Understanding the Role of Secondary Metabolites and Endophytic Fungi in White Spruce Defenses Against Eastern Spruce Budworm

by

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Abstract

As a boreal tree species, white spruce (*Picea glauca* Moench Voss) is under a constant threat by outbreaks of defoliators (*foliar feeders*) including eastern spruce budworm (*Choristoneura fumiferana*). Historically, applications of biological insecticides and silvicultural treatments have been used to improve white spruce resistance against the eastern spruce budworm. More recently, variations in chemical defenses, such as monoterpenes, phenolics, and fungal endophytes of white spruce have received a particular attention due to their potential roles in herbivory resistance. However, how such variations in white spruce defenses affect insect herbivores is largely unknown in part due to limitations in replicating the complete defense profile of trees under controlled conditions. Furthermore, little is known about the role of fungal endophytes alone or in combination with defense compounds in tree resistance to insect herbivores.

In the first study (Chapter II), I sampled the foliage of 80 white spruce families and clustered them into two chemotypes (chemotypic phenotypes) based on their foliar monoterpene concentrations. I investigated the role of phenolics and monoterpenes in white spruce defense by observing how budworms respond to media amended with these metabolites. I found that the higher proportions and concentrations of monoterpenes and phenolics in the white spruce foliage negatively affected the survival of eastern spruce budworms. Families that had greater proportions and concentrations of monoterpenes were more fatal to the budworm, while families that contained large proportions of phenolics and low monoterpene concentrations were less fatal. I concluded that both monoterpenes and phenolics play essential roles in the defenses of white spruce, with monoterpenes being responsible for the overall toxicity and phenolics

inhibiting the growth of the budworms. This study shows that different classes of defense metabolites are being coordinated against the same insect herbivore.

In the second study (Chapter III), I characterized the composition of fungal endophytes and terpenes (monoterpenes and sesquiterpenes) in the needles of 30 white spruce genotypes at two progeny locations. I found that the fungal community and terpene compositions varied between locations and among spruce genotypes, and those with higher sesquiterpene abundance also had higher fungal endophyte abundance. I also tested the effects of selected fungal morphotypes on the eastern spruce budworm performance and found that some fungi reduced the feeding and caused significant mortality to the budworms. Interestingly, fungal metabolites contained the same class of terpenes as those characterized in spruce trees including monoterpenes and sesquiterpenes. Overall, I concluded that foliar fungal endophytes play a pivotal role in enhancing white spruce defenses through the modification of host defense metabolites and providing direct anti-herbivore resistance. I suggested that the plant-associated fungal endophytes are crucial in the arms-race between host plants and herbaceous insects.

In the third study (Chapter IV), I grew seedlings from 30 different white spruce families (20 trees/family) from seeds in a growth chamber. After ten months of vegetative growth, I inoculated five fungal endophytes into the needles of half of the seedlings in each family, *Cladosporium halotolerans, Cladosporium cladosporioides, Chalara* sp.*, Geopyxis* sp.*,* and *Didymella* sp. The remaining half of the seedlings were not inoculated and served as the control. After two months of inoculation, I harvested the seedlings and weighed the above ground fresh biomass from both inoculated and control plants. I found that the fungal inoculation was successful since the fungal endophytic communities were different between inoculated and control seedlings. Overall, spruce families with the higher endophytic fungal abundance also had

higher monoterpene and sesquiterpene concentrations and needle biomass. All together, these results demonstrate that fungal endophytes can increase the concentrations of the defense metabolites of spruce trees along with improving growth.

Preface

This thesis is Aziz Ullah's original work and follows the format specified by the University of Alberta's Graduate Studies and Research Faculty. The concept and idea of this work originated from Aziz Ullah, his supervisor Dr. Nadir Erbilgin of the University of Alberta. There are a total of five chapters in the thesis.

Chapter II of this thesis was submitted for publication as Aziz Ullah, Jennifer Klutsch, and Nadir Erbilgin “Complementary roles of two classes of defense chemicals protecting white spruce trees from spruce budworm”.

Chapter III of this thesis was submitted for publication as Aziz Ullah, Jean C. Rodriguez-Ramos, Shih-hsuan (Ethan) Chen, Aftab Shah, Rashaduz Zaman, Ateeq Shah, Nadir Erbilgin “Foliar fungal endophytes alter tree defense metabolites and provide direct anti-herbivore resistance”.

Chapter IV of this thesis will be submitted for publication as Aziz Ullah, Ateeq Shah, Federico Antonioli, Shih-hsuan (Ethan) Chen, Rashaduz Zaman, Nadir Erbilgin, “Fungal endophytes of white spruce modulate tree defenses”.

