# Host Plant Chemistry Affects Fungal Interactions, Which Influence the Production of Volatile Fungal Metabolites

By

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**Abstract**

Mountain pine beetle (MPB) has recently expanded its host range to novel jack pine forests in Alberta. Invasion success of MPB may depend on the outcome of interactions between its primary symbiotic fungus *Grosmannia clavigera* with other organisms sharing the same host. Among resources-sharing organisms, the pine engraver beetle and its vectored fungus *Ophiostoma ips* are likely to interact with *G. clavigera* as the pine engraver beetle and its vectored fungus are one of the major disturbance agents on jack pine. On MPB historical host lodgepole pine, beetle brood production significantly decreased in response to the presence of the pine engraver beetles, likely as a result of competition between two fungal species. We hypothesized that the outcome of these interactions between two fungal species will likely affect MPB development in jack pine trees. However, lodgepole pine and jack pine differ in both primary and secondary phytochemistry. Whether such variation could affect the fungal growth as well as the fungal interaction is unknown. Besides phytochemistry, volatile organic compounds (VOCs) produced by beetle symbiotic fungi can also affect fungal growth and reproduction, and function as infochemicals to mediate fungal interaction. However, not much research has focused on the VOC of phytopathogenic fungi, especially how the presence of one fungal species could affect the VOC profile of a given fungal species.

We investigated whether: 1) different concentrations of host nitrogen and monoterpenes affect the growth of and interaction between the beetle symbiotic fungi; 2) the presence of a resource-sharing fungus qualitatively and quantitively affect the VOCs production of a given fungal species. We found that jack pine phloem nitrogen levels did not alter the growth of either fungal species as well as their interactions. In the absence of monoterpenes, *O. ips* had a positive impact on the growth of *G. clavigera* suggesting commensalism. However, the presence of

monoterpenes either promoted or inhibited the growth of both fungi, and furthermore altered the outcome of species interactions from commensalism to amensalism. These finding show that MPB could still obtain the benefit from its symbiotic fungus on jack pine as fungi can establish on jack pine. However, the benefits could be suppressed on jack pine which is also attacked by the pine engraver beetle and *O. ips*. As for the VOC production, two fungal species share similar VOCs profile revealing their similar ecological function as phytopathogenic fungi. However, each fungus produced qualitatively and quantitively less VOCs in response to the presence of another fungus. The reduction emission of FVOC shows that phytopathogenic fungi could interact at distance by sensing VOC produced by another fungus.

Overall, our study is the first to demonstrate how host phytochemistry shifts fungal interaction from facilitation to no-effect. Another interesting finding is how the presence of potential competitor fungus qualitatively and quantitively affect the VOC production of a given fungal species.

## Preface

This document presents two studies intended for publication and represent collaborative work led by Fuai Wang, Dr. Nadir Erbilgin and Dr. Jonathan A. Cale of the University of Alberta. The first chapter has been submitted to *Fungal Ecology*, whereas the second chapter will be submitted soon. This work investigates the adaptability of mountain pine beetle symbiotic fungus on a novel host jack pine as well as how a resource-sharing fungus could potentially affect the fungus in both growth and metabolite production, which will in turn affect MPB development and success in boreal forests.

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# Chapter One

## Thesis Introduction

Mountain pine beetle (*Dendroctonus ponderosae* Hopkins; MPB) is one of the most destructive bark beetles in western North America (Safranyik et al. 2010). During outbreaks, they have killed millions of hectares of pine (*Pinus* spp.) forest that have resulted in cascading ecological impacts. The beetle's success is facilitated by mass attack and tree infections by blue stain ophiostomatoid fungi (Ophiostomataceae and Ceratocistidaceae) (Raffa et al. 2005, 2008; Whitney 1982). Mass attacks are initiated by pioneer female beetles by releasing aggregation pheromone that attracts more beetles to the same host tree (Borden 1982). Blue stain fungi are carried by beetles to the new host tree in a special structure called mycangia. In return, fungi provide benefits to MPB in different ways. These benefits include: 1) providing essential nutrients that are necessary for beetle larval development (Adams & Six 2007; Ayres et al. 2000; Bleiker & Six 2007; Six & Klepzig 2004); 2) overcoming tree defenses by detoxifying tree defensive compounds that adversely affect beetles (Cale et al. 2017; DiGuistini et al. 2007; Raffa et al. 2005); 3) protecting the beetle against their antagonistic microorganisms (Therrien et al. 2015). In general, MPB's development is largely dependent on its symbiotic fungi, such as its primary fungus *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wing, 2006. Due to warmer temperatures and drought conditions, MPB and its symbiotic fungi have expanded into jack pine (*Pinus banksiana* Lambert) forests (Cullingham et al. 2011; Erbilgin et al. 2014). The developing establishment of these native invasive species in jack pine forests provided us an opportunity to study blue stain fungi dynamics in a novel host species (Rice et al. 2007).

Jack pine is one of the most abundant and important pulpwood species in the Canadian boreal forest, with a geographical range extending from eastern Alberta to the Atlantic Provinces in Maritime Canada. The historical host of MPB, lodgepole pine (*P. contorta* Douglas var. *latifolia* Engelmann) and the novel host jack pine differ in both primary and secondary phytochemicals (Erbilgin 2018). Such variation could potentially terminate the beetle range shift, as the beetle and its symbiotic fungi may not sufficiently adapt to the jack pine host environment. Among secondary phytochemicals, monoterpenes are essential chemical defenses in pine trees that can exceed the biological tolerance of both beetles and fungi after attack (Cale et al. 2017; Raffa et al. 2005; Raffa & Smalley 1995). Besides the variation in host phytochemistry, competition with other organisms can also impact MPB's symbiotic fungi, which could in turn affect beetle development and success. Among organisms associated with jack pine, the pine engraver beetle (*Ips pini* Say) and its primary symbiotic fungus *Ophiostoma ips* (Rumbold), 1931 are likely to experience novel interactions with invading MPB and *G. clavigera* (Kegley et al. 1997; Kopper et al. 2004, 2005; Schenk & Benjamin 1969). In lodgepole pine, MPB and the pine engraver beetle co-exist and compete with each other, therefore we suspect the negative impact of one bark beetle species on another species is will also occur in the jack pine trees. However, the outcome of interactions between *G. clavigera* and *O. ips* in the novel host jack pine is largely unknown. Understanding the outcome of these interactions can be important in predicting MPB development and invasion success in jack pine forests.

Inter-specific competition between fungi can occur when one fungal species negatively affects another species by consuming a common limited resource to the detriment of the other species, therefore inter-fungal competition for nutrients is usually driven by the competition for space (Boddy 2000; Young et al. 1995). However, besides water soluble metabolites, fungi could

communicate and interact at distance by sensing the volatile compounds produced by the same or different species of fungi (Macias-Rubalcava et al. 2012). Fungal volatile organic compounds (FVOCs) can function as infochemicals and mediate interactions among different organisms in many ecosystems. For example, FVOCs can defend territory by inhibiting the growth of fungi sharing the same niche (Lee et al. 2009; Mitchell et al. 2010; Wheatley et al. 1997; Zhang et al. 2010). As a consequence, FVOCs can be important factors mediating inter-fungal interactions. However, not much research has focused on the VOCs of phytopathogenic fungi, especially how the presence of other species could affect the VOC profile of a given fungal species.

In this thesis, I investigated the adaptability of *G. clavigera* in their novel host jack pine by testing, in Chapter 2, the effects of jack pine nutrients and secondary phytochemicals as well as the presence of another fungus on fungal growth and, in Chapter 3, the FVOC profiles of *G. clavigera* and *O. ips* and how the presence of another fungus quantitively and qualitatively affects FVOC profiles.

# Chapter Two

# Induced defenses of a host tree similarly affect novel and native beetle-vectored fungi and mediate their interactions

## 2.1 Introduction

Fungi are important contributors to ecosystem functions including nutrient cycling, decomposition, and symbiosis (Ingham et al. 1985; Read & Perez-Moreno, 2003; van der Heijden et al. 1998). These contributions can occur through various interactions between a fungal species and other organisms, including insects, plants, and different species of fungi. The outcomes of these complex inter-specific interactions can be mutualistic, facilitative, or competitive, and potentially shape the entire fungal community dynamics (Rayner & Boddy 1988). For example, competition between two fungal species for a resource (i.e., when sharing the same host plant substrate) results in the growth reduction of at least one of the species depending on the ability of a particular fungal species to capture and defend the resource (Boddy 2000; Young et al. 1995). Furthermore, the outcome of such interactions can also depend on the quality of resource consumed (Bleiker & Six 2009; Klepzig et al. 2004). These inter-specific interactions could be especially important to the host range expansion of invading species that share an ecological niche with species native to the invaded range. However, how the quality of shared resources can affect interactions between novel and native fungal species are poorly understood.

Ophiostomatoid fungi (Ascomycota: Ophiostomataceae and Ceratocystidaceae) are commonly associated with conifer-infesting bark beetles (Coleoptera: Curculionidae, Scolytinae) (Paine et al. 1997). These fungi infect and develop in the vascular tissues of host trees and thus their growth is strongly influenced by host quality, which is characterized by the composition of

nutrients and secondary compounds (Bleiker & Six 2007; Boone et al. 20011; Keeling & Bohlmann 2006; Paine et al. 1997; Raffa et al. 2005,2008). Among the nutrients available in host tissues, nitrogen is especially important as it generally occurs at low levels in plants and can have a strong influence on fungal growth (Adam & Six 2007; Ayres et al. 20000; Young et al. 1995). Likewise, host secondary compounds, such as monoterpenes, can also influence fungal growth and reproduction depending on their concentrations (Cale et al. 2017; Raffa et al. 2005; Raffa & Smalley 1995). While different species of ophiostomatoid fungi frequently co-occur in host trees, little is known about how host quality, especially various concentrations of nitrogen and monoterpenes, affect the outcome of their interactions.

