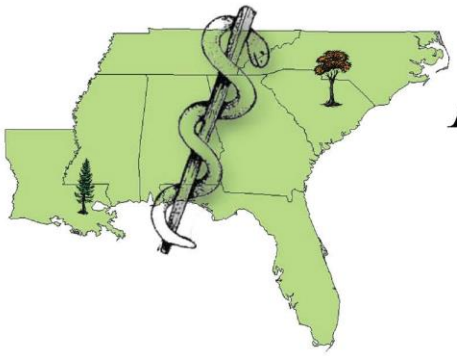

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PRODUCTION OF VOLATILE ORGANIC COMPOUNDS FROM OPHIOSTOMATOID FUNGI: SINGLE AND COMBINATION

By

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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.

2.1. 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.

2.2. MATERIALS AND METHODS

2.2.1. *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.

2.2.2. *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.*

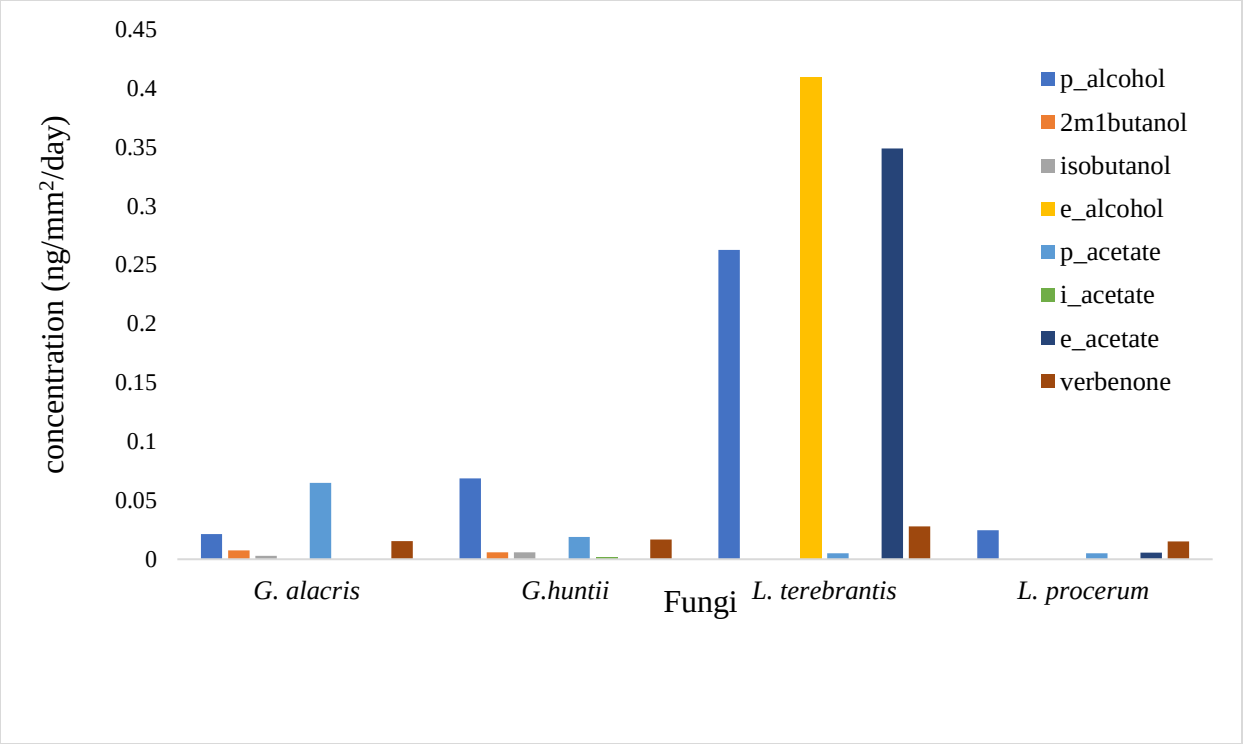

Figure 2.1. Graph of compounds detected in *G. alacris, G. huntii, L. terebrantis,* and *L. procerum.*

