	GALT	GHGA	GHLP	GHLT	LPGA	LPGH	LPLT	LTGA	LTGH	LTLP			
Phenylethyl alcohol	ND	0.03± 0.007	0.01± 0.004	ND	0.02± 0.005	0.03± 0.005	0.02± 0.007	0.01± 0.02	0.02± 0.004	0.03± 0.007	0.03± 0.007	0.03± 0.006	9	4.5	<0.0001
Phenylethyl acetate	ND	0.03± 0.007	0.03± 0.007	ND	0.03± 0.006	0.02± 0.004	0.02± 0.005	0.02± 0.006	0.02± 0.005	0.02± 0.005	0.02± 0.007	0.02± 0.007	9	3.6	<0.0001
2-methyl-1-butanol	0.03± 0.006	0.02± 0.004	0.03± 0.006	0.02±0 .006	0.02± 0.006	ND	0.02± 0.006	0.03± 0.007	ND	0.02± 0.006	ND	ND	7	6.9	<0.0001
Isobutanol	0.03± 0.006	0.02± 0.008	ND	0.03±0 .005	ND	0.03± 0.008	0.03± 0.006	ND	0.02± 0.004	ND	0.03± 0.005	0.03± 0.005	7	8.43	<0.0001
Verbenone	0.03± 0.007	0.02± 0.005	0.02± 0.006	0.03±0 .007	0.03± 0.007	0.03± 0.008	0.02± 0.006	0.02± 0.007	0.02± 0.006	0.03± 0.006	0.03± 0.005	0.03± 0.006	11	0.34	0.9738
Isoamyl acetate	0.02± 0.008	0.02± 0.005	0.02± 0.007	0.02±0 .006	0.03± 0.006	0.03± 0.006	0.02± 0.006	0.02± 0.004	0.04± 0.007	0.02± 0.004	0.02± 0.006	0.03± 0.007	11	0.68	0.7541
Ethyl acetate	ND	0.04± 0.008	0.03± 0.008	ND	0.02± 0.004	ND	0.03± 0.008	0.03± 0.006	0.03± 0.008	0.03± 0.006	ND	0.03± 0.005	7	7.15	<0.0001
Ethyl alcohol	0.03± 0.007	ND	ND	0.02±0 .007	0.02± 0.004	ND	ND	0.03± 0.009	0.03± 0.008	ND	ND	0.04± 0.006	5	9.77	<0.0001

3.5 Discussion

Fungal VOCs emitted by a given fungus can be influenced by those from another fungal species. Our study showed that fungal VOC profiles from the double treatments differed quantitatively from *G. alacris, G. huntii, L. terebrantis* and *L. procerum* growing alone. Despite the difference in fungal VOC profiles, compounds detected in the double treatment had lower concentrations ((50% less) compared to fungi growing alone. There is strong evidence that ophiostomatoid fungi can affect and alter the fungal VOC profiles of other fungal species. Studies have shown that communication between closely related fungal species involves not only water-soluble chemicals, but also the emission and detection of volatile organic compounds (Hofstetter et al., 2005; Schmidt et al., 2015). Our study demonstrates that fungal VOCs can be used as a semiochemical to inhibit the production of VOCs by another fungus, as the compounds emitted by the combination treatments were modified. This supports other studies showing that, ophiostomatoid fungi can interact and communicate through VOCs emitted by a different ophiostomatoid species (Wang et al., 2020). This suggests that the VOCs emitted by a fungus may alter the fungal community composition by having an antagonistic relationship with competitive fungi (Reino et al., 2007; Hynes et al., 2007; El Ariebi et al., 2016; Cale et al., 2016).

Fungi that occupy the same ecological niche can share similar VOC profiles (Müller et al., 2013). In our study, the fungal VOCs detected were similar among the ophiostomatoid fungal species used. Ethyl acetate, isoamyl acetate, phenylethyl acetate, isobutanol, 2-methyl-1-butanol, ethyl alcohol and phenylethyl alcohol were dominant components of fungal VOC profiles of other ophiostomatoid fungi: *G. clavigera, L. longiclavatum*, *Endoconidiophora polonica*, *G. clavigera* and *Ophiostoma montium* (Cale et al., 2019; Kandasamy et al., 2016; Wang et al.,

2020). Similarities in fungal VOCs among different ophiostomatoid fungal species may reflect a common ecological niche (Cale et al., 2016; Wang et al., 2020).

Fungal VOCs can act as semiochemicals that function as attractants and repellents to insects and other organisms (Morath et al., 2012). Generally, alcohols (2-methyl-1-butanol, ethyl alcohol, isobutanol, and phenylethyl alcohol) are highly attractive to several bark beetles. Phenylethyl alcohol and 2-methyl-1-butanol are particularly attractive to some bark beetles (Renwick et al., 1976; Pureswaran et al., 2000; Zhao et al., 2015; Cale et al., 2016). Some species of bark beetles are more attractive to a pheromone blend (frontalin-trans-verbenol-turpentine) containing a mixture of esters (phenethyl acetate, 3-methyl-1-butyl acetate and isoamyl acetate) than the blend itself (Cale et al., 2016; Kandasamy et al., 2019). Isoamyl acetate is known to be toxic to some fungi and bacteria (Strobel et al., 2001). Ethyl acetate can be used as insect deterrent or repellent in the field because of its strong smell or vapor. It is used as a killing agent during insect collection in the field.