This dissertation is dedicated to my beloved family

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Finally, I pray for my late grandmother and uncle, who passed away in Pakistan while I was in Canada; may their souls rest in peace. I will always miss having you around.

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Chapter I

Thesis Introduction

1.1 Range expansion by eastern spruce budworm (*Choristoneura fumiferana*)

The eastern spruce budworm (*Choristoneura fumiferana*) can cause significant defoliation on trees during outbreaks, resulting in mortality at the landscape level (Blais, 1983; Sanders, 1991). The eastern spruce budworm has a relatively large geographical range from Alaska to Newfoundland in North America. In this range, white spruce (*Picea glauca* Moench Voss) and balsam fir (*Abies balsamea* L. Mill.) trees are defoliated by the eastern spruce budworm (Sutton, 1969). The budworm has a one-year lifecycle; this means the budworm completes its development from egg to adulthood in a year. It spends the winter on the host tree as a first instar larva, then emerges in the spring to feed on current-year needles throughout the second to fourth instars (Pureswaran *et al.*, 2016; Fig. 1.1). It pupates in the summer. Adult moths emerge in late summer or early autumn, mate, and deposit eggs on the foliage of a host tree, and then die. Those trees that are not killed by the eastern spruce budworm often become susceptible to other pests and diseases (Lumley *et al.*, 2020; Fig. 1.1).

Figure 1.1. Pictures of budworm (*Choristoneura fumiferana*) and its damage. (a) Aerial view of large-scale defoliation caused by eastern spruce budworm. (b) Budworm

feeding pattern. (c) to (f) 3rd and 6th instar larva, pupa, and adult respectively (Lumley *et al.,* 2020).

Due to climate change (Netherer & Schopf, 2010; Pureswaran *et al.*, 2015), the present outbreak of the eastern spruce budworm has expanded farther north than in the past, and in regions where outbreaks have not previously occurred (Pureswaran & Borden, 2005; Régnière *et al.*, 2012). The eastern spruce budworm has traditionally been found in eastern Canada and the northern United States, but it has recently been spotted extending its historical distribution. This spread has been ascribed to a number of causes, including shifts in weather and moisture trends, woodland management practices, and the entry of the budworm into new regions *via* human activity (Bellemin-Noël *et al.*, 2021). Because of the budworm's cyclical population patterns, their outbreaks are known to happen every 30 to 40 years (Royama *et al.*, 2017). These outbreaks can last anywhere from 4 to 16 years, during which time they cause serious defoliation of their preferred host trees, balsam fir and spruce (Hennigar *et al.*, 2008) and can have an enormous impact on the forest products industry (Chang *et al.*, 2012). Loss of carbon sinks and feedback on climate change have also raised some concerns when large numbers of trees are killed by the spruce budworm outbreaks in North America (Hicke *et al.*, 2012). This can have severe repercussions for both biodiversity and the ability of ecosystems to perform their functions (Drever *et al.*, 2018).

Bacillus thuringiensis (Bt) is frequently used to kill the eastern spruce budworm and other defoliators (van Frankenhuyzen, 2000; Bravo *et al.*, 2011). Bt is usually applied aerially and then foliage contaminated by Bt is consumed by the budworm larvae as they feed. However, the use of Bt does not provide long-term budworm management; it is not cost-effective and it has

a harmful influence on the environment (Nester *et al.*, 2002). There is an urgency to develop cost-effective natural pest management practices targeting the eastern spruce budworm.

Since the eastern spruce budworm has extended its distribution (Candau & Fleming, 2011), it is also especially critical to gain an understanding of the relationship between this species and white spruce resistance. White spruce relies on different classes of defense compounds against defoliators including phenolics and terpenes. More recently, researchers reported the role of foliar endophytes in white spruce resistance to the eastern spruce budworm (Sumarah *et al.*, 2005a; Quiring *et al.*, 2019b). I discuss plant defenses and endophytes with respect to the white spruce resistance to the eastern budworm below.

1.2 White spruce chemical defenses

Over millions of years, plants developed the capacity to respond to threats by actively regulating their morpho-physiological responses, which included changes in gene expression, metabolic pathways, and resource allocation patterns (Condamine *et al.*, 2016). From plant-insect coevolution complicated relationships have often emerged, such as herbivory by specialist insects (Agrawal, 2007) and pollination and seed dispersal by selective animals (Guimaraes Jr. *et al.*, 2017).

