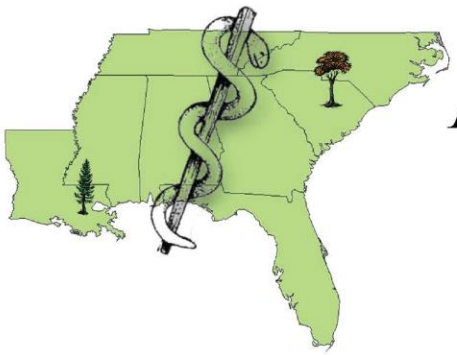

AUBURN UNIVERSITY
FOREST HEALTH
COOPERATIVE

REASEARCH REPORT 22-02

THE INFLUENCE OF DIFFERENT FUNGAL INTERACTION ON THE PRODUCTION OF FUNGAL METABOLITES

by

Sylvester Menanyih and Lori Eckhardt

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.

3.1. 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.

3.2. MATERIALS AND METHODS

3.2.1. *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 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.

3.2.2. *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 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.

3.3. 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).

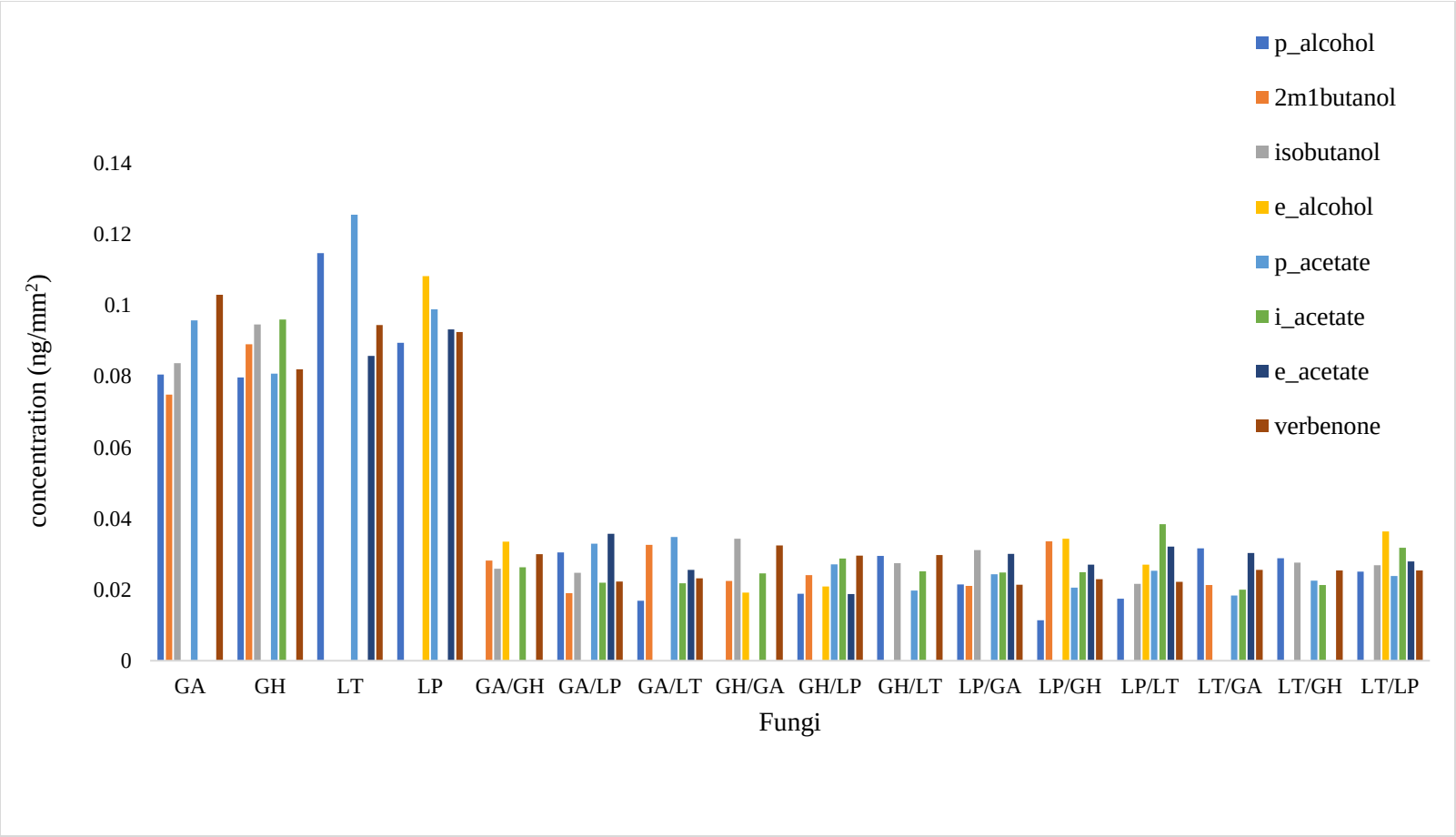

Figure 3.1. Graph of compounds detected in *G. alacris*, *G. huntii*, *L. terebrantis*, and *L. procerum* and their combination treatments.

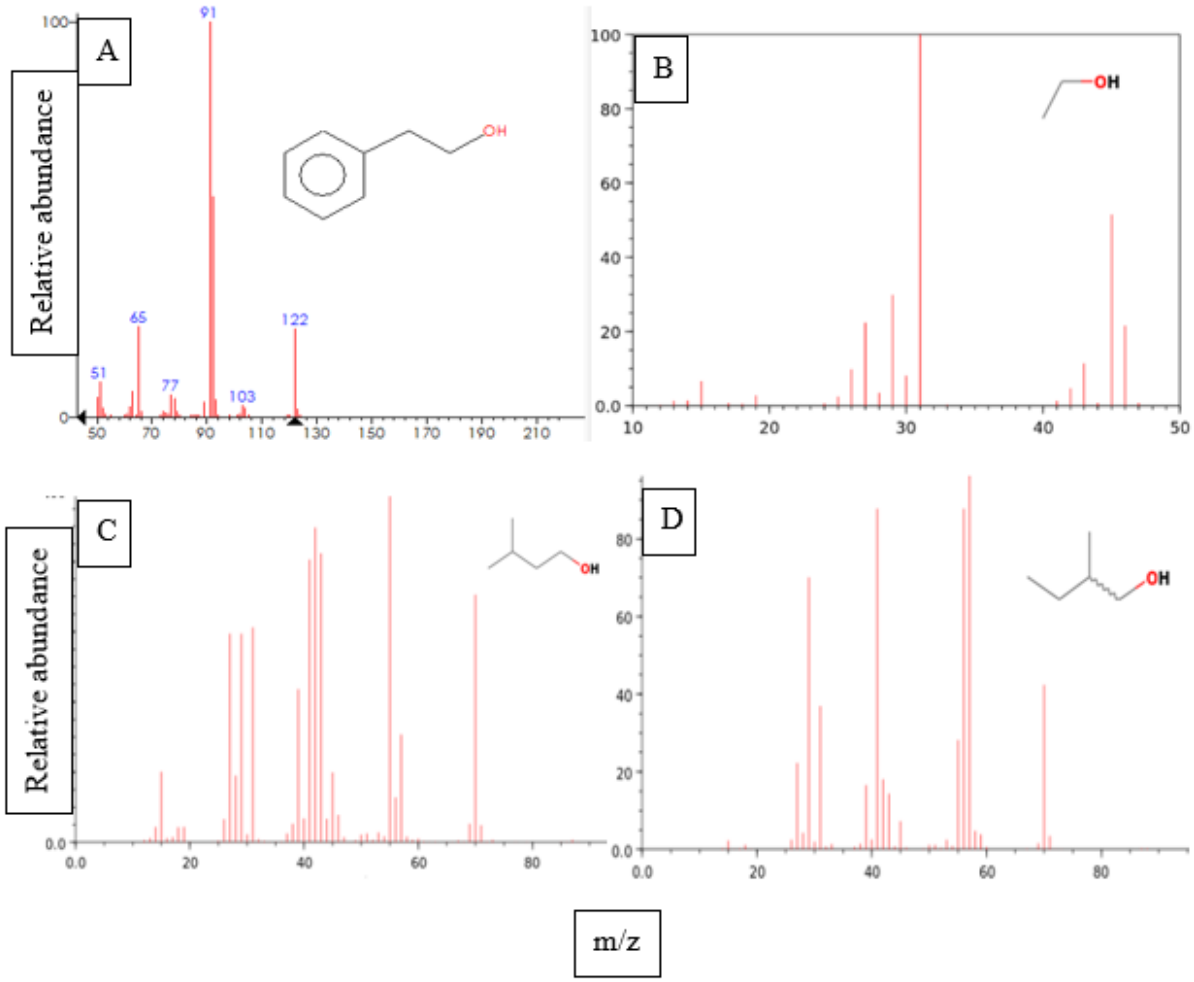

Figure 3.2. Spectral diagrams of abundant monoterpenes phenylethyl alcohol (A), ethyl alcohol (B), isobutanol (C) and 2-methyl-1-butanol (D).