3.6 Conclusion

This study demonstrates that the presence of resource-sharing fungi may affect the production of fungal VOC profiles as well as the interactions between fungi occupying the same niche, inhibiting the production of fungal VOCs. There is also a suggestion that the root-feeding bark beetle still maintain the relationship with their symbiotic ophiostomatoid fungi with the aid of fungal VOCs. Field experiment is recommended to test whether fungal VOC can be used either as an attractant or repellent.

CHAPTER IV

Allelochemicals production from loblolly seedlings inoculated with ophiostomatoid fungi

4.1 Abstract

Root-feeding bark beetles are considered pests of *Pinus taeda* in the southeastern United States. The bark beetles and their associated ophiostomatoid fungi contribute to decline disease in pine trees. Trees produce monoterpenes as a defense mechanism when they are infected by insect pests and pathogens. *Pinus taeda* seedlings were inoculated with four ophiostomatoid fungi: *Grosmannia alacris, Grosmannia huntii, Leptographium terebrantis,* and *Leptographium procerum.* Monoterpenes were extracted and analyzed with GC-MS after 4, 8 and 12 weeks after inoculation. Fourteen monoterpenes were identified and quantified: α-pinene, camphene, β-pinene, limonene, myrcene, terpineol, p-cymene, bornyl acetate, ocimene, γ-terpinene, *trans*-verbenol, 3-carene, camphene, *cis*-verbenol, and borneol. Higher quantities of compounds were produced in week 8 when compared to weeks 4 and 12. Monoterpenes produced can be used as attractant or deterrent either individually or synergistically.

4.2 Introduction

The root-feeding bark beetles are considered pest of loblolly pine tree in the southeastern United States (Eckhardt, 2007). The insect bores into the roots of the host tree allowing its associated fungi to colonize the phloem and overwhelm the tree's defense system. Root-feeding bark beetles and their fungal associates are biotic factors associated with pine decline (Eckhardt et al., 2007). Ophiostomatoid fungi are carried either in the mycangia or on the exoskeleton of the bark beetle and enhances the decline (Bridges and Moser, 1986; Six, 2003). The role played by ophiostomatoid fungi during bark beetle colonization can be complicated. The fungi can have antagonistic effect on the beetle's larval stage (Barras, 1970) or be involved in the success of the

beetle in the early stages of the attack due to the fungi's rapid growth and phloem colonization that compromises host defenses (Klepzig et al., 2005; Lieutier et al., 2009).

When bark beetles and their associated fungi rapidly invade a pine tree, the attackers trigger a defense system. A major chemical defense system of conifers is the production of oleoresin which consists primarily of a complex mixture of different volatile monoterpenes, non-volatile diterpenoids, and sesquiterpenes (Keeling and Bohlmann, 2006; Kolosova and Bohlmann, 2012; Zulak and Bohlmann, 2010). Constitutive and induced defense responses are crucial for the survival of pine trees after infection (Karban and Baldwin, 1997). Conifers continuously produce baseline amounts of some monoterpenes that form constitutive defenses to repel, kill or contain invaders such as pathogens or insects (Bonello et al., 2006). They may be the first line of defense to inhibit the initial growth of pathogenic fungi that infect conifer trees (Bridges 1987; Michelozzi et al., 1995; Lombardero et al., 2006). Monoterpenes can also form inductive defenses when trees synthesize or up-regulating compounds in response to specific stressors (Evenesen et al., 2000). In addition to phenolics, monoterpenes are also important chemical defense of conifers (Franceschi et al., 2005). The majority of monoterpenes produced are secondary compounds, that is, they are mainly required for communication and tree defense but not for growth (Gershenzon and Dudareva, 2007). In addition, these monoterpene-based defenses play an important role in constraining fungi from transitioning from endemic to epidemic population densities (Boone et al., 2011). Apart from defense, conifer terpenes have several ecological functions. Conifer terpenes can function as insect-attracting odors in conifers (Nordlander, 1991), or as building blocks for *in vivo* synthesis of pheromones by wood-boring beetles (Martin et al., 2003).

The present study was conducted to test the hypothesis that, comparing *P. taeda* monoterpene responses to infection by different fungi (*G. alacris, G. huntii, L. terebrantis,* and *L. procerum*). To test this hypothesis, seedlings were inoculated with *G. alacris, G. huntii, L. terebrantis,* and *L. procerum,* monoterpenes were extracted, and were qualitatively and quantitively assessed.

4.3 Materials and methods

4.3.1 Potting and inoculation of seedlings

A total of 225 1-year-old bare-root *P. taeda* seedlings from a single genetic family were grown in one-gallon pots filled with ProMix BX® peat-based potting media. The seedlings were allowed to grow under natural conditions in an open field of the College of Forestry and Wildlife Sciences, Auburn University, located in Auburn, Alabama. Seedlings' stems were artificially inoculated with mycelial agar plugs of fungal isolates taken from the leading edge of 14-day-old malt extract agar plate. Forty-five seedlings were assigned for each of four different pathogens (*L. terebrantis, L. procerum, G. alacris* and *G. huntii*). Control seedlings were inoculated with a sterile agar plug without fungus. Inoculations were made by making a small (1 cm) vertical slit in the stem was made with a sterile razor blade extending into the vascular tissues, followed by placing a 3 mm diameter plug of colonized MEA in the wound. Inoculation points were covered with sterile moist cotton balls to prevent desiccation of the fungal media and then wrapped with parafilm to prevent further contamination.