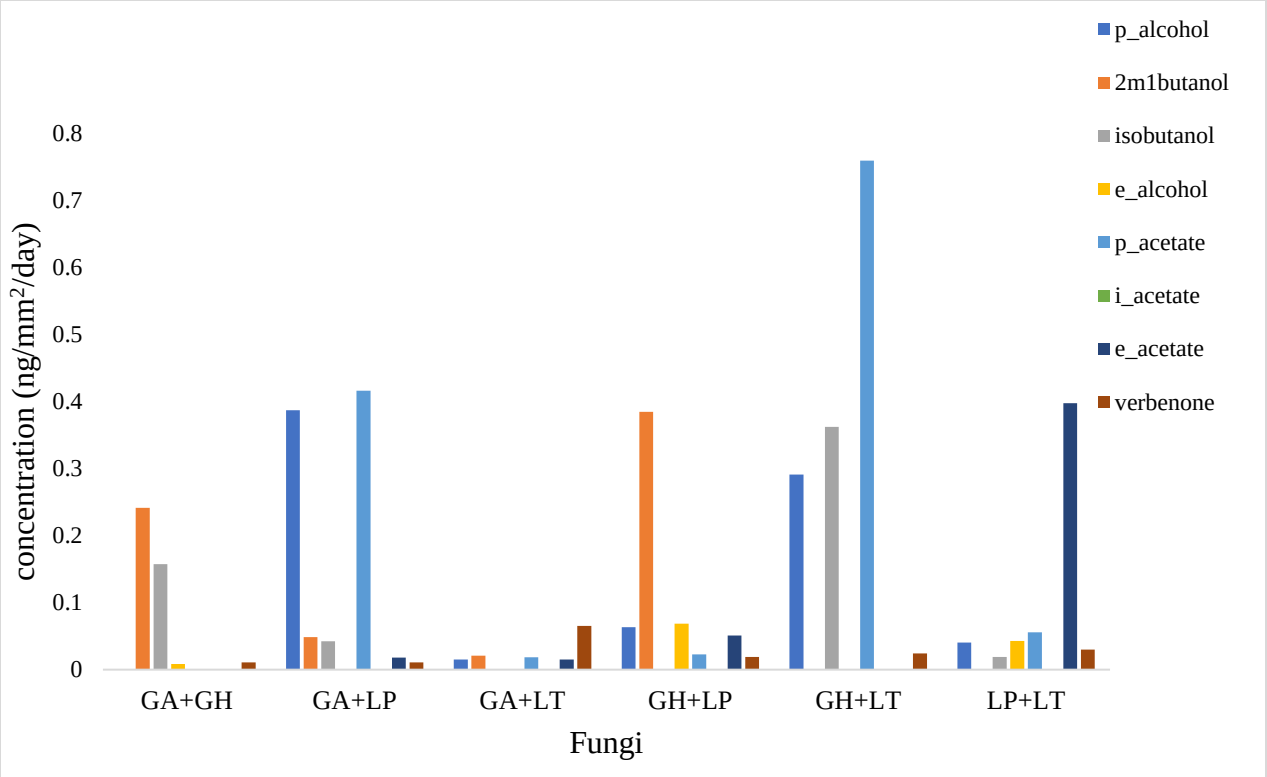

Figure 2.2. Graph of compounds detected in combined fungal treatments.