Bark beetles feed on nutritionally-poor host substrates such as the vascular tissues of woody plants (also called phloem) (Ayres et al 2000; Klepzig & Six 2003). While most bark beetle species colonize weak or recently killed host trees (Raffa & Berryman 1983; Rudinsky 1962), only a few species can attack and complete their development in living hosts. The ability of beetles to kill and utilize the phloem of living trees is facilitated by mass attacks and infections by their mutualistic ophiostomatoid fungi that are inoculated into the trees during host aggregation (Raffa et al. 2005, 2008; Whitney 1982). These fungi benefit their associated beetles in a variety of ways (Paine et al. 1997; Six & Wingfield 2011), including overwhelming tree defenses by detoxifying and metabolizing host defense chemicals such as monoterpenes (Cale et al. 2017; DiGuistini et al. 2007; Raffa et al. 2005) and improving host quality by concentrating nitrogen on or near beetle galleries, which can be critical for larval development (Adams & Six 2007; Ayres et al. 2000; Bleiker & Six 2007; Six & Klepzig 2004). Similarly, fungal hyphae are a rich source of ergosterol, an essential dietary lipid that beetles require to produce hormones and viable eggs (Bentz & Six 2006; Clayton 1964; Norris et al. 1969). Finally, symbiotic fungi

provide protection for bark beetles by competing and deterring bark beetle antagonistic microorganisms (Therrien et al. 2015). Thus, by association with symbiotic fungi, beetles are more likely to successfully complete their development and be more robust by gaining more nutrients and requiring less phloem tissue for development (Bleiker & Six 2007; Goodsman et al. 2012; Ishangulyyeva et al. 2016; Myrholm & Langor 2015; Six & Paine 1998; Therrien et al. 2015).

Mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) is one of the most destructive pests of mature pine forests in western North America (Bentz et al. 2010; Safranyik et al. 2010). During the recent outbreak, MPB has killed millions of hectares of mostly lodgepole pine (*Pinus contorta* Douglas var. *latifolia* Engelmann) forests. More recently, warmer temperatures have allowed MPB to expand their geographical range eastward from lodgepole pine- to jack pine (*Pinus banksiana* Lambert)-dominated forests (Cullingham et al. 2011; Erbilgin et al. 2014; Lusebrink et al. 2013). Apparently, phytochemicals played critical role in such range expansion (Erbilgin 2018). The ability of MPB to successfully colonize trees is largely dependent on symbiotic relationships with several ophiostomatoid fungal species, of which including *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wing, 2006 the most frequent associate (Lee et al. 2005, 2006; Roe et al. 2011; Six 2003; Whitney and Farris 1970). Vectored by MPB, *G. clavigera* has expanded into the novel host jack pine and will likely experience novel interactions with other ophiostomatoid fungi that share the same niche under bark. The pine engraver beetle (*Ips pini* Say) is one the most widely distributed bark beetle species in North America and a major beetle species attacking jack pine (Erbilgin et al. 2002; Erbilgin & Raffa 2002; Kegley et al. 1997). Successful colonization of jack pine by *I.*

*pini* is in part due to the beetle's primary symbiotic ophiostomatoid fungus *Ophiostoma ips* (Rumbold), 1931 (Kegley et al. 1997; Kopper et al. 2004, 2005; Schenk & Benjamin 1969).

Since MPB and *G. clavigera* share the same niche in tree vascular tissue as *I. pini* and *O.ips* the fungi and their beetles are likely to interact in jack pine (Colgan & Erbilgin 2010), as such interactions commonly occur in lodgepole pine trees (Rankin & Borden 1991). In lodgepole pine, interactions between MPB and *I. pini* often result in a reduced number of emerging broods of both species (Rankin & Borden 1991). While interactions between *G. clavigera* and *O. ips* could have cascading effects on the development and fitness of their associated beetles, such interactions have not been investigated. Furthermore, because jack and lodgepole pines have quantitatively different profiles of monoterpenes in their phloem (Erbilgin 2018), jack pine-mediated interactions between *G. clavigera* and *O. ips* are expected (Colgan & Erbilgin 2010; Cale et al. 2017).

We conducted an *in vitro* experiment to determine how different concentrations of nutrients and secondary chemicals of jack pine affect the growth of and interactions between *G. clavigera* and *O. ips*. Considering the importance of induced host monoterpenes in MPB biology (Erbilgin 2018), we focused on this group of defense compounds in our experiments. Jack pine induced (post-attack) defenses substantially differ in terms of composition and concentrations of individual compounds from those in constitutive (pre-attack) defenses (Cale et al. 2017; Erbilgin 2018; Erbilgin et al. 2017; Klutsch et al 2016, 2017, 2018; Lusebrink et al. 2016). Fungi were grown alone or in combination on artificial media amended with different concentrations of nitrogen or monoterpenes (α-pinene, limonene, myrcene) to address the following three research questions: (1) Does the presence of *O. ips* affect the growth of *G. clavigera*? (2) Does the

variation of nitrogen in jack pine phloem affect interactions between the two fungi? (3) Do the monoterpenes of jack pine phloem affect interactions between the two fungi?

## 2.2 Methodology

### 2.2.1 Fungal cultures

Two isolates of *G. clavigera* (NOF2894 and NOF2969) and two isolates of *O. ips* (NOF1205 and NOF1284) were used in the experiments described below. Two isolates of *G. clavigera* were collected from different locations in Alberta: One isolate was isolated from mountain pine beetle infested lodgepole pine phloem at Banff; the other isolate was collected from mountain pine beetle gallery on infested-lodgepole pine × jack pine hybrid at Grande Prairie. Two isolates of *O.ips* were collected from different locations in British Columbia, Canada: NOF 1205 was collected from mountain pine beetle larvae on infested lodgepole pine; NOF 1284 was collected from mycangia of adult mountain pine beetle on infested lodgepole pine. These isolates were originally provided by the Northern Forestry Center in Edmonton.

All isolates were identified using fungal morphology and confirmed using DNA barcoding. Cultures used for barcoding were prepared on sterilized cellophane overlaid on malt extract agar (MEA, 20 g mart extract, 15 g agar, 1 L distilled water) and grown for 10-days at 22 ºC in darkness. After this time, approximately 0.2 g (fresh weight) of mycelium of each culture was scrapped from the cellophane and freeze-dried for two days. Fungal DNA was extracted from the dried mycelium using a CTAB (cetyl trimethyl ammonium bromide) extraction method. The genomic region spanning from the internal transcribed spacer region 3 (ITS3) to the large subunit of the ribosomal DNA was sequenced for all isolates using the forward primer ITS3 and reverse primer TW13 (White et al 1990; Brunslab 2018). Reaction mixture contained 10-20 µg

of template DNA, 2.5 µL 10x Standard *Taq* Reaction Buffer, 0.5µl deoxynucleotide triphosphates, 1 µL each of 10 µM primer and 0.125 µL *Taq* DNA polymerase. Amplification were performed using the following PCR conditions: 94 ºC for 2 min, 30 cycles at 94 ºC for 30 s, 45.5 ºC for 40 s, 72 ºC for 90 s, and finally 72 ºC for 10 min. Amplicons were electrophoresed in 2% agarose gels with 0.5x TBE (Tris/Borate/EDTA) buffer and stained with GelRed and bands were visualized using UV light. PCR products were treated with ExoSAP and Sanger sequenced. Extracted DNA was quantified using a spectrophotometer prior to sequencing. The fungal sequences were matched to those in GeneBank (National Library of Medicine) using a BLAST search. All amplification, quantification, and sequencing were performed using equipment provided by the Molecular Biology Services Unit at the University of Alberta.

2.2.2 Nitrogen-amendment treatments

To evaluate the interactions between *G. clavigera* and *O. ips* in media amended with varying concentrations of nitrogen in the laboratory, we first collected phloem samples (each 3x3 cm) from both north and south sides of 120 mature (> 28 cm dia. at breast height) jack pine trees at 1.3 m above the ground in eight different sites in Lac la Biche, Alberta, to determine the natural variation in nitrogen. Tissues were stored on dry ice in the field, transported to the laboratory, ground in liquid nitrogen, and stored at -40 ºC. These samples were then oven dried at 40 °C for 24 h before nitrogen analysis. Total nitrogen in phloem was analyzed in a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany) at the University of Alberta. Samples from the north and south sides were pooled to get more accurate natural variation of phloem nitrogen for each tree (Hussain, unpublished data).

Fungal growth media was amended with three nitrogen concentrations that reflected the natural variations in mature jack pine trees in Alberta: 3.9% (low; 2.5% percentile), 4.5%

(medium: 50% percentile), and 5.3% (high; 97.5% percentile). Nitrogen concentrations in the media were manipulated by the addition of peptone (Difco Bacto peptone) to a basal media otherwise devoid of usable nitrogen. The basal media contained 3.5% dextrose (Difco Potato Dextrose Broth), 1.4% agar (Difco Bacto Agar), 1.75% YNB (Sigma Yeast nitrogen base without amino acids and ammonium), and 1 L distilled water. The YNB and nitrogen portion was filter-sterilized and added into an autoclaved mixture of the dextrose and agar components prior to pouring media plates.