Figure 4.1: Seedlings inoculated with *G. alacris, G. huntii, L. terebrantis,* and *L. procerum.*

4.3.2 Terpene extraction

Seedlings were selected at three different time points (four, eight- and twelve-weeks post-inoculation) for terpene extraction. Fifteen seedlings were randomly selected from each treatment during each time point. Same was done for control seedlings. For terpene extraction, inoculation point, or stem of seedlings were destructively extracted and ground into powder in liquid nitrogen. Lesion length, width and depth were measured before seedlings were destructive sampled. Grinding was done to prevent damage to gas chromatography (GC) columns and equipment. Samples were freeze dried at -40ºC. One hundred milligram of ground sample was weighed and extracted with dichloromethane together with 0.004% of tridecane as the internal standard. The mixture was vortexed for 30 sec, sonicated for 10 min, and centrifuged (at 30,000 rpm) for 15 minutes. Extracts were transferred to glass vials for GC analysis. One microliter of each extract was injected from GC vials into an Agilent 7890A/5062C gas chromatograph-mass spectrometer (GC-MS, 7890A/5975C, Agilent Tech., Santa Clara, CA, USA) with a DB-5MS UI column 30 m x 0.25 mm ID x 0.25 μm film. Helium was used as a carrier gas flowing at 1 mL

min^{-1} with initial temperature of 50ºC for 2 minutes, increased to 60ºC and held for 1 minute. The temperature was increased again from 20ºC to 250ºC and held for 1 minute. The standards used for quantification were α-pinene, β-pinene, 3-carene, myrcene, limonene, p-cymene, camphor, 4-allyanisole, borneol, γ-terpinene, α-terpinene, pulegone, terpineol, ocimene, terpinolene, bornyl acetate, and camphene.

4.3.3 Data analysis

The lesion area was calculated to standardize the concentration of analyte as the amount of compound (ng/mm^2). Data were analyzed using STATA (version 14) statistical software. A multivariate analysis of variance (MANOVA) was conducted to determine if there were differences between the monoterpenes produced on fungal and time treatments. Data were first checked for normality and equal variance using Wilks' lambda. Pair-wise comparisons were undertaken using the Tukey Honest Significant Difference (HSD) on the four fungal treatments and their interaction with time at $\alpha = 0.05$. Graphs were created in Microsoft Excel.

4.4 Results

A multivariate analysis of variance (MANOVA) was conducted to determine whether the different compounds identified and quantified by the GC analysis differed based on time and fungal group as well as the interaction between time and fungal treatments. From the analysis, significant differences were observed between fungi, time, and their interactions at ($p<0.001$), ($p<0.001$) and ($p<0.001$) respectively (Table 4.1).

Fourteen monoterpenes were identified and quantified after GC analysis. In decreasing abundance from species, the compounds are α-pinene, camphene, β-pinene, limonene, myrcene, terpineol, p-cymene, bornyl acetate, ocimene, γ-terpinene, trans verbenol, 3-carene, camphene, *cis* verbenol, and borneol (Fig 4.2). Nine compounds (α-pinene, β-pinene, limonene, p-cymene,

camphene, bornyl acetate, γ-terpinene, terpineol and trans verbenol) were produced by *G. alacris. Grosmannia huntii* produced 8 compounds: α-pinene, β-pinene, limonene, p-cymene, camphene, bornyl acetate, terpineol and myrcene. Ten compounds were detected in *L. procerum* (α-pinene, β-pinene, 3-carene, myrcene, limonene, p-cymene, camphene, borneol, ocimene and bornyl acetate). α-pinene, β-pinene, 3-carene, myrcene, limonene, p-cymene, camphene, γ-terpinene, terpineol, ocimene, and *cis* verbenol were detected from seedlings inoculated with *L. terebrantis.* Increased quantity of compounds was observed in all the experiments after inoculation when compared with control, though the quantities differed across compounds. Six compounds were visible in all fungal treatments (α-pinene, β-pinene, camphene, limonene, bornyl acetate, and p-cymene). Statistical significances were observed in α-pinene, limonene, bornyl acetate, and p-cymene. However, β-pinene (p=0.1162) and camphene (p=1.72) were not statistically different. Terpineol was only present in *G. alacris, G. huntii* and *L. terebrantis* showed significant difference at (p= 0.0259). Ocimene ($p<0.001$) and 3-carene (p=0.0036) were compounds produced in only *L. procerum* and *L. terebrantis.* No significant difference (p=0.0605) was seen in the production of myrcene. Myrcene was only present in *G. huntii, L. procerum* and *L. terebrantis.* However, *trans*-verbenol, borneol and *cis*-verbenol were only present in *G. alacris, L. procerum* and *L. terebrantis* (Table 4.2).