Plant secondary compounds are critical against insect herbivores and pathogens, attract natural enemies of herbivorous insects, and mediate interactions with pollinators (Moore *et al.*, 2014; Moreira *et al.*, 2014). Plant defenses can be constitutive or induced (Franceschi & Krekling, 2005; Zulak & Bohlmann, 2010). Constitutive defenses are pre-formed in plants such as specialized cell walls, waxy epidermal cuticles, trichomes, thorns, different bark types, and chemicals. Inducible plant defenses are temporary chemical attributes initiated by the initial

stress thereby altering plant suitability for further attack by insects or pathogens (Agrawal, 1998; Wittstock & Gershenzon, 2002).

Plant defenses have vast diversity (Dixon, 2003) and can have a range of adverse effects on insect herbivores (Delvas *et al.*, 2011; Kumbasli & Bauce, 2013; Ullah *et al.*, 2021), including mortality, fecundity reduction, growth inhibition, and altered insect behavior (Gatehouse, 2002; Mertens *et al.*, 2021). These defenses are widely considered the most important component of plant resistance to insects, and thus they have been well characterized for many plant species including coniferous trees (Clancy, 2002; Mumm & Hilker, 2006). In fact, the variation in defenses in conifers is often viewed as a result of co-evolution with its herbivorous insect enemies (Moore *et al.*, 2014; Moreira *et al.*, 2014).

In North America, conifer trees are widely distributed (Lyons & Darrah, 1989). Due to their long-life span, they are attacked by large numbers of herbivorous insects and pathogens. During periodic outbreaks, defoliators and bark beetles cause heavy losses to both natural and plantation conifer forests around the world. Conifer trees produce different secondary (chemical defense) compounds to fight against these biotic disturbances. This is particularly true for one of the most abundant and widespread conifer species occurring in Alberta and across the Canadian boreal forests, white spruce (Halliday & Brown, 1943).

White spruce is an ecologically and economically important tree species in the Canadian boreal forests. It has a wide distribution in North America, from Newfoundland and Labrador, extending west across Canada along the northern limit of trees to Hudson Bay, Northwest Territories, and Yukon. It almost reaches the Arctic ocean at latitude 69 °N. White spruce growth occurs from sea level to around elevation of 1,524 meters (Sutton, 1969). Trees can live up to 50

to 200, but a few trees have been found to reach 500 years. White spruce is under a constant threat by insects throughout its range in Canada (Royama *et al.*, 2017). One of the primary threats of white spruce is the eastern spruce budworm (Blais, 1983; Sanders, 1991).

White spruce foliage contains two major groups of secondary compounds against the eastern spruce budworm, including terpenoids and phenolics (Johnson & Felton, 2001; Mumm & Hilker, 2006). Terpenoids and phenolics show both positive and negative effects on the biology of spruce budworm depending on their concentrations (Johnson & Felton, 2001; Daoust *et al.*, 2010; Delvas *et al.*, 2011; Kumbasli & Bauce, 2013). Although studies are lacking, like many other conifer species both composition and concentrations of terpenes and phenolics likely vary throughout the range of the species depending on the co-evolutionary interactions with pest species as well as differences in growing conditions. For instance, Delvas *et al.* (2011) investigated the phenolic compounds in white spruce foliage in Quebec (Canada) and found four distinct phenolic compounds, piceol, pungenol, picein, and pungenin among different populations of white spruce trees. They further tested these phenolic compounds against the performance of the eastern spruce budworm larvae and found that they can reduce the performance of budworms. Likewise, Daoust *et al.* (2010) profiled the monoterpene composition of white spruce foliage in southern Quebec and found that the foliage from eastern spruce budworm-resistant trees contained higher concentrations of some monoterpenes than susceptible ones, suggesting that monoterpenes can also play an important role in white spruce defenses to the budworm.

1.3 White spruce foliar fungal endophytes

Fungal endophytes of conifer species represent substantial unknown biodiversity (Ganley *et al.*, 2004; Ganley & Newcombe, 2006). Endophyte diversity, distribution, and interactions with their hosts have been extensively studied on some of the conifer species, but not all (Müller *et al.*, 2001). The term, "endophyte" was first coined by de Bary (1866) about 150 years ago for fungi entering inside leaves (De Bary & Engelmann, 1866). The tissues and organs of plants known to have symptomless infections are considered endophytes (Carroll, 1988; Clay, 1988). Transfer of endophytic fungi in plants occurs in two ways: 1) horizontally (transferred through sexual or asexual spores) and 2) vertically (transmission from plant to offspring by host seeds) (Clay *et al.*, 2002). Endophytic fungi could be both plant mutualists and insect pathogens (Moonjely *et al.*, 2016). These endophytes (mainly fungi) protect plants from herbivorous insects by producing toxic metabolites (Clay, 1988; de Souza *et al.*, 2011). The relationship of these endophytes with their host plants varies from symbiotic to semi-parasitic.