The master cultures of the *G. clavigera* and *O. ips* isolates were sub-cultured onto the amended media in a full factorial experiment, involving the three nitrogen-amendment treatments and five fungal treatments for a total of 15 treatments. The fungal treatments consisted of *G. clavigera* growing alone (Gc Control), *O. ips* growing alone (Oi Control), G. *clavigera* growing along with a *O. ips* culture at the same age (Gc-Oi Concurrent), *G. clavigera* growing on media where *O. ips* has already established (Oi Priority), and *O. ips* growing on media where *G. clavigera* has already established (Gc Priority). For the Gc and Oi Control treatments, a culture plug (4mm diam.) from a master culture was placed culture-side down onto the edge of a plate (100 mm in diam) containing the amended media. For the Gc-Oi Concurrent treatments, one plug from each of the master cultures of *G. clavigera* and *O. ips* was concurrently placed at opposite edge of a plate (100 mm in diam). For the Gc Priority treatment, plates were initially inoculated with a plug from a master culture of G. *clavigera* and then inoculated with *O. ips* at the edges when the plate was half covered with the initial *G. clavigera* culture. This method of staggered inoculations was similarly applied to the Oi Priority treatment except *G. clavigera* was inoculated after the plate was half covered by a *O. ips* culture. All culture plates were grown in darkness at 22 ºC for four days, after which time culture area was

measured by image analysis using ImageJ (National Institutes of Health, Bethesda, MD, USA; Abramoff 2007). The area of cultures in each treatment was measured in this manner except for the Gc Priority and Oi Priority treatments for which only the younger cultures (i.e., later inoculated fungus) were measured. Each of the fifteen treatments (3 nitrogen concentrations x 5 types of fungal inoculations) was replicated 10 times. Different isolates of *G. clavigera* and *O. ips* were randomly assigned to replicates of a given treatment such that each treatment consisted of an even number of replicates of each isolate.

2.2.3 Monoterpene-amendment treatments

To evaluate the interactions between *G. clavigera* and *O. ips* in media amended with varying concentrations of major monoterpenes of jack pine (α-pinene, myrcene, and limonene) in the laboratory, we selected low and high concentrations reflecting the natural variation of these monoterpenes in induced jack pine phloem (Cale et al. 2017) (concentrations were listed in Table 2.1). Separate media amendments were made using the low and high concentrations of each monoterpene. This resulted in a total of six amendment treatments (two concentrations for each of three monoterpenes). For the limonene and α-pinene amendments, concentrations represented the total concentration of both positive and negative enantiomers in a racemic mixture. Each amendment treatment was prepared by adding an amount of monoterpene (analytical standard at >99% purity) to autoclaved malt extract agar (MEA). Non-amended MEA was used as a control treatment.

These six amendment treatments, including control, were crossed in a full factorial design with three fungal treatments: *G. clavigera* alone (Gc Control), *O. ips* alone (Oi Control), and the two fungi growing together (Gc-Oi Interaction). This cross resulted in a total of 18 treatments, which were replicated 15 times each. Each of the two isolates for a given fungus were randomly

assigned to a given treatment replicate, resulting in seven to eight splits in isolates for all treatment replicates together. Fungi were inoculated from 10-day old master cultures onto the edge of plates containing amended or non-amended media. For the Gc-Oi Interaction treatment, culture plugs of each fungus were placed at opposite edges of the plate. Fungal growth in all monoterpene amendment treatments was measured after four days as described above for the nitrogen experiment.

2.2.4 Data analysis

The area (mm2) of fungal cultures was used for statistical comparisons among treatments in each of the amendment experiments (nitrogen and monoterpenes). Two-way ANOVA was used to test the statistical significance of the main effects of fungal and nitrogen treatments, as well as their interaction, on fungal growth. Differences in the growth of *G. clavigera* and *O. ips* alone and together were tested using two-sample t-test. Two-way ANOVA was also used to test the main effects, and their interactions, of monoterpene and fungal treatments on fungal growth separately for each monoterpene-fungal species combination. Each of the analyses in the monoterpene experiments used the same set of controls (i.e., fungal growth on non-amended media). Significant ANOVA results were followed by multiple comparisons using Tukey Honest Significant Difference test. Statistical assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk's and Levene's tests, respectively. Data were log- or rank-transformed to satisfy assumptions, as needed. Tables and figures were constructed using non-transformed data. All data analysis was performed using the R software environment version 3.4.3 (R Core Team 2017).

## 2.3 Results

### 2.3.1 Nitrogen-amendment treatments

All treatments produced cultures with measurable growth except for the Gc Priority treatments, where *O. ips* did not have enough space to grow as *G. clavigera* culture grew up to the edge of the plate. Thus, this treatment was removed from the analysis of *O. ips* data.

Growth of *G. clavigera* and *O. ips* showed that neither species responded to variation in nitrogen concentrations or the presence of the other fungus as the statistical main and interaction effects of nitrogen and fungal treatments were not significant (Table 2.2). However, *G. clavigera* growth in the Gc-Oi Concurrent treatment was 27% and 25% larger than when it grew alone (Gc Control) or in the presence of an established *O. ips* culture (Oi Priority) respectively (Table 2.2).

### 2.3.2 Monoterpene-amendment treatments

Overall, in absence of monoterpenes, cultures of *G. clavigera* were on average 48% larger when growing in the presence of *O. ips* than when growing alone ($t(22.98) = -3.58$, $P = 0.040$; Fig. 2.1). However, the growth of *O. ips* did not vary with the presence of *G. clavigera*.

Both cultures of *G. clavigera* and *O. ips* grew on media amended with low or high concentrations of myrcene, limonene, and α-pinene. For amendments with α-pinene, the growth of *G. clavigera* was depended on the presence of *O. ips*, as indicated by a significant amendment-fungus interaction effect ($F(2,83)=50.28$, $P < 0.001$; Fig. 2.2). Overall, amendments inhibited the growth of *G. clavigera*, with growth in the low or high concentration being 145% or 87% less, respectively, than that on non-amended media regardless of the presence of *O. ips*. However, the growth of *G. clavigera* did not vary with the presence of *O. ips* in the either low or high amendment treatments. Furthermore, fungal growth was similar between low and high concentration amendments, independent of *O. ips* presence. For the *O. ips* on α-pinene-amended

media, fungal growth varied with the amendments but not with the presence of *G. clavigera*, as a significant main effect of amendment was detected ($F(2,78)=3.752$, $P < 0.001$; Fig. 2.3). α-Pinene inhibited *O. ips* growth, with growth being 67% or 47% lower in the low or high amendment, respectively, than in the non-amended control treatments. However, fungal growth was similar between the low and high α-pinene amendment treatments.

For amendments with limonene, the growth of *G. clavigera* varied with the presence of *O. ips,* as indicated by a significant amendment-fungus interaction effect ($F(2,81)=3.752$, $P = 0.028$; Fig. 2.2). Overall the high concentration of limonene stimulated the growth of *G. clavigera*; however, its growth did not vary with the presence of *O. ips* in either the low or high amendment treatments. Furthermore, a low concentration of limonene stimulated growth of *G. clavigera* only when *O. ips* was present. For *O. ips* on limonene-amended media, its growth varied only with the amendments but not with the presence of *G. clavigera*, as indicated by a significant amendment main effect ($F(2,78)=3.752$, $P < 0.001$; Fig. 2.3). *Ophiostoma ips* grew 34% or 33% larger on media amended with low or high limonene concentrations, respectively, than on non-amended media. However, the growth did not differ between the limonene treatments.

The growth of *G. clavigera* on myrcene-amended media varied with the presence of *O. ips* as indicated by a significant amendment-fungus interaction effect ($F(2,82)=6.06$, $P = 0.004$; Fig. 2.2). Overall *G. clavigera* growth was stimulated by myrcene amendments but was similar between low and high treatments relative the growth when the fungus grew alone. However, the growth of *G. clavigera* did not vary with the presence of *O. ips* in either the low or high amendment treatments. For *O. ips* on myrcene-amended media, fungal growth varied with amendments ($F(2,78)=3.89$, $P = 0.030$; Fig. 2.3), with growth on media amended with the low concentration being 36% greater than that on non-amended media.

## 2.4 Discussion

Interactions between two ophiostomatoid fungi sharing the same niche can be altered by induced monoterpenes of their host plants. This is the first study showing that induced host monoterpenes can modify the interactions between two niche-sharing fungal species associated with two competing bark beetle species in jack pine. We demonstrated that, in the absence of monoterpenes, *G. clavigera* grew more when the niche-sharing fungus *O. ips* was present; however, this *G. clavigera* growth response was consistently nullified when it grew in the presence of all three host monoterpenes (α-pinene, limonene, and myrcene) tested at different concentrations. Furthermore, the magnitude of *G. clavigera* growth varied depending on the individual monoterpene concentration. In contrast, the growth of *O. ips* was not affected by the presence of *G. clavigera* but was strongly influenced by the all three host monoterpenes. Species of ophiostomatoid fungi commonly interact with each other within the vascular tissue of bark beetle-infested trees (Solheim 1995; Klepzig & Wilkens 1997). However, the outcome of their interactions and the ability of the fungi to capture resources can be mediated by abiotic factors simulating host tree conditions (Bleiker & Six 2009; Klepzig et al. 2004). For example, *O. minus* (Hedgcock) Sydow & P. Sydow, 1919 and *Ceratocystiopsis ranaculosus* T.J. Perry & J.R. Bridges, 1987 fungi associated with the southern pine beetle (*D. frontalis* Zimmermann), capture similar amounts of primary resouses (uncolonized media) when growing on media with low water potentials (-10 and -20 MPa) used as a proxy for different vascular moisture conditions, but the former fungus captured significantly more resources than the latter on media with higher potentials (0 and -5 MPa). Our findings indicate that induced levels of certain individual monoterpenes can also influence the outcome of interactions between two ophiostomatoid fungi,

and that such compounds can reduce the primary resource-capture abilities of certain species (e.g., *G. clavigera*) but not others (e.g., *O. ips*). Such reductions likely influence the amount of nutrients the fungi can concentrate in their hyphae and, in turn, is available for feeding beetle larvae. Limitations in hyphal nutrient concentrations could adversely affect beetle development as well as brood success and size.