The quantity of compounds produced differed across time. From the analysis, the results showed that higher quantities of compounds were produced in week 8 when compared to weeks 4 and 12 (figure 4.3). Quantitative analysis showed that the production of compounds was induced due to fungal inoculation. All fungal treatments had significantly higher quantities of the measured compounds when compared with the control group. The most abundant compounds in all treatments were α-pinene, β-pinene, camphene, and limonene. A-pinene, β-pinene and

limonene showed statistical significance at (p=0.0414), (p<0.001) and (p<0.0026), respectively. Camphene, however, did not show significant (p=0.2025) increase over time although absolute values increased gradually. Most compounds concentration in control treatments were low often below detection limits. Myrcene was an exception as it was not detected during the fourth weeks but was seen in week 8 and marginally increased in week 12. There was no significant difference (p= 0.1619) of myrcene across time. Also, γ-terpinene and *trans*-verbenol was not produced in week 4 but was detected abundantly in week 8 but decreased in week 12. γ-terpinene and *trans*-verbenol differed significantly at (p=0.0161) and (p=0.0025) respectively. There was statistical significance seen in the concentration of compounds p-cymene, terpineol, bornyl acetate and ocimene, and their *p*-values were (0.0147), (0.0226), (<0.001) and (0.01) respectively. Terpineol, p-cymene, bornyl acetate and ocimene compounds were produced during all the time points. *Cis*-verbenol was only produced in weeks 4 and 12 and there was a statistical difference (p=0.0414) between the production of *cis* verbenol in weeks 4 and 12. Production of 3-carene was only detected in week 12 (Table 4.3).

Significant differences were observed in compounds produced for time and fungal interactions except compounds camphene and myrcene. *Grosmannia alacris* and *L. procerum* produced in week 8 had relative higher interactions with all fungi at different periods and were statistically significant (Table 4.4).

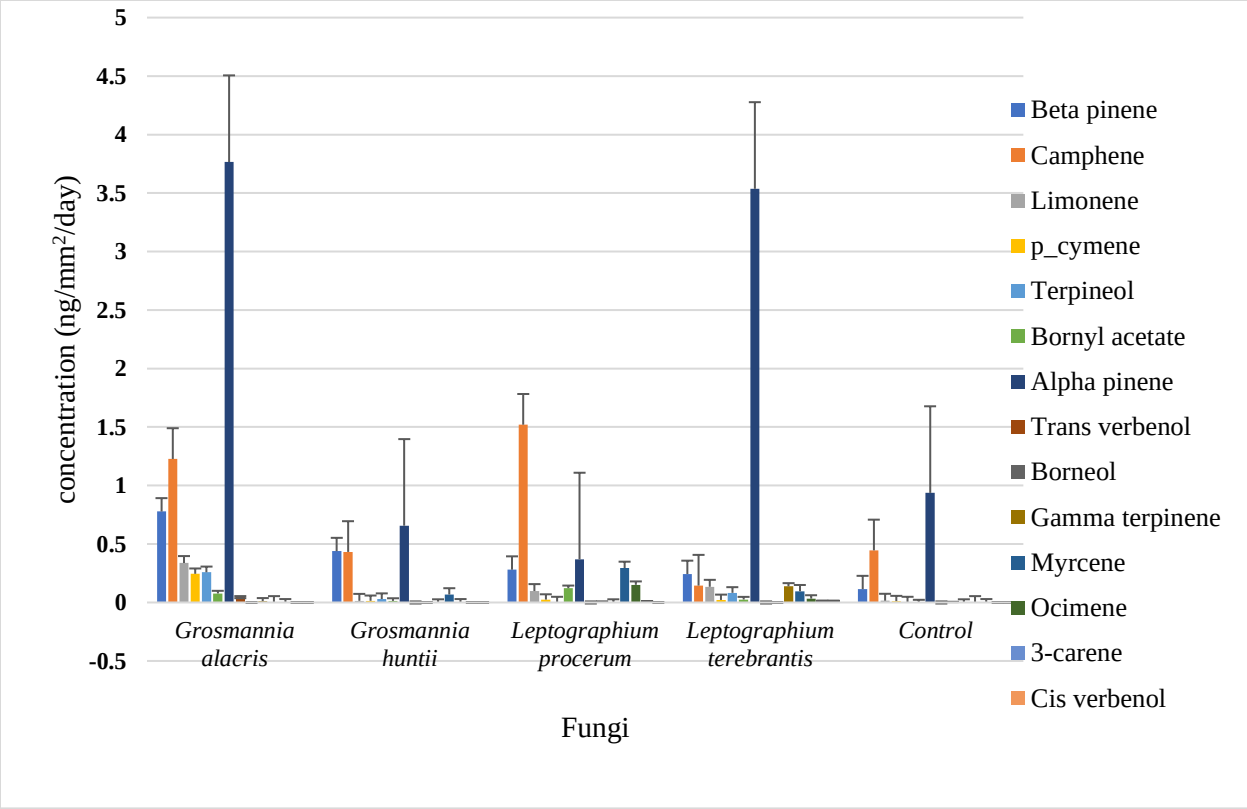

Figure 4.2 Graph of compounds detected in seedlings inoculated with *G. alacris*, *G. huntii*, *L. terebrantis*, and *L. procerum*.