Taxonomically, most of the endophytic fungi belong to the phylum Ascomycota and its associated anamorphs (Arnold *et al.*, 2001). There is a growing interest among the scientific community in the ecological roles of fungal endophytes, particularly in plant resistance to insect pests or diseases. Since fungal endophytes are heterotrophic organisms and use the same host substrate as other heterotrophs such as insect herbivores, they all compete for the same resources. As a result, many fungal endophytes can produce bioactive compounds that are antagonistic to other plant consumers and may defend the host from their damage (Zhang *et al.*, 2006; Jaber & Ownley, 2017). The potential interaction between fungal endophytes and insect herbivores can be either direct or indirect (Moonjely *et al.*, 2016). Direct interactions may occur through resource competition, antagonisms, or even parasitism. Indirect interactions take place through the changes in host plant metabolism induced by endophytes that harm insects. For instance,

fungal endophytes can alter or enhance the production of secondary metabolites (Strobel & Daisy, 2003; Zhang *et al.*, 2006), which may deter insect herbivory (Sumarah *et al.*, 2005a,b; Miller *et al.*, 2020). Currently, little information is available on the abundance and diversity of fungal endophytes associated with white spruce and their roles in host resistance.

White spruce foliage contains a diversity of endophytic fungi that may play an important role in spruce resistance to the eastern spruce budworm (Quiring *et al.*, 2019b). Based on the limited studies white spruce hosts different species of fungal endophytes in its foliage and apparently, the diversity of endophytes shows significant differences among different populations even in the same province (Stefani & Bérubé, 2006a). Based on the outcome of these studies, *Lophodermium piceae* is the most abundant fungal endophyte, followed by *Mycosphaerella* spp*., Hypoxylon* spp*.,* and *Phomopsis* spp. Depending on the species, some endophytes show strong host specificity (Koukol *et al.*, 2012). Recently Quiring *et al*. (2019b) reported that the foliar endophytic fungus, *Phialocephala scopiformis,* that is native to white spruce from eastern Canada, reduced the performance of eastern spruce budworm. In the same study, endophytic fungi also reduced the performance and survival of gall insects on white spruce (Lasota *et al.*, 1983). Relevant studies tested rugulosin, a secondary metabolite produced by *P. scopiformis*, and showed that it inhibited the growth of the eastern spruce budworm (Sumarah *et al.*, 2005b). We need a more comprehensive undertaking to determine the endophyte diversity of white spruce across its population in Canada as well as to assess whether such diversity can differentially influence the eastern spruce budworm fitness.

Thesis aims

The overarching purpose of this thesis research is to understand the role of endophytes in plant resistance to insect herbivores. Specifically, I characterized the foliar endophytic fungal communities and terpene defenses of different white spruce families/genotypes and determined whether these characteristics vary between locations (spatially) across different white spruce genotypes. I was also interested in determining the relationship between the foliar terpene composition and fungal endophyte composition; in particular whether the foliar endophytic fungal communities can explain the abundance of terpenes in white spruce foliage. Finally, I inoculated the foliar endophytic fungal communities of white spruce foliage to determine whether changes in foliar endophytes can alter the composition of foliar terpenes in white spruce. I specially addressed the following specific research objectives:

(1) To determine whether foliar endophyte community and terpene defenses show variations among different plant genotypes and needle types (current vs. older) across two sampling locations in Alberta,

(2) To evaluate whether there is a relationship between foliar endophytes and terpene defenses in mature white spruce trees,

(3) To examine whether the terpenes and phenolics of white spruce affect the performance of eastern spruce budworm,

(4) To determine whether white spruce foliar endophytic fungi synthesize terpenes,

(5) To determine whether fungal endophytes affect the performance (feeding and growth) of eastern spruce budworm, and

(6) To observe whether changes in foliar fungal endophytes affect terpene profiles of white spruce seedlings.

In Chapter II, I examined how monoterpene and phenolic compounds vary among mature white spruce tree genotypes and how they affect eastern spruce budworm performance. I tested budworm performance in plant-based media amended with phenolics or monoterpenes based on the actual composition of selected white spruce genotypes. Based on these results, I developed "susceptibility indices" to eastern spruce budworm for white spruce families tested.