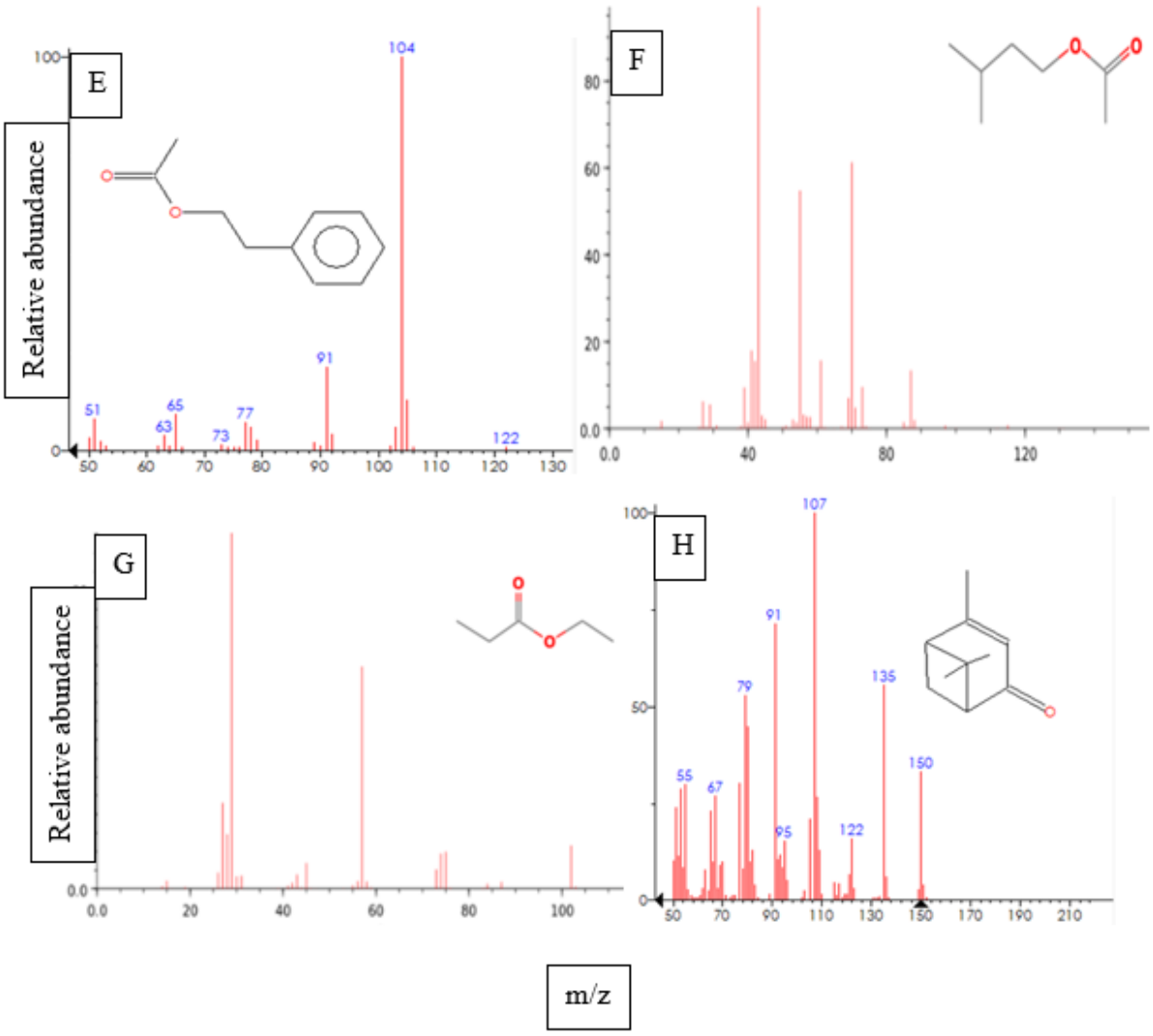

Figure 3.3. Spectral diagrams of abundant phenylethyl acetate (E), isoamyl acetate (F), ethyl acetate (G) and verbenone (H).

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

3.4. 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.

3.5. 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.