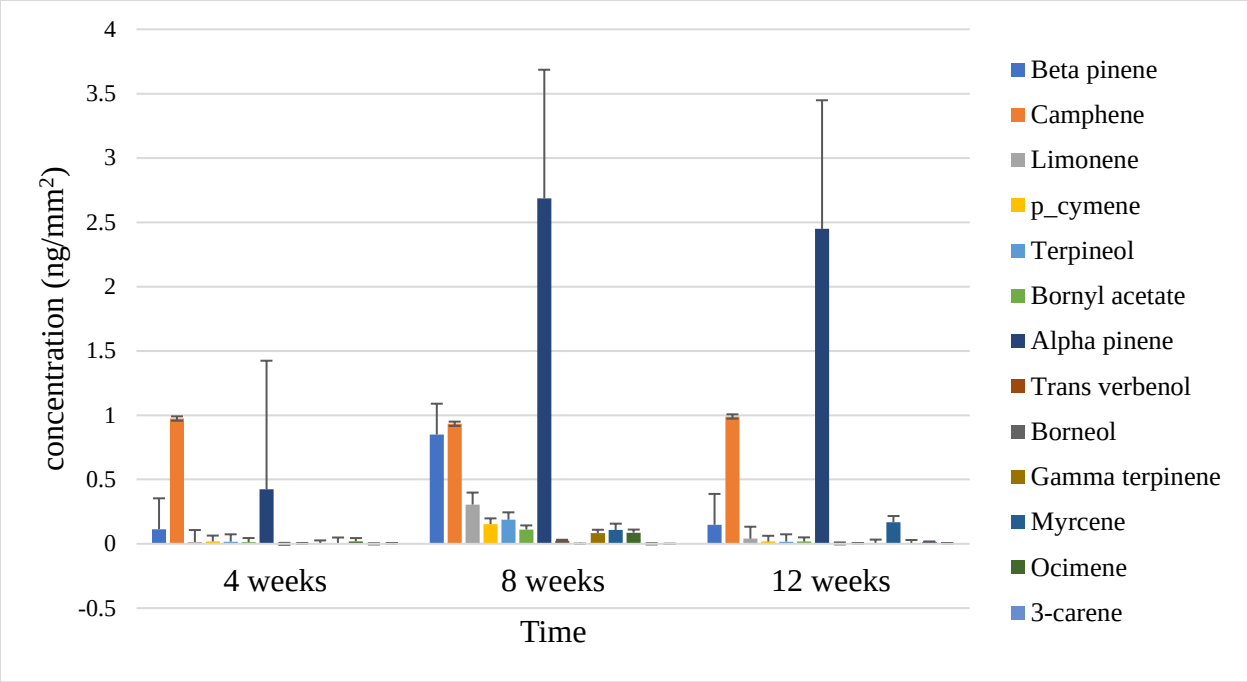

Figure 4.3 Graph of compounds detected at time points.