Induced levels of certain monoterpenes from jack pine can differentially affect the growth of fungi associated with bark beetles. While the growth of both *G. clavigera* and *O. ips* was promoted by both concentrations of limonene and myrcene, fungal growth was inhibited by both concentrations of α-pinene. Monoterpenes are a major component of defense-related oleoresin in conifer trees and can affect bark beetles and their associated phytopathogenic fungi (Boone et al 2011; Erbilgin 2018; Paine & Hanlon 1994; Raffa et al. 2005, 2008; Keeling & Bohlmann 2006). Work by Chiu et al. (2017) assessed and compared the toxicity of individual monoterpenes, including those tested in the current study, to adult MPB. They found that both enantiomers of limonene were the most toxic to the beetle while both (–)- and (+)-α-pinene were the least toxic. Similar results were also reported by Reid et al. (2017). However, these compounds do not seem to have the same general toxicity to MPB-associated fungi. Cale et al. (2017) tested three fungal associates of MPB and showed that the growth of *G. clavigera* were stimulated by induced levels of limonene but inhibited by α-pinene and myrcene, whereas the growth of *O. montium* (Rumbold), 1952 and *Leptographium longiclavatum* (S.W. Lee, J.J. Kim & C. Breuil, 2005) were stimulated by α-pinene and limonene but tended to be inhibited by myrcene. Except in response to myrcene, our results for *G. clavigera* support those of Cale et al. (2017). The stimulatory responses observed for *G. clavigera* are likely attributable to the species' ability to detoxify and metabolize certain monoterpenes (Wang et al 2013, 2014) and use the resulting

carbon binds as a source of resource (Cale et al. 2017). *Ophiostoma ips* is likely capable of detoxifying and metabolizing monoterpenes as this fungus and *G. clavigera* were stimulated by the same monoterpenes; however, this needs to be verified. Thus, individual host monoterpenes can affect the primary resource capture abilities of these fungi. In nature, variation in the amounts of individual monoterpenes in tree vascular tissues could potentially affect the overall effects of induced tree secondary metabolites on fungal growth and resource acquisition, which could in turn affect the growth and development of feeding beetles.

In contrast to the effects of host monoterpenes, the growth and interactions of *G. clavigera* and *O. ips* were not influenced by the availability of nitrogen in jack pine phloem even though *G. clavigera* growth tended to be highest on media with the highest nitrogen amendment regardless of whether *O. ips* was present. Nitrogen is a key component of fungal diets, with ample availability of nitrogen promoting healthy mycelial development (Ayres et al. 2000; Bleiker & Six 2007). Thus, the availability of nitrogen in jack pine may not affect the growth and development of *G. clavigera* in this novel host. This will likely support MPB development in jack pine. While dietary nitrogen is essential for development, pine phloem is a relative low-quality food for phloem-feeding insects (Mattson 1980). Small increases of nitrogen in the host substrate can have proportionally larger effects on beetle performance (Ayres et al. 2000; Mattson 1980; Paine et al. 1997). The fungal symbionts of bark beetles promote beetle development by concentrating phloem nitrogen in their hyphae, which can then be fed on by developing beetles (Ayres et al. 2000; Bleiker & Six 2006). Taken together, our results suggest nitrogen levels in jack pine may not limit the nutritional support of *G. clavigera* to MPB development in jack pine.

In conclusion, since the invasion of jack pine forests by MPB began, studies have mainly focused on the quality of jack pine as a host for MPB and its fungal symbionts. How organisms that commonly occur in jack pine (e.g., *O. ips*) could affect the invasion is poorly understood (Colgan and Erbilgin 2011; Klutsch et al. 2016, 2018). The results of the current study showed that interactions between two co-occurring ophiostomatoid fungal species can be mediated by host defense compounds. While some host monoterpenes such as limonene promote the growth of *G. clavigera*, other compounds reduce the growth. Interestingly, *O. ips*, which is associated with a bark beetle species co-evolved with jack pine, responded to the host monoterpenes in a manner similar to that of *G. clavigera*. Thus, there appears to be a consistency in the responses of at least some ophiostomatoid fungi associated with different bark beetles and host trees to monoterpenes (Adhikari et al. 2013; Jankowiak and Bilański, 2013; Raffa et al. 2017). Future studies should investigate how the outcomes of *G. clavigera*-*O. ips* interactions could affect interactions between MPB and pine engraver beetle.

## Figures

Figure 2.1 Mean (±SE) growth (culture area; $mm^2$) of *Grosmannia clavigera* and *Ophiostoma ips* in response to the presence of another fungal species on MEA media. Bars with different letter were statistically different as indicated by two sample t test.

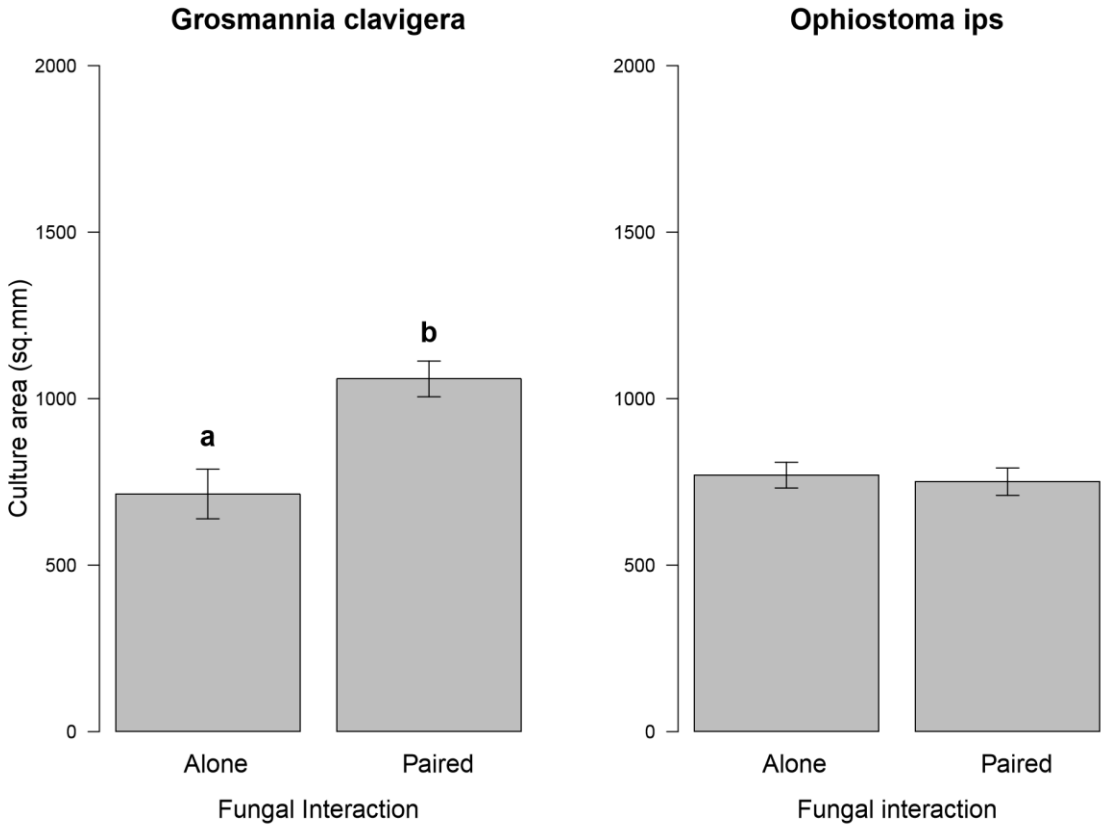

Figure 2.2 Interaction plot of the mean (±SE) growth (culture area; $mm^2$) of *Grosmannia clavigera* in response to the media amendments simulating different concentrations of jack pine (*Pinus banksiana*) induced monoterpenes as well as the presence of the other fungal species.

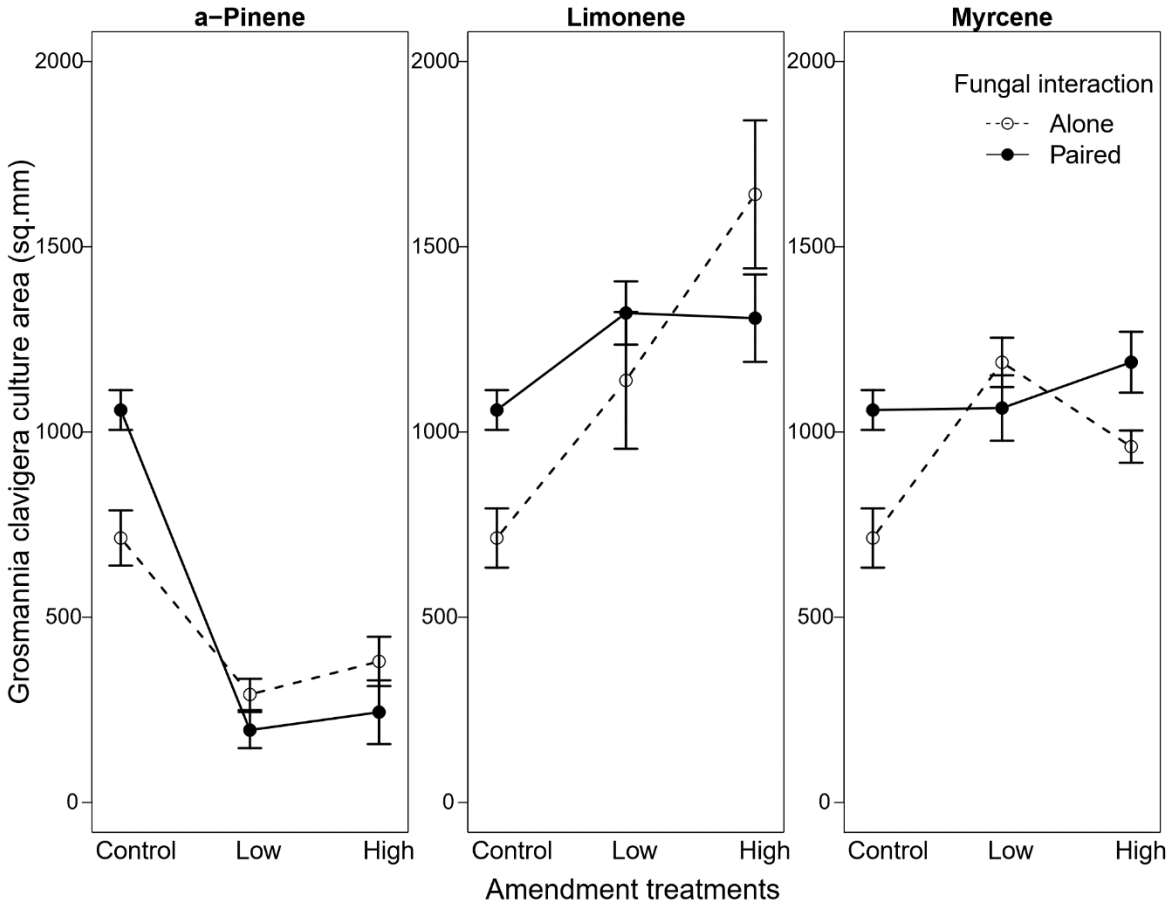

Figure 2.3 Mean (±SE) growth (culture area; $mm^2$) of *Ophiostoma ips* in response to the media amendments simulating different concentrations of jack pine (*Pinus banksiana*) induced monoterpenes. Bars with different letters were statistically different as indicated by Tukey's Honest Significant Different test.