In Chapter III, I investigated whether endophytic fungi in the white spruce foliage contribute to the host tree resistance to eastern spruce budworm and suggested some possible mechanisms explaining the role of fungal endophytes in white spruce resistance. In particular, I found out that fungal endophytes can indirectly alter white spruce defense metabolites and can directly affect the budworm performance. Here, I evaluated the composition of monoterpenes, sesquiterpenes, and endophytic fungi of 30 different white spruce genotypes established in two locations in Alberta. In addition, I isolated and identified several endophytic fungal morphotypes from white spruce foliage and examined their metabolite profiles. I further examined the impact of these morphotypes on the eastern spruce budworm fitness by evaluating the budworm attractiveness and feeding response to either fungal volatile organic compounds or be part of budworms diet.

In Chapter IV, I manipulated the foliar endophyte communities of one-year old white spruce seedlings by inoculating seedlings with five distinct fungal endophytes isolated from mature trees and measured the seedlings' terpene composition and biomass. I grew seedlings from 30 spruce tree families from seeds, each containing 20 seedlings. During the ten months, I inoculated half these seedlings with a mixture of five endophytic fungi and left the other half untreated as a control. Two months post-inoculation, I collected leaves from treated and control

seedlings and compared their fungal endophytic abundance, monoterpenes, sesquiterpenes, and fresh biomass.

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Chapter II

Complementary roles of two classes of defense chemicals protecting white spruce trees from spruce budworm

2.1 Introduction

Plant secondary metabolites are critical components of determining plant resistance to herbivorous insects (Züst & Agrawal, 2017). They can have diverse adverse impacts on a broad range of phytophagous insects (Futuyma & Agrawal, 2009; Mithöfer & Boland, 2012; Raffa *et al.,* 2013; Lämke & Unsicker, 2018) ranging from causing direct mortality to reducing fecundity, inhibiting growth, altering insect behaviour, and affecting host plant preference (Dudareva *et al.,* 2006; Howe & Jander, 2008; Arimura *et al.,* 2009; War *et al.,* 2012; Jamieson *et al.,* 2017; Bittner *et al.,* 2019). Although plants produce a substantial structural diversity of secondary metabolites (Dixon, 2003; Wink, 2003; Lattanzio *et al.,* 2006; Mithoför & Boland, 2012; Moore *et al.*, 2014), they may vary in their impacts on the same herbivore species (Lankau, 2007; Orians & Ward, 2010; Ali & Agrawal, 2012). In particular, some classes of defense metabolites can be toxic to one insect species while others in the same plant may not have a similar impact on the same pest species (Franceschi *et al.,* 2005; Fürstenberg-Hägg *et al.,* 2013; Feeny, 2021). Thus, studies incorporating multiple classes of defense metabolites against a single pest species can help explain their roles in the evolutionary and ecological processes that take place between plants and insect herbivores (War *et al.,* 2012; Beyaert & Hilker, 2014).

Coniferous trees use several classes of secondary metabolites including terpenes and phenolics to defend themselves against a broad range of insect species (Franceschi & Krekling, 2005; Keeling & Bohlmann, 2006; Kolosova & Bohlmann, 2012; Raffa *et al*., 2017). Often the variation in secondary metabolites in conifers is viewed as a result of co-evolutionary

interactions with its major insect enemies (Moore *et al.*, 2014; Moreira *et al.*, 2014). In conifers, resistance to bark beetles generally depends on a higher activity of monoterpenes than phenolics, which has led to a greater focus on monoterpenes (Raffa *et al*., 2013; Erbilgin, 2019; Liu *et al*., 2022; Ullah *et al.,* 2021 and references therein), whereas more generally consistent effects of phenolics than monoterpenes on lepidopteran folivores has led to a greater research focus on phenolics (Zou & Cates, 1997; Abou-Zaid, 2000; Johnson & Felton, 2001; Roitto *et al*., 2009; Delvas *et al.,* 2011; Lamara *et al*., 2018; Donkor *et al.,* 2019; Beulieu *et al*., 2020).

White spruce ((*Picea glauca* Moench) Voss) is an ecologically and economically important conifer species in the Canadian boreal forests. It has a wide distribution, from Newfoundland and Labrador west across Canada along the northern limit of trees to Hudson Bay, Northwest Territories, and Yukon. It almost reaches the Arctic Ocean at latitude 69 °N (Halliday & Brown, 1943). White spruce is under constant threat by phytophagous insects throughout its range (Eveleigh *et al.,* 2007; Larrogue *et al.,* 2020). One of the primary threats is the eastern spruce budworm (ESB), *Choristoneura fumiferana* Clemens, (Lepidoptera: Tortricidae), a univoltine tortricid moth that feeds primarily on current-year needles (Halliday & Brown, 1943; Blais, 1983; Mattson *et al.,* 1988; Sanders, 1991). During an outbreak, millions of hectares of spruce trees can be severely defoliated (Boulanger & Arseneault, 2004; Gray & MacKinnon, 2006; Berguet *et al.,* 2021) causing mortality at the landscape scale. While several factors can affect ESB defoliation including host phenology and quality, natural enemies, and forest stand composition, the role of host defense metabolites can also be important (Delvas *et al.,* 2011; Kumbasli & Bauce, 2013; Ennis *et al.,* 2017; Beulieu *et al*., 2020).