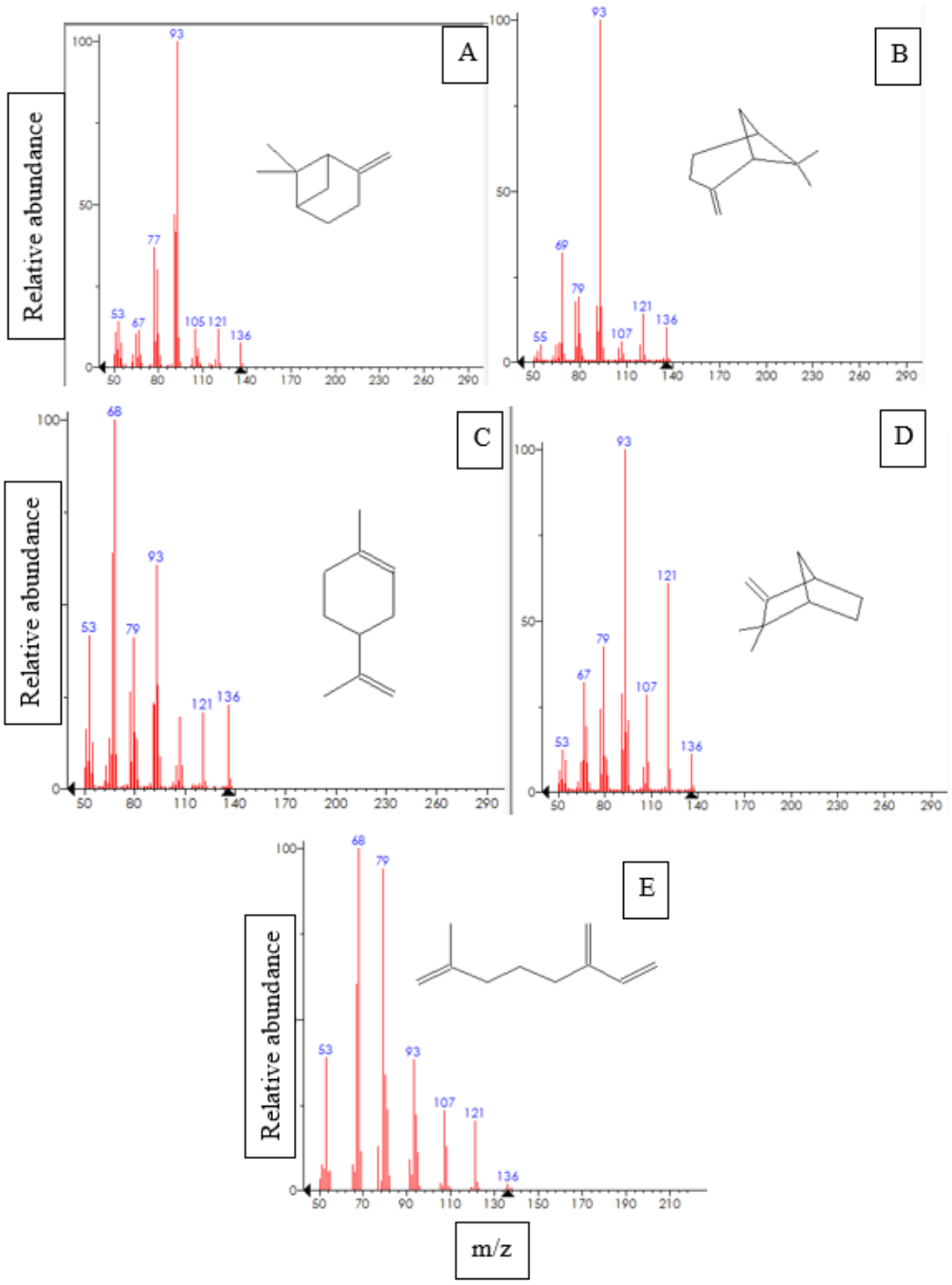

Figure 4.4 Spectral diagrams of abundant monoterpenes α-pinene (A), β-pinene (B), limonene (C), Camphene (D) and myrcene (E).

Table 4.1: Summary of descriptive statistics of compounds based on fungi treatments, time and as well as their interactions.

Variable	**df**	**F**	**P-value**
Fungi	14	4.92	<0.001
Time	2	3.2	<0.001
Interaction	14	4.92	<0.001

Table 4.2 Mean and standard error concentrations (ng/mm^2/day) of compounds detected from seedlings inoculated with *G. alacris*, *G. huntii*, *L. terebrantis*, and *L. procerum.* Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Fungal treatments**				**df**	**F**	**P-value**
	Grosmannia alacris	*Grosmannia huntii*	*Leptographium procerum*	*Leptographium terebrantis*			
β-pinene	0.78±0.35	0.44±0.19	0.28±0.08	0.24±0.07	4	1.88	0.1162
Camphene	1.23±0.73	0.43±0.23	1.52±0.83	0.14±0.05	4	1.72	0.21
Limonene	0.34±0.18	0.01±0.005	0.1±0.03	0.13±0.04	4	2.44	0.0494
p_cymene	0.25±0.1	0.01±0.004	0.02±0.009	.022±0.005	4	4.71	<0.001
Terpineol	0.26±0.14	0.03±0.006	ND	0.08±0.02	3	2.85	0.0259
Bornyl acetate	0.08±0.03	0.01±0.004	0.12±0.04	0.03±0.005	4	4.84	<0.001
α-pinene	3.77±1.67	2.69±0.18	2.45±0.13	3.54± 0.94	4	3.6	0.0079
Trans-verbenol	0.05±0.01	ND	ND	ND	-	-	-
Borneol	ND	ND	0.007±0.002	ND	-	-	-
γ-terpinene	0.01±0.004	ND	ND	0.14± 0.06	2	4.65	<0.001
Myrcene	ND	0.07±0.06	0.29±0.16	0.1±0.04	3	2.31	0.0605
Ocimene	ND	ND	0.15±0.05	0.03±0.02	2	7.29	<0.001
3-carene	ND	ND	0.01±0.004	0.01±0.006	2	4.09	0.0036
Cis-verbenol	ND	ND	ND	0.01±0.003	-	-	-

Table 4.3 Mean and standard error concentrations (ng/mm^2) of compounds detected from volatiles of seedlings at periods 4, 8 and 12 weeks. Compounds not detected during headspace collection are indicated with "ND".

Volatile emission (ng/mm^2)	**Time (weeks)**			**df**	**F**	**P-value**
	4	8	12			
β-pinene	0.11±0.03	0.85±0.29	0.15±0.03	2	8.98	<0.001
Camphene	0.09±0.04	0.93±0.55	0.99±0.64	2	1.61	0.2025
Limonene	0.01±0.01	0.31±0.14	0.04±0.01	2	6.21	0.0026
p_cymene	0.02±0.01	0.15±0.08	0.02±0.003	2	4.35	0.0147
Terpineol	0.02±0.01	0.19±0.11	0.02±0.003	2	3.89	0.0226
Bornyl acetate	0.01±0.003	0.11±0.04	0.02±0.004	2	9.7	<0.001
α-pinene	0.42±0.16	2.69±1.21	2.45±0.82	2	3.25	0.0414
Trans-verbenol	ND	0.024±0.01	0.003±0.002	1	10.65	<0.001
Borneol	ND	ND	0.004±0.002	-	-	-
γ-terpinene	ND	0.08± 0.05	0.006±0.004	1	4.25	0.0161
Myrcene	ND	0.11±0.04	0.11±0.13	1	1.84	0.1619
Ocimene	0.02±0.01	.086±0.04	0.005±0.002	2	4.75	0.01
3-carene	ND	ND	0.01±0.005	-	-	-
Cis-verbenol	0.004±0.001	ND	0.004±0.002	1	3.25	0.0414

Table 4.4 Significant compounds detected across fungi and time interactions.