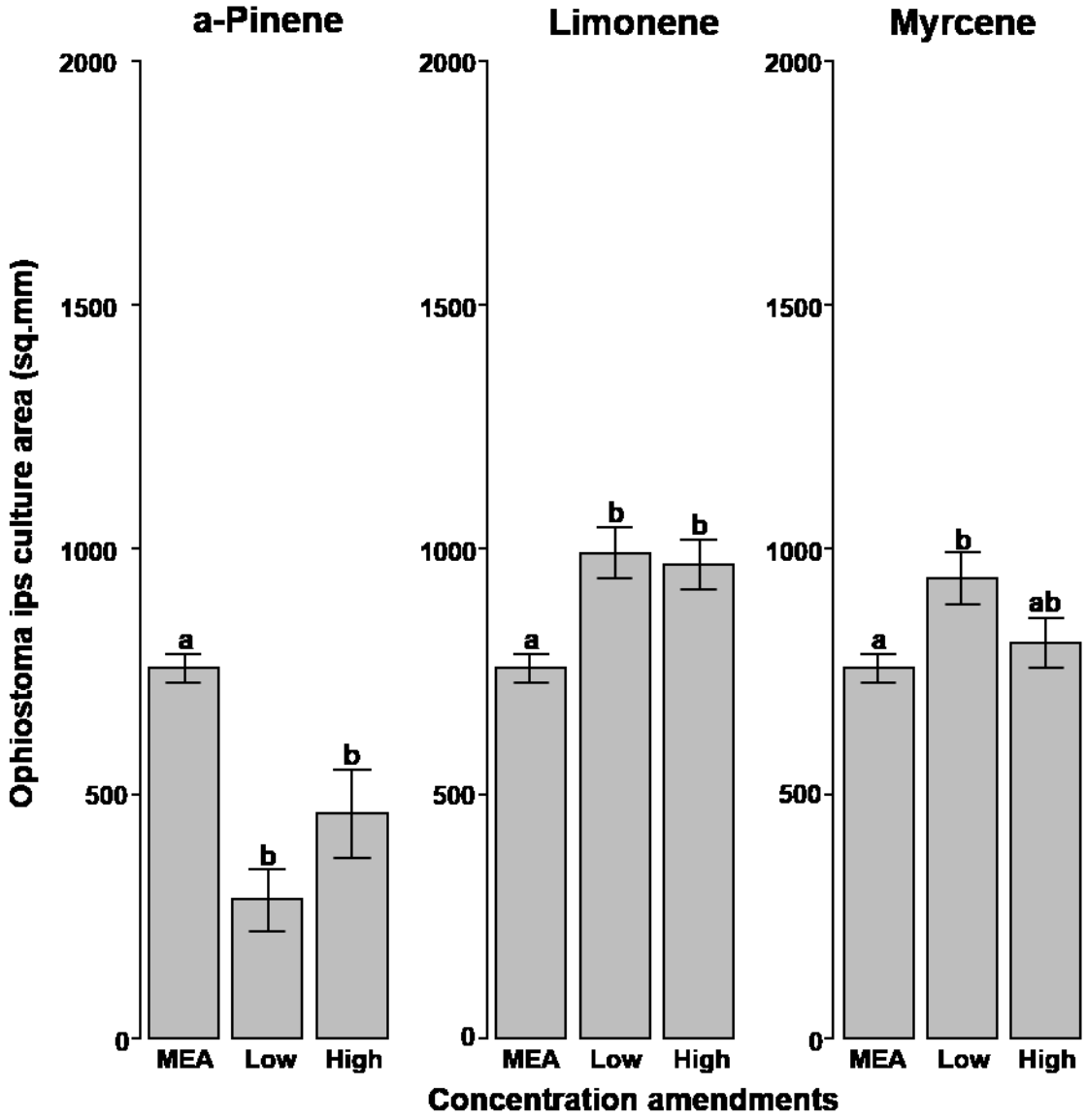

Table 2.1 Monoterpene concentrations used to amend fungal growth media. Amendments with limonene and α-pinene were prepared by using a racemic mixture of each compounds present in the jack pine (*Pinus banksiana*) phloem. Control media was non-amended MEA media. Amendments were based on concentrations reported by Cale et al. (2017)

| Monoterpene | Control | Concentration (μg/mg FW phloem) | |
|---|---|---|---|
| | | Low | High |
| α-Pinene | 0.00 | 9.28 | 21.39 |
| Limonene | 0.00 | 0.09 | 0.36 |
| Myrcene | 0.00 | 0.07 | 0.26 |

Table 2.2. Mean (±SE) growth (as culture area; $mm^2$) of *Grosmannia clavigera* and *Ophiostoma ips* in response to media amendments simulating different levels of jack pine (*Pinus banksiana*) phloem nitrogen as well as the presence of the other fungal species.

| Nitrogen level (% fresh weight) | Culture area ($mm^2$ ±SE) | | | | |
|---|---|---|---|---|---|
| | *Grosmannia clavigera (Gc)* | | | *Ophiostoma ips (Oi)* | |
| | *G. clavigera* alone (Gc-Control) | Concurrent (Gc-Oi concurrent) | Staggered (Oi Priority) | *O. ips* alone (Oi Control) | Concurrent (Gc-Oi concurrent) |
| Low (3.9%) | 1421.4(±125.2) | 1629.8(±247.6) | 1316.9(±179.2) | 790.9(±156.8) | 754.7(±157.7) |
| Medium (4.5%) | 1359.2(±126.0) | 1745.5(±381.6) | 1391.1(±224.9) | 665.1(±95.9) | 564.3(±154.3) |
| High (5.3%) | 1444.1(±105.2) | 2011.2(±404.9) | 1575.1(±258.3) | 685.4(±103.4) | 927.7(±288.8) |

# Chapter Three

# Interaction between two fungal species can affect the production of volatile fungal metabolites

## 3.1 Introduction

Fungal volatile organic compounds (FVOCs) are low molecular weight carbon-based compounds formed in the metabolism of fungi that are able to enter the gas phase by vaporizing at normal atmospheric pressure and temperature (Herrmann 2010). During the metabolism process, fungi produce a mixture of various molecular carbons which vary in types and sizes (Korpi et al. 2009). Fungal VOCs comprise many different chemical classes, primarily alcohols, aldehydes, ketones, amines, benzene derivatives, furanes, hydrocarbons, and terpenes (Ortiz-Castro et al. 2009). So far, more than 300 fungal VOCs have been identified (Morth et al. 2012; Piechulla & Degenhart 2014). Because of their unique properties, FVOCs can be important factors in interspecific communications. In particular, they are critical in functioning as infochemicals in interactions between physically-separated organisms. Fungal VOC profiles may vary depending on species (Corcuff et al. 2010). Consequently, FVOCs emitted by one species may have different impact on organisms occupying the same ecological niche. Fungal VOCs can regulate antagonistic interactions with other microorganisms (Macias-Rubalcava et al. 2012). For example, fungal VOCs could defend territory by inhibiting the growth and reproduction of other species fungi or even bacteria (Lee et al. 2009; Mitchell et al. 2010; Wheatley et al. 1997; Zhang et al. 2010). On the other hand, FVOCs can have positive impacts on organisms occupying the same niche (Briard et al. 2016; Schmidt et al. 2015). For example, FVOCs emitted by phytopathogenic fungi can be used as carbon resources to support fungal growth on carbon resource-limited substrate (Cale et al. 2016). Currently, FVOCs functioning as infochemicals in ecological interactions has

been investigated in many systems (Biere et al. 2013; Schmidt et al. 2015; Schulz-Bohm et al. 2017). However, how fungal interactions adversely affect the FVOC production is largely unknown. Understanding such effects could be important to clarifying potential VOC-mediated feedback between co-occurring fungi and fungi, and vectoring animals.

Conifers are often infected with a range of blue-stain ophiostomatoid fungi (Ascomycota: Ophiostomataceae) associated with and vectored by bark beetles (Coleoptera: Curculionidae, Scolytinae) (Paine et al. 1997). Among ophiostomatoid fungi associated with beetles, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wing, 2006 is a widely spread fungus mostly associated with the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Lee et al. 2006; Roe et al. 2011; Whitney and Farris 1970), whereas *Ophiostoma ips* (Rumbold) is the fungus primarily in association with *Ips pini* (Say) (Kegley & Wilkens 1997; Kopper et al. 2004; Schenk & Benjamin 1969). These fungi form mutualisms with their host beetles. The beetle carries the fungus to a host plant. In return, fungi enhance beetle success by providing critical nutrients for developing beetles, exhausting tree defense by detoxifying defensive chemicals, and providing protection for a beetle against antagonistic organisms (Ayres et al. 2000; Bleiker & Six 2007; Cale et al. 2017; DiGuistini et al. 2007; Raffa et al. 2005; Therrien et al. 2015). Overall, beetle success is largely dependent on its vectored fungi.