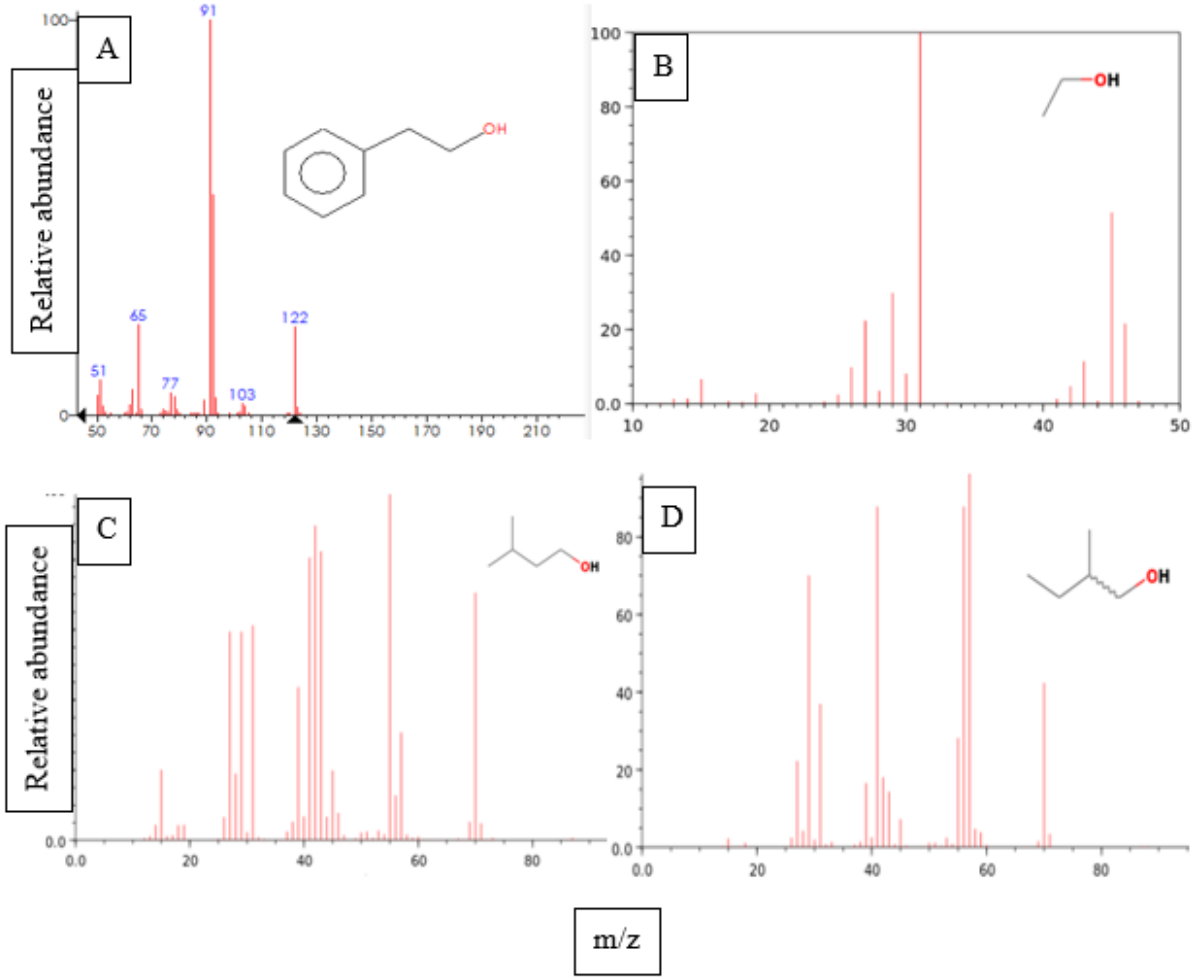

Figure 2.3. Spectral diagrams of abundant monoterpenes phenylethyl alcohol (A), ethyl alcohol (B), isobutanol (C) and 2-methyl-1-butanol (D).