Compound	Interaction of fungi and time	t-stat	P-value
β-pinene	8 weeks Ga vs 4 weeks Ga	4.96	0.000
	8 weeks Ga vs 4 weeks Gh	5.24	0.000
	8 weeks Ga vs 4 weeks Lp	4.96	0.000
	8 weeks Ga vs 4 weeks Lt	4.75	0.000
	8 weeks Ga vs 8 weeks Lp	4.07	0.007
	8 weeks Ga vs 8 weeks Lt	4.27	0.003
	8 weeks Ga vs 12 weeks Gh	5.18	0.000
	8 weeks Ga vs 12 weeks Lp	4.71	0.001
	8 weeks Ga vs 12 weeks Lt	5.02	0.000
p_cymene	8 weeks Ga vs 4 weeks Ga	6.65	0.000
	8 weeks Ga vs 4 weeks Gh	6.9	0.000
	8 weeks Ga vs 4 weeks Lp	6.95	0.000
	8 weeks Ga vs 4 weeks Lt	6.53	0.000
	8 weeks Ga vs 8 weeks Gh	7.00	0.000
	8 weeks Ga vs 8 weeks Lp	6.46	0.000
	8 weeks Ga vs 8 weeks Lt	7.00	0.000
	8 weeks Ga vs 12 weeks Ga	6.89	0.000
	8 weeks Ga vs 12 weeks Gh	6.9	0.000
	8 weeks Ga vs 12 weeks Lp	6.68	0.000
	8 weeks Ga vs 12 weeks Lt	6.85	0.000
Bornyl acetate	8 weeks Ga vs 4 weeks Gh	4.5	0.001
	8 weeks Ga vs 4 weeks Lp	4.76	0.000
	8 weeks Ga vs 8 weeks Lt	4.76	0.006
	8 weeks Ga vs 12 weeks Ga	4.76	0.000
	8 weeks Ga vs 12 weeks Gh	4.1	0.006
	8 weeks Ga vs 12 weeks Lp	4.41	0.002
	8 weeks Ga vs 12 weeks Lt	3.687	0.025
	8 weeks Lp vs 4 weeks Ga	7.51	0.000
	8 weeks Lp vs 12 weeks Lt	7.43	0.000
	8 weeks Ga vs 12 weeks Lp	6.68	0.000
	8 weeks Ga vs 12 weeks Lt	6.85	0.000
	8 weeks Lp vs 8 weeks Gh	8.09	0.000
	8 weeks Lp vs 8 weeks Lt	8.09	0.000
	8 weeks Lp vs 12 weeks Ga	8.09	0.000
	8 weeks Lp vs 12 weeks Gh	7.43	0.000

	8 weeks Lp vs 12 weeks Lp	7.74	0.000
	8 weeks Lp vs 12 weeks Lt	7.01	0.000

4.5 Discussion

Fungal infections increase the production of monoterpenes in conifers, a set of primary defense compounds against bark beetles (Munro et al., 2020; Cale et al., 2017; Boone et al., 2011; Erbilgin et al., 2017a, b). Monoterpenes are generally toxic to insects and may increase bark beetle mortality during host tree colonization (Gershenzon and Dudareva, 2007). Monoterpenes can also inhibit the growth of fungal pathogens, for example blue stain fungi vectored by bark beetles (Novak et al., 2014). Bark beetle associated fungi when inoculated into Norway spruce (*Picea abies* (L.) Karst) increased drastically terpene concentration. The monoterpene increase inhibited the colonization by the spruce bark beetle (*Ips typographus* Linnaeus) in a dose-dependent manner (Zhao et al., 2011). Same pattern was observed for this study when *Grosmannia alacris, G. huntii, Leptographium procerum,* and *L. terebrantis* were inoculated into *Pinus taeda.* The observation suggests that loblolly pine can activate appropriate defenses when infected.

In the southeastern United States, emission of monoterpenes is dominated by α-pinene, β-pinene, camphene, limonene and myrcene (Geron et al., 2000; Klepzig et al., 1995). The same compounds are also known to be dominant compounds of pines though terpineol, p-cymene, bornyl acetate, ocimene, γ-terpinene, *trans*-verbenol, 3-carene, camphene, *cis*-verbenol, and borneol are produced by pine species (Chiu et al., 2017; Jost et al., 2008). This study supports previous findings that the compounds α-pinene, β-pinene, camphene, limonene and myrcene are the abundant monoterpenes produced after fungal infections in pine. Several studies have demonstrated that 3-carene was produced in increased quantities when lodgepole and pines were

inoculated with *Grosmannia clavigera* (Lusebrink et al., 2011; Sadof and Grant, 1997), however, our study showed otherwise. Only small quantities of 3-carene were detected when loblolly pine seedlings were inoculated with the four ophiostomatoid fungi used for the studies. Production of the compound is only seen in seedlings inoculated with *L. procerum* and *L. terebrantis*.