In the past 20 years, mountain pine beetle has killed millions of lodgepole pine (*Pinus contorta* Douglas var. *latifolia* Engelmann) trees during periodic outbreaks in western Canada. Due to warmer temperatures and drought conditions, mountain pine beetle and *G. clavigera* have expanded their host and geographical ranges from the historical host lodgepole pine east into jack pine (*Pinus banksiana* Lambert) forests (Safranyik et al. 2010; Cullingham et al. 2011;

Erbilgin et al. 2014; Erbilgin 2018). *Grosmannia clavigera* is likely to experience novel interactions with other ophiostomatoid fungi associated bark beetle species commonly affecting jack pine, such as *O. ips* associated with *I. pini* (Kopper et al. 2004). While Wang et al. (submitted) reported that *O. ips* positively affects the growth of *G. clavigera* on media representing jack pine phloem chemistry, how the interaction between these species could affect FVOC production is unknown. Knowing the outcome of this question is important in understanding the development of fungi in the novel host jack pine, and thus understanding the success and persistence of their associated beetles in the Canadian boreal forest.

In this study, *G. clavigera* and *O. ips* were used in a laboratory experiment to test the hypothesis that the chemical profile of FVOCs can be influenced by the presence of another ophiostomatoid fungal species. To test this hypothesis, we collected the headspace FVOCs emitted by the cultures of *G. clavigera* and *O.ips* growing in the absence or presence of the other fungus. In particular, we wanted to determine whether *G. clavigera* and *C. ips* emit qualitatively and quantitatively different VOC profiles when the fungi are growing alone or in the presence of the other fungus.

## 3.2 Methodology

### 3.2.1. Fungal volatiles collection and quantification *in situ*

To determine the VOCs profiles of two fungal species when they grow alone or concurrently, we used a push-pull head space fungal volatile collecting system reported in Cale et al. (2016). This system allows us to collect FVOCs emitted from *G. clavigera* (Gc), *O. ips* (Oi), and *G. clavigera* and *O. ips* combined (Gc + Oi) without any physical contact between the two. Briefly, this

volatile collecting system contained a glass jar (473mL) covered with a metal cap that had two holes. Two Teflon tubes were fitted through each hole into the glass chamber. For the first tube, we packed glass wool halfway down to the 10 cm long tube with activated carbon to filter the incoming air. Then the tube was connected to another tube that was attached to a metal gang-valve. This metal gang-valve was connected to a tube that jointed the outlet spigot of a bellows vacuum/pressure pump to generate the consistent air flow into the glass jar. The second tube inserted into the metal cap hosted a volatile trap which consisted 150 mg activated carbon as an adsorbent with glass wool to absorb the volatiles releasing from fungi. The volatile trap was connected to a tube that attached to another gang-valve. This gang-valve was connected to the tube jointed the inlet spigot of a bellows vacuum/pressure pump through which air was drawn by suction (Fig. 3.1). By using this collecting system, fungal volatiles are absorbed effectively by the activated carbon adsorbent. In total, each gang-valve manifold was connected to four tubes with four jars. A flowmeter was used (approximately 450 mL $min^{-1}$) to generate the constant flow through each glass chamber.

Two isolates of *G. clavigera* (NOF2894 and NOF2969) and two isolates of *O. ips* (NOF1205 and NOF1284) were used in the experiments described below. These isolates were provided by the Northern Forestry Center in Edmonton. All isolates were identified using fungal morphology and confirmed using DNA barcoding. Two isolates of *G. clavigera* were collected from different locations in Alberta: One isolate was isolated from mountain pine beetle infested lodgepole pine phloem at Banff; the other isolate was collected from mountain pine beetle gallery on infested-lodgepole pine × jack pine hybrid at Grande Prairie. Two isolates of *O.ips* were collected from different locations in British Columbia, Canada: NOF 1205 was collected

from mountain pine beetle larva on infested lodgepole pine; NOF 1284 was collected from mycangia of adult mountain pine beetle on infested lodgepole pine.

Each fungal isolate used in the following experiment was sub-cultured with a 4 mm diameter plug of a 10-day old actively growing margins of a master culture. Fungal isolates were incubated in the permanent darkness at 22 ºC until the growing margin covered around 80% of the whole plate (culture area of *G. clavigera* and *O. ips* was approximately 2.12 and 1.98 $cm^2$ respectively). After this period, *G. clavigera* isolate alone, *O. ips* isolate alone, *G. clavigera* and *O. ips* isolates combined, or control treatments without any fungal cultures made of potato dextrose agar (24g potato dextrose broth (Difco™ Potato Dextrose Broth), 15g agar (Difco Bacto™ Agar), and 1 L distilled water; PDA) media resulting in four treatments in total was placed in the fungal volatile collecting system with the Petri-dish lid open to accelerate the volatile diffusion. For the combination treatment, a piece of steel wire was coiled, bent along the horizontal plane, and placed inside the glass jar to hold two fungal isolates. One isolate from one fungal species was selected randomly and placed on the bottom of the chamber while another isolate from another fungal species was selected randomly and held by the wire shelf above. Each treatment was replicated 10 times.

Guided by the preliminary experiments, we extracted volatiles after 48 h by transferring the activated carbon to a microtube (2 mL), which was the optimal time to reach the fungal volatile peak in mass spectrum before the media dry out. Dichloromethane (1 mL) containing a tridecane internal standard (0.001%) was added to the activated carbon. This mixture was vortexed for 30 s, sonicated for 10 min, and centrifuged (at 30,000 rpm) at 0 ℃ for 30 min before the extract was collected and transferred to a 2 mL gas chromatograph (GC) vial. The same procedure was repeated a second time before chromatographic separation. Extracts were

analyzed using a GC coupled to a mass spectrometer fitted with a DB-5MS UI column (GC-MS; GC: 7890A, MS: 5062C, 30 m x 0.25 mm ID x 0.25 μm film, product: 122-5532UI; Agilent Tech, USA). Helium was used as a carrier gas flowing at 1 mL $min^{-1}$ with a temperature program beginning at 45°C (held for 2 mins) then increased by 3°C $min^{-1}$ to 70°C, 10°C $min^{-1}$ to 200°C followed by an increase of 25°C $min^{-1}$ to 300°C (held for 2 min). 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 isolates to determine peaks unique to the media to eliminate the volatiles released by the PDA 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), except *cis*-grandisol which was purchased from Alpha Scents (West Linn, OR, USA). Analyte concentrations was standardized by the culture area of fungal isolates, which *G. clavigera* and *O. ips* were 2119.5 $mm^2$ and 1978.5 $mm^2$, prior to data analysis.

3.2.2. Data analysis

The concentration of each compound detected in fungal treatments were calculated as the amount (μg) of compound per unit ($mm^2$) of fungal culture area per day (μg/$mm^2$/day). Qualitative and quantitative differences in the profiles of VOCs were analyzed for each fungal treatment. The quantitative differences in FVOC profile were compared by permutational multivariate analysis of variance (PerMANOVA) and principle component analysis (PCA) after Herrlinger data transformation. The total VOCs (the summation of concentrations of individual

compounds) of each fungal treatment were compared among treatments using one-way analysis of variance (ANOVA), with significant results following by Tukey Honest Significant Difference (HSD) multiple comparisons. In addition, one-way ANOVA was used to determine the significance of variation in chemical concentrations among fungal treatments separately for each individual chemical. For chemicals that were only detected in two of the treatments, two-sample t-test was used to compare the difference. Data were rank transformed to satisfy statistical assumptions for normality and heteroscedasticity. Tables and figures were constructed using non-transformation data. All data analysis was conducted by using R version 3.5.1 (R Core Team., 2018).

**3.3 Result**

Eight FVOCs, representing three classes of chemicals, were detected in extractions of headspace volatiles of the *G. clavigera*, *O. ips*, and *G. clavigera* and *O. ips* combination treatments during the 48h sampling period: acetoin (ketone), ethyl acetate, and phenylethyl acetate (esters), *cis*-grandisol, isobutanol, 2-methyl-1-butanol, isoamyl alcohol, and phenylethyl alcohol (alcohols) (Fig. 3.1).

*Grosmannia clavigera, O.ips,* and the combination treatments produced qualitatively and quantitatively different VOCs. Overall, more individual VOCs were detected from the *G. clavigera* and combination treatments than the *O. ips* treatment, with eight compounds detected from *G. clavigera* and combination treatments and six from the *O. ips* treatment (Fig. 3.2). Total concentrations of FVOCs significantly differed among treatments ($F_{2, 27} = 3.67$, $P = 0.04$). The *G. clavigera* treatment produced the greatest amounts of VOCs, with total concentrations being 39.8% and 83.9% greater than those in the *O. ips* and combination treatments (Table 3.1). Similarly, the

*G. clavigera* treatment produced a profile of eight individual compounds whose overall concentrations were significantly higher than those of the *O. ips* and combination treatments (PerMANOVA $F_{2,28} = 3.34$, $P = 0.002$; Fig. 3.3).

For individual chemicals, *cis*-grandisol and phenylethyl acetate were detected exclusively from the *G. clavigera* and combination treatments. Among individual chemicals, isobutanol was the most dominant VOC in the *G. clavigera*, *O. ips*, and combination treatments, accounting for 63.1%, 43.1%, and 46.3%, respectively, of the total concentration of VOCs. Isoamyl alcohol was the second dominant VOCs, accounting for approximately 20.8%, 38.6%, and 25.6% perceptively of total VOCs from each treatment (Table 3.1).

The individual chemicals detected from each treatment also exhibited quantitative differences among fungal treatments. The *G. clavigera* treatment produced significantly higher concentrations of isobutanol, isoamyl alcohol, and 2-methyl-1-butanol than those detected from the combination treatment (Table 3.1). The concentration of phenylethyl alcohol from the *O. ips* treatment was significantly higher than those from the combination treatment (Table 3.1).