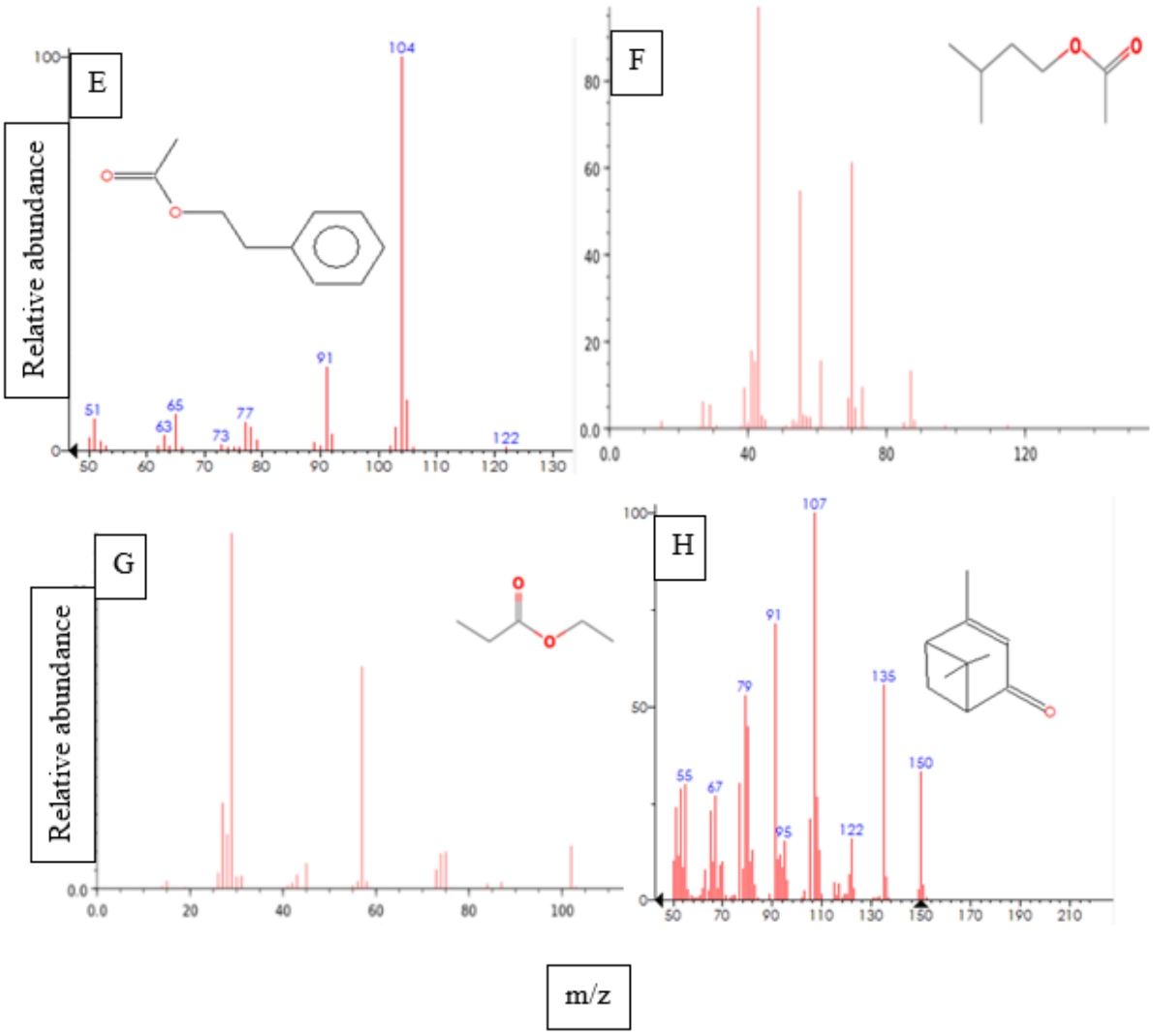

Figure 2.4. Spectral diagrams of abundant phenylethyl acetate (E), isoamyl acetate (F), ethyl acetate (G) and verbenone (H).

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

2.4. 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).

2.5. 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.