Some monoterpenes such as β-pinene, 3-carene and limonene have antifungal properties (Himejima et al., 1992). In the southern United States coniferous system, the growth and germination of bark beetle- associated fungi are inhibited by monoterpenes and other compounds in the resin of host pine (Klepzig et al., 1996; Eckhardt et al., 2009). Limonene is known to inhibit bark beetle attack (Cale et al., 2017). α-pinene is a precursor to aggregation pheromone *trans*-verbenol is toxic to some bark beetles. *Trans*-verbenol can be converted into verbenone by some ophiostomatoid fungi contributing to the anti-aggregation chemicals emitted by bark beetle when it colonizes trees (Cale et el., 2019). *Trans*-verbenol is known to be the primary compound of mountain pine beetle aggregation pheromone (Borden et al., 2008). Additionally, both α-pinene and β-pinene have been identified as attractants or attractant synergists for many beetles infesting conifers, serving as host susceptibility to the insects (Hofstetter et al., 2008; Miller and Rabaglia, 2009). Also, α-pinene and β-pinene reduce fungal growth (Eckhardt et al., 2009). Certain bark beetles (e.g., *Dendroctonus frontalis*) exploits myrcene synergistically to produce its aggregation pheromone (*exo*-brevicomin and frontalin) to mass attack pine trees (Miller and Lindgren, 2000; Boone et al., 2008; Borden et al., 2008). Although when myrcene and limonene are combined with α- and β-pinene, they become directly toxic to some adult bark beetles in loblolly pine (Raffa and Berryman, 1987). Studies have shown that, myrcene can also inhibit the growth of fungi associated with bark beetles (Hofstetter et al., 2005). Induction of myrcene and β-pinene may also promote beetle aggregation (Cale et al., 2017; Clark et al., 2012). Limonene is

a monoterpene identified as a resistance factor against many bark beetles by acting as a toxicant and oviposition deterrent (Sadof and Grant, 1997), and reduces fungal growth (Eckhardt et al., 2009). There is strong evidence that 3-carene also synergizes with some bark beetles' aggregation pheromone response (Borden et al., 2008). 3-carene showed strongly attractive to mountain pine beetle during an olfactory test (Conn, 1981). This compound sometimes functions as an indicator of a weakened tree thus supporting aggregation of beetles on susceptible hosts (Lusebrink et al., 2011). In adult *Dendroctonus valens,* 3-carene is a major volatile attractant in flight response (Erbilgin et al., 2007). *Cis* verbenol has been identified as an attractant to some bark beetles, and isolated from female *Dendroctonus* species. It is an attractive component in the pheromone blend of mountain pine beetle as well as several *Ips* spp. (Sullivan, 2005; Cognato, 2011). γ-terpinene is also known to inhibit the growth of fungal pathogens, as well as playing an active role in constitutive and inducive defenses against the bark beetles (Faccoli et al., 2005; Seybold et al., 2006). Additionally, γ-terpinene plays an indirect role to attract or repel beetles or their enemies (Villari et al., 2012).

Generally, pine trees respond to fungal infection by producing terpenes within 3 – 14 days and the rise is drastic (Litvak and Monson, 1998; Raffa and Smalley, 1995). Subsequently, monoterpenes level off or decline after reaching an initial peak (Raffa and Smalley, 1995). Similarly, monoterpenes were recorded at week 4, increased significantly at week 8 and declined during week 12. Faldt et al., (2006) reported that, high quantities of α-pinene, β-pinene and limonene were observed 17 weeks after fungal inoculation. Consistent with our experiment, previous studies conducted showed α-pinene, camphene, β-pinene, limonene, myrcene and 3-carene were compounds produced 1 – 4 weeks after inoculation with *L. terebrantis* and *Ophiostoma minus* indicating a strong induced response against fungal attack (Klepzig et al.,

1995; Arango-Velez et al., 2018; Delorme and Lieutier, 1990). Significant increase in monoterpenes were observed during the 9th week when *Pinus strobus* Linnaeus was inoculated with blue stain fungi.

The four ophiostomatoid fungi: *G. alacris, G. huntii, L. procerum,* and *L. terebrantis* we studied are pathogenic to loblolly pines (Eckhardt et al., 2004; Pan et al., 2018). *Grosmannia alacris* previously *L. serpens* has been described as the most virulent compared to *G. huntii, L. procerum,* and *L. terebrantis* (Eckhardt et al., 2004; Matusick et al., 2010). This can be attributed to why *G. alacris* had significant interactions with other fungi used for the studies. Although *L. procerum* is considered a weak pathogen (Eckhardt et al., 2008), it had considerable interactions across fungi and time.

4.6 Conclusion

Pine trees produce monoterpenes as defense mechanism when they are infected by bark beetles or pathogens. After several weeks of fungi inoculation, seedlings produce a significant number of monoterpenes. In this study, the monoterpenes produced by seedlings can be used to manage both bark beetle and its fungal symbiont. A field experiment should be conducted to determine whether compounds can be used either as an attractant or repellent or as precursor.

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