### 3.4 Discussion

The profiles of VOCs emitted by ophiostomatoid fungi are similar between species that share the same ecological niche. Here, we showed that *G. clavigera* and *O. ips* emit qualitatively and quantitatively similar VOC profiles. Six compounds were common between the species, whereas two compounds were detected only when *G. clavigera* grew alone. Of these eight compounds, the six have been detected from emissions of many species of ophiostomatoid fungi, likely because they are byproducts of primary metabolism during vegetative growth (Cale et al. In

review). This is also reflected in the similar quantitative profiles of *G. clavigera* and *O. ips*. Furthermore, the similarities in profiles may also reflect a common ecological niche as phytopathogenic fungi with a common ecological function or niche have been shown to emit similar VOCs (Muller et al. 2013).

An increasing number of studies have shown that communication between microbial species involves not only water-soluble compounds but also the release and detection of volatile organic compounds (Schmidt et al. 2015). Our study shows that phytopathogenic fungi can interact and communicate at distance through sensing volatiles emitted by another phytopathogenic fungus. In general, our study is the first to demonstrate that the presence of phytopathogenic fungus strongly modifies VOCs emitted by another potential competing phytopathogenic fungus. The total concentration of VOCs decreased in response to another phytopathogenic fungus and was only about 27.2% compared with it growing alone. Our results emphasize that the presence of a fungus may have different impact on FVOC production of a given fungal species. The presence of another fungal species could stimulate the growth and at the same time suppress the VOC production of a given fungal species. Reducing the emission of VOC may have different effects on different species of fungi and bark beetles.

Fungal VOCs emitted by bark beetle-vectored fungi may affect other organisms including fungi and bark beetles occupying the same niche. Phenylethyl alcohol is attractive to several other bark beetles (e.g. pine engraver beetles) other than mountain pine beetle and southern pine beetle (Rewick et al. 1976; Pureswaran & Borden. 2004). *Ophiostoma ips* emitted significantly larger concentrations of phenylethyl alcohol compared *G. clavigera* or the combination treatment. One hypothesis we can draw from this finding is that *O. ips* produces less concentration of phenylethyl alcohol on trees also attacked by MPB symbiotic fungus *G. clavigera,* therefore less

pine engraver beetle will be attracted to the same tree suggesting less competition for MPB. In this case, MPB may benefit from its associated fungus by reducing beetle kairomones produced by other beetle-vectored fungus. The predominant volatile isoamyl alcohol, which has been identified as the chemical produced by bacterium *Enterobacter agglomerans* associated with Caribbean fruit fly, are attractive to female *Anastrepha suspensa* (Epsky et al. 1998). It also has been identified as a chemical in the pheromone used by hornets to attract other members of the hive to attack (Wilson et al. 2017). *cis*-Grandisol is also an important aggregation pheromone of several *Antonomus* weevil species (Tewari et al. 2014). 2-Methyl-1-butanol is a common fungal VOC that attract many species representing several insect phyla (Davis et al. 2013). Based on the known ecological function of these three chemicals on other insect phyla, we suspect them to have similar attractive function on bark beetles such as MPB and the pine engraver beetles. The chemical ethyl acetate has been wildly used as an entomological killing agent in insect collection. Because the vapors of ethyl acetate can kill the insects quickly without destroying it and keep the insect soft enough to allow proper mounting suitable for a collection. The property of ethyl acetate may act as a deterrent against bark beetles in the field. Overall, the concentration of individual chemical produced by a fungus can decrease in response to the presence of another fungus, suggesting relatively reduced impact on both fungi and bark beetles.

**Figures**

Figure 3.1 Illustration of the general process of the push-pull head space volatile collection and analysis system.

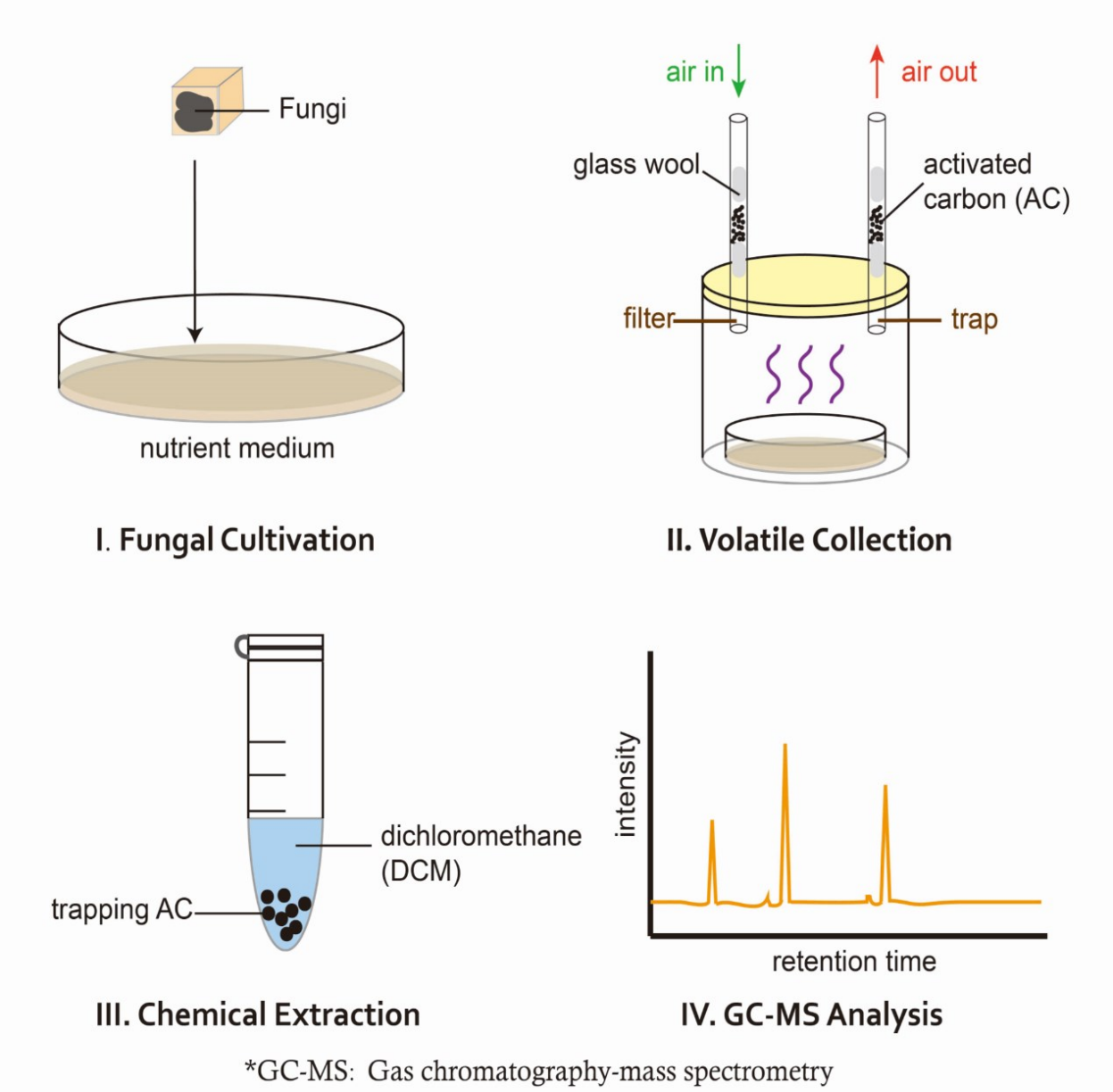

Figure 3.2 Heatmap of the proportion of fungal volatile organic compounds detected from *Grosmannia clavigera* (Gc), *Ophiostoma ips* (Oi), and combination treatment (Gc+Oi).