White spruce foliage contains two major classes of defense metabolites, monoterpenes, and phenolics that can affect ESB defoliation (Lawrence *et al.,* 1997; Ralph *et al.,* 2006; Daoust *et*

al., 2010; Delvas *et al.,* 2011). However, both classes show variations across the range of white spruce (Daoust *et al.,* 2010; Mageroy *et al.,* 2015; Parent *et al.,* 2020) and can have both positive and negative impacts on the spruce budworm biology (reviewed in Table 2.1; Abou-Zaid *et al.,* 2000; Daoust *et al.,* 2010; Delvas *et al.,* 2011). For instance, Delvas *et al.* (2011) reported four distinct phenolic compounds, piceol, pungenol, picein, and pungenin among different populations of white spruce trees sampled in Quebec (Canada) that reduced the performance of ESB in laboratory bioassays. Likewise, Daoust *et al.* (2010) profiled the monoterpene composition of white spruce foliage in southern Quebec and found that the foliage from budworm-resistant trees contained higher concentrations of some monoterpenes compared susceptible trees, suggesting that monoterpenes can play a critical role in white spruce defenses to the budworms. Yet, studies integrating both defense classes against the ESB are lacking in white spruce. Thus, studies incorporating the complete monoterpene and phenolic profiles of mature white spruce trees based on field data and how the proportions of phenolics and monoterpenes in white spruce foliage affect the budworms are urgently needed.

The objevtive of this study was to examine whether the terpenes and phenolics of white spruce affect the performance of eastern spruce budworm. Here, I studied the proportions and concentrations of the monoterpenes and phenolics of different populations of mature white spruce trees and tested how such variations impact ESB performance. I first sampled 638 trees in 80 spruce families from a progeny trial located in Alberta, Canada and characterized the monoterpene and phenolic profiles of each family. I then grouped the families in phenotypically different clusters (chemotypes) based on their monoterpene profiles, using cluster analysis, and selected representative families, and thereby their defense profiles, in each cluster. I further tested ESB performance in plant-based media amended with phenolics alone or with

monoterpenes. Finally, I characterized the white spruce families that had the most negative or positive consequences on the budworm performance and developed white spruce "susceptibility indices" based on the proportions and concentrations of monoterpenes and phenolics. Investigating the contributions of different classes of defense metabolites can deepen the understanding of the ecological and co-evolutionary interactions between plants and phytophagous insects (Whitham *et al.,* 2008). Furthermore, the outcome of this study could potentially result in the selection of ESB -resistant white spruce phenotypes across its range in Canada.

2.2 Materials and Methods

2.2.1 White spruce needle collection

I sampled foliage from 34 yr-old white spruce trees in a progeny trial in Red Earth, Alberta, Canada (56°34' N, 115°19' W; 518 m) to quantify and characterize needle secondary metabolites. Trees from 150 open-pollinated half-sib families were spaced 2.5 m apart in a randomized complete block design organized into six trees per family per row with six replications in a row. The 80 representative families were selected from the range of height growth from the progeny trial. On June 20-23, 2018, I collected branch ends with current-year needles from the upper 1/3rd canopy and south-facing aspect of eight trees in each family (n=638 total due to two missing trees). Foliage samples were wrapped in aluminum foil, and submerged in liquid nitrogen in the field until transported to -40°C freezers in the laboratory. Current-year needles were removed and ground into a fine powder using liquid nitrogen in a mortar and pestle. Ground samples were stored in -40°C freezers until chemical analyses as described below.

2.2.2 Monoterpene analysis

I used 100 (± 5) mg (fresh weight) of frozen ground samples to identify and quantify hexane-extractable compounds (mainly monoterpenes). I extracted the fresh needle tissue twice with 0.5 ml hexane using 0.004% pentadecane as an internal standard (Ullah *et al.,* 2021). Samples were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 0°C for 15 min. I combined the supernatant from each extraction and identified monoterpenes in a sub-sample of extracts with a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) using authentic standards. To quantify monoterpenes in each sample, I used a GC/Flame Ionization Detector (GC/FID, Agilent 7890B). The method used for both GC/MS and GC/FID was as follows: a 1 µl of sample extract was injected with a split injection (10:1) into the GC equipped with an HP-Innowax column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) with helium carrier gas flow at 1.1 ml min^{-1}, and a temperature of 40 °C for 1 min, increased to 55 °C by 30 °C min^{-1}, and held for 0.5 min, increased to 122 °C by 8 °C min^{-1}, and held for 2 min, increased to 200 °C by 10 °C min^{-1}, and then to 260 °C by 20 °C min^{-1}, and held for 1 min. I used 11 authentic standards (mainly monoterpenes, one bornyl acetate (acetate ester of borneol)) to determine the concentrations of individual compounds: borneol (chemical purity: 99%), α-terpineol (90%) (Sigma-Aldrich, St. Louis, MO, USA), terpinolene (90%), α-pinene (98%), β-pinene (98%), limonene (99%), myrcene (90%), camphene (90%), camphor (95%) (Fluka, Sigma-Aldrich, Buchs, CHE), bornyl acetate (97%), (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (83%) (Erbilgin Lab). For the concentrations of monoterpene compounds (ng mg^{-1}) were expressed on the fresh weight (FW) basis.

2.2.3 Cluster analysis of monoterpenes

Using the monoterpene profiles, I determine the chemotypes (phenotypic clusters) of 80 spruce families. I calculated the best linear unbiased predictors (BLUPs) with the natural log transformation for monoterpene concentrations using a mixed linear model with replicate as fixed effects and the half-sib pedigree as a random effect (Butler *et al.,* 2017). By calculating BLUPS, I was able to use genetic variation in monoterpenes to identify clusters of family monoterpenes. Using unstandardized estimated breeding values calculated from the BLUPs, I performed a partitioning around medoid clustering analysis (*pamk*) in the *fpc* package in R (Hennig, 2018) using the same methodology as Ullah *et al.* (2021). This clustering method minimizes the sum of dissimilarities. Using the average silhouette width, I estimated the optimum number of clusters, which was tested with the Duda-Hart test ($P<0.05$). I identified two clusters composed of white spruce family estimated breeding values for monoterpenes (Fig. S1). From each chemotype (cluster), I selected five families based on 5^{th}, 25^{th}, 50^{th}, 75^{th}, and 95^{th} percentile of total monoterpenes. Furthermore, from each family, I averaged monoterpene concentrations from three individual trees based on the 5^{th}, 50^{th} and 95^{th} percentile of total monoterpenes to define the family mean profiles. Overall, 10 families from both clusters were selected (Figs. S2.1, S2.2). Tables 2.1 and 2.2 show the monoterpene and phenolic profiles of selected families.

2.2.4 Polyphenolic analysis

I used the solvent extraction method for the extraction of polyphenols. Flavonoids and phenolic acids have a wide range of polarity with the majority being semi-polar to non-polar with log P in the range of 1-4. Spruce needles also have certain polar secondary metabolites such as glycosides of phenolic acids with log P in the range of -1 to -3. Therefore, in the present study, I extracted

targeted polyphenols by adding 500 µL of 80 % MeOH to 5 mg (dried weight) of spruce needles. To facilitate the extraction, I used a multi-tube vortex at 1,500 rpm for about 30 min followed by centrifugation at 12,000 rpm for 5 min. I then transferred the clear supernatant and filtered through a 0.22 µm Acrodisc® syringe filter with the GHP membrane for ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) analyses.

I purchased Optima™ LC/MS grade formic acid, Optima™ LC/MS grade water, and methanol from Fisher Scientific (Ottawa, ON, CAN) and NIST® SRM® 1575A pine needle material from Sigma-Aldrich (Oakville, ON, CAN). I purchased chemical standards gallic acid monohydrate, 3,4-dihydroxybenzoic acid, caffeic acid, vanillin, p-coumaric acid, ferulic acid, protocatechuic aldehyde, catechin hydrate, gallocatechin, piceol, and pungenol from Sigma-Aldrich (Oakville, ON, CA), vanillic acid from Fluka (Mexico City, MEX), quercetin from Cayman Chemical (Ann Arbor, MI, U.S.A.), and apigenin from AK Scientific (Union City, CA, USA). I prepared the stock solutions in MeOH:H_2O (4:1) at a concentration between 10-30 mM except for isorhamnetin and naringenin which were prepared in Dimethyl sulfoxide (DMSO). I purchased isotopically labeled internal standards ($^{13}C_3$-Ferulic acid and $^{13}C_3$-Catechin from TRC (Toronto, On, CAN). I obtained a ready-to-use negative ion calibration solution for the calibration of Q-Exactive HF from Fisher Scientific. I prepared working solutions in MeOH:H_2O (80:20) by diluting the stock solutions. Working solutions were used to spiked into sample powder (spruce needle) in preparation for standard addition calibration and the fortified samples for optimization and validation.