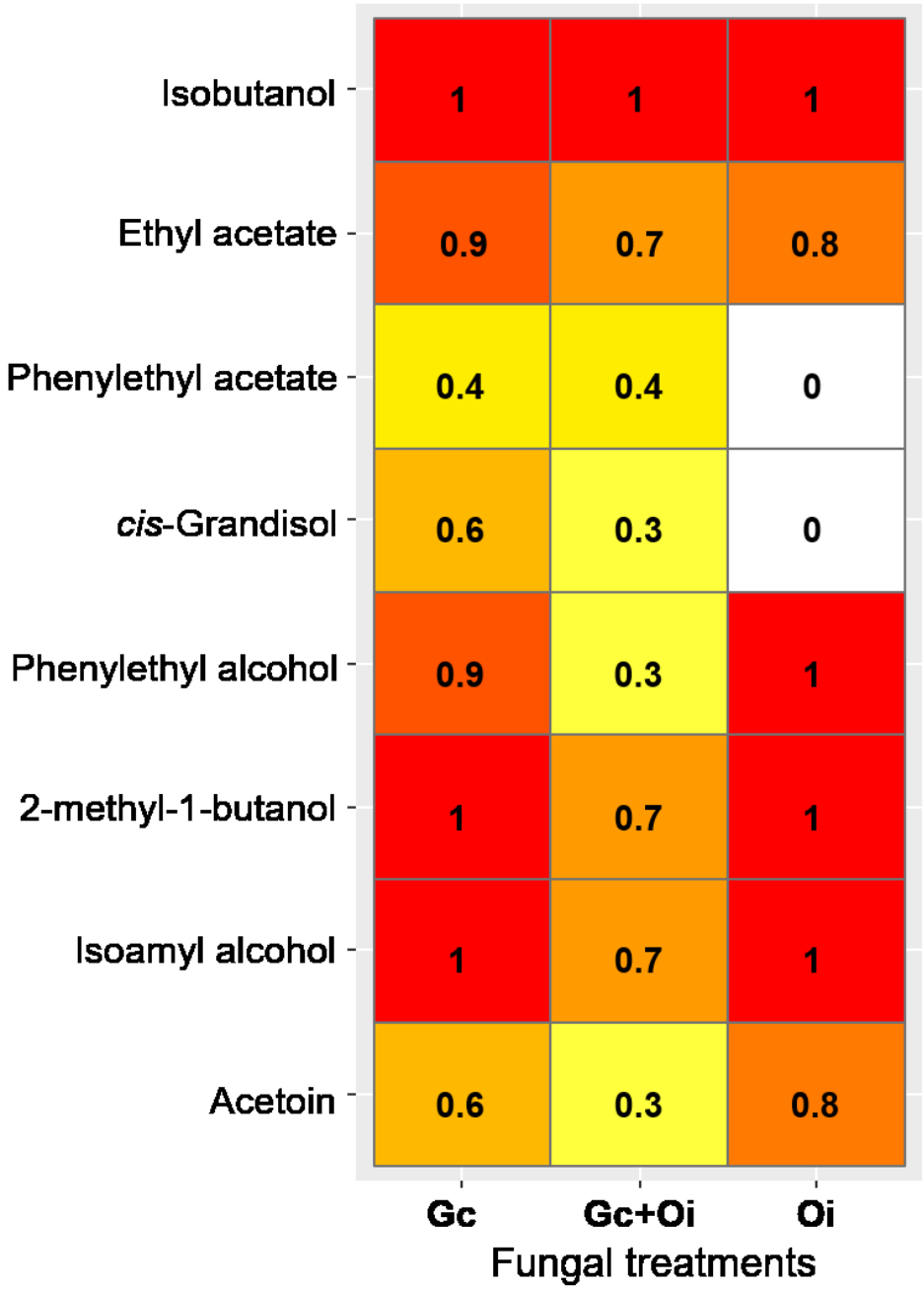

Figure 3.3 Principle component analysis result showing the separation of *Grosmannia clavigera*, *Ophiostoma ips*, and combination treatment based on concentrations (ng/mm$^2$/day) of eight headspace volatiles: acetoin (ATN), isoamyl alcohol (ISAL), 2-methyl-1-butanol (2MB), phenylethyl alcohol (PEAL), phenylethyl acetate (PEA), *cis*-grandisol (GRD), isobutanol (IBA), and ethyl acetate (ETA).

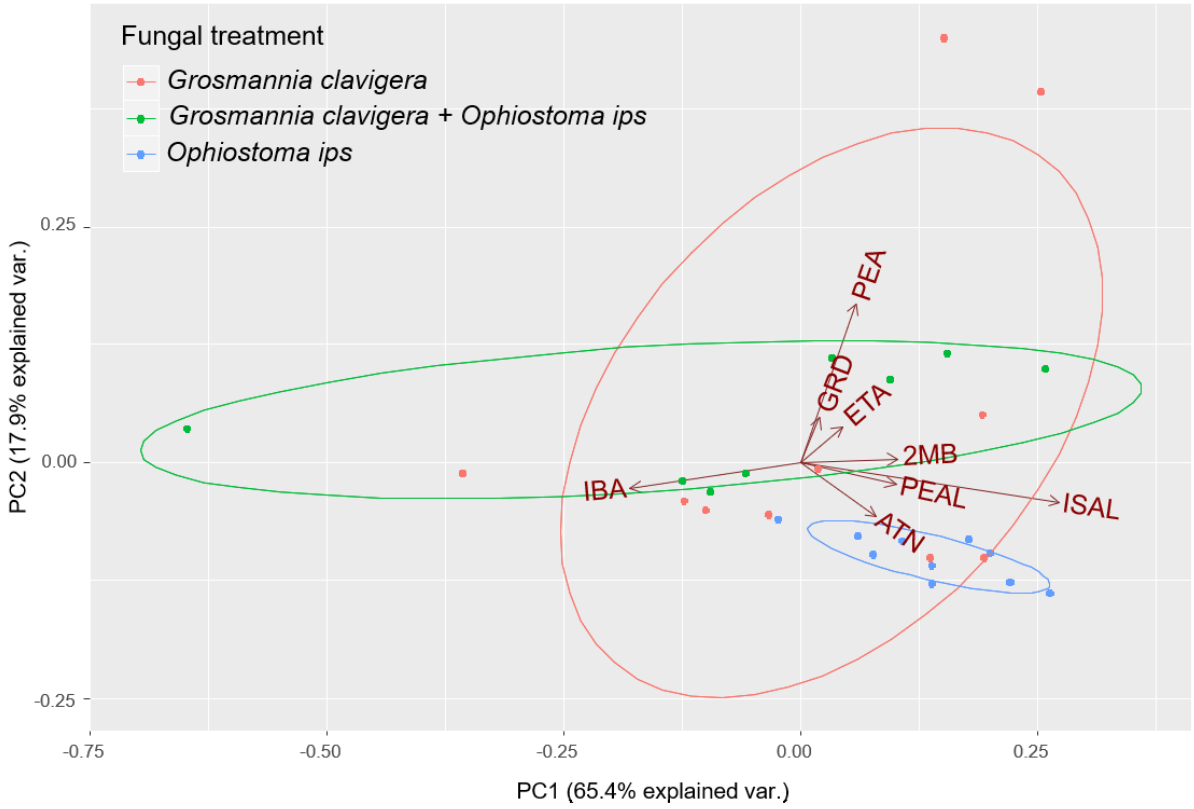

Table 3.1 Mean (± SE) concentrations (ng/mm$^2$/day) of detected headspace volatiles from four-day old cultures of *Grosmannia clavigera*, *Ophiostoma ips,* and *Grosmanna clavigera* and *Ophiostoma ips* combined. Analysis of Variance on rank transformed data and two sample t test result for species difference in mean concentrations for each individual chemical. Mean with different letter superscripts are significantly different as indicated by Tukey Honest Difference tests. Compounds not detected in headspace collections of a given fungus are indicated with "ND".

| **Volatile emission (ng/mm$^2$/day)** | **Fungal treatments** | | | **df** | **F** | **P-value/t** |
|---|---|---|---|---|---|---|
| | *G. clavigera* | *O. ips* | *G. clavigera* + O. *ips* | | | |
| Acetoin | 2.04 ± 0.64 | 2.50 ± 0.71 | 0.70 ± 0.41 | 2,27 | 2.05 | 0.147 |
| Isoamyl alcohol | 27.3 ± 7.24 [a] | 31.64 ± 7.49 [a] | 9.15 ± 4.10 [b] | 2,27 | 5.26 | 0.01 |
| 2-methyl-1-butanol | 6.48 ± 1.93 [a] | 4.35 ± 1.38 [ab] | 1.54 ± 0.68 [b] | 2,27 | 4.45 | 0.02 |
| Phenylethyl alcohol | 1.91 ± 0.78 [ab] | 6.42 ± 2.42 [a] | 0.45 ± 0.27 [b] | 2,27 | 4.27 | 0.02 |
| Phenylethyl acetate | 2.23 ± 1.23 | ND | 0.80 ± 0.42 | 12 | 2.18 | 0.23 |
| *cis*-Grandisol | 0.24 ± 0.12 | ND | 0.19 ± 0.10 | 17 | 2.11 | 0.56 |
| Isobutanol | 82.90 ± 38.17 [a] | 35.29 ± 7.35 [a] | 16.55 ± 27.73 [b] | 2,27 | 5.40 | 0.01 |
| Ethyl acetate | 1.66 ± 0.55 | 0.52 ± 0.15 | 0.49 ± 0.21 | 2,27 | 1.74 | 0.20 |
| **Total concentration** | 131.40 ± 10.91 [a] | 81.94 ± 10.71 [b] | 35.75 ± 6.08 [b] | 2,27 | 3.67 | 0.04 |

## Chapter Four

## Thesis Discussion

The presence of another phytopathogenic fungus can affect the fungal growth and FVOC emissions of a given fungal species. The fungal growth and FVOCs production respond differently to the presence of another fungus and/or induced host monoterpenes. In absence of monoterpenes, the presence of *O. ips* had a positive impact on the growth of *G. clavigera,* while *O. ips* did not respond to the presence of *G. clavigera*. Both fungal species grew on media amended with different concentrations of jack pine induced monoterpenes suggesting that they are able to establish and develop in jack pine trees. Only monoterpene α-pinene inhibited the fungal growth, whereas limonene and myrcene promoted fungal growth regardless of the concentrations of monoterpenes. The positive impact of *O. ips* on the growth of *G. clavigera* reduced or nullified in response to different concentrations of monoterpenes. These findings indicate that jack pine monoterpenes can mediate fungal interactions shifting from commensalism to amensalism. A hypothesis I can draw from this study is that mountain pine beetle may obtain less benefit from *G. clavigera* on jack pine that is also attacked by the pine engraver beetle and its vectored fungus *O. ips*.

*Grosmannia clavigera* and *O. ips* share similar FVOCs profiles with having six compounds in common. The similarity of their FVOCs profiles reveals their similar ecological function as phytopathogenic fungi (Muller et al. 2013). However, the presence of another fungus modifies their FVOCs profile quantitively and qualitatively. The total and individual concentration of FVOCs decreased significantly in response to a resources-sharing fungus. The reduction of FVOCs emission suggests less impact of FVOCs, regardless harmful or beneficial, on organisms occupying the same niche. However, more research is needed on the correlation

between FVOCs and bark beetles in order to understand how FVOC profiles affect beetle development, which will determine the beetle success in boreal forest.

Overall, our study is the first to demonstrate how the presence of a resources-sharing fungus differently affects the fungal growth and FVOCs production. We also demonstrate that fungal interaction can occur at distance by sensing FVOCs produced by other organisms. Based on the induction of fungal growth and reduction of FVOCs production, we suspect that there is a potential tradeoff between FVOC production and fungal growth in response to a potential competitor. Taken together, our work suggests that MPB may still maintain the relationship with their symbiotic fungi on jack pine. However, the benefits may be suppressed on the jack pine also attacked by the pine engraver vectoring fungus. As for the FVOCs production, fungus produce less concentration of VOCs in response to the presence of a resource-sharing fungus, suggesting less impact of FVOCs on organisms sharing the same niche.

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