I performed LC-MS analyses on Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled with Vanquish UHPLC pump and equipped with a HESI-II probe (Thermo Scientific, USA). The chromatographic separation

was achieved on reversed-phase Agilent Eclipse XDB C18 column (3.5 µm particle size; 3.0 x 100 mm; Agilent, Santa Clara, CA, USA) connected to ACQUITY UPLC CSH C18 VanGuard Pre-column, (1.7 µm, 2.1 mm X 5 mm). Solvent A was 0.1 % (v/v) formic acid in LC-MS grade water, and solvent B was 0.1 % (v/v) formic acid in LC-MS grade methanol. The binary gradient elution program was as follows t = 1 min, 1% B; t = 0.5 min 0 % B; t = 3.5 min, 60% B; t = 4.5 min 99% B; t = 5.71 min, 1% B; t = 8 min, 1% B. The sample injection volume and flow rates were 5 µL and 0.5 mL/min, respectively, while the column temperature was maintained at 30 °C. For MS analysis, the LC eluents were allowed to enter heated electrospray ionization (HESI-II) probe with the typical operating conditions of 370 °C auxiliary gas heater temperature, 320 °C capillary temperature, 2.70 kV spray voltage. Nitrogen was used as both auxiliary and sheath gas at a flow rate of 50 and 12 mL/min, respectively. Mass range m/z 70 to 1000 and resolution of 30,000 (FWHM) was employed. Data acquisition and processing were done using Thermo Scientific Xcalibur software (Version 4.1.31.9). Data acquisition was done using a parallel reaction method (PRM) and the data processing (peak identification and integration) was done using a dedicated quantification software Trace Finder 4.1 software (Thermo Scientific).

2.2.5 Testing the effects of host monoterpenes and phenolics on ESB performance

I freeze-dried the spruce needles for 72 h. This drying process removed almost all of the volatile and semi-volatile compounds from the needles and retained mainly phenolic compounds (Table S2.1). I ground the dried needles using TissueLyser II (Qiagen, Montreal, QC, CAN). I dissolved 2 g of Bacto-agar in 60 ml boiling water. Then I added McMorran diet (McMorran diet was purchased from the Great Lakes Forestry Centre, Canadian Forest Service (Sault Ste. Marie, ON, CAN) at 25% standard concentration plus 4.7 g ground needles. I divided the mixture into two groups. The first group was amended with monoterpenes and the second was without

monoterpenes. The first group was considered phenolics plus monoterpenes and the second group was phenolics alone. For each group, the sample size was five per family. The freeze-dried needles contained a total 19 of phenolic aglycon/flavonoid and glycosides (Fig. S3; hereafter "phenolics"). In this way, I prepared a stock diet for each family. I developed a blend of metabolites representing each of the ten families based on the constitutive monoterpene and phenolic concentrations (Tables 1 and 2). The stock diet of each family contained 11 monoterpenes (α-pinene, camphene, myrcene, terpinolene, bornyl acetate, α-terpineol, camphor, borneol, (−)-β-pinene, limonene, and β-phellandrene), and 19 phenolics (gallic acid, gallocatchin, 3,4-dihydroxybenzoic acid, catechin, protocatechuic aldehyde, pungenol, caffeic acid, vanillic acid, vanillin, piceol, taxifolin, p-coumaric acid, ferulic acid, myricetin, quercetin, naringenin, kampferol, apigenin, and isorhamnetin). I used concentrations of each monoterpene in an artificial diet according to the monoterpene profile of the ten families. The monoterpenes were dissolved in 1 ml ethanol before pouring into the media. Since phenolics were already present in the foliage, I did not mix and add them to the media (Table 2.3, Fig. S2.3). In addition to the two main treatments described above, I also included four control treatments: (1) 100% McMorran diet, (2) 100% McMorran diet with ethanol, (3) 25% McMorran diet, and (4) 25% McMorran diet with ethanol (n=5 for each of the four control treatments). The aim of the 100% McMorran diet was to see the development of budworms with a highly nutritious diet and without chemicals. Since I mixed ethanol in family blends so I also mixed ethanol in a 100% McMorran diet to see the impact of ethanol on budworm development. I also mixed a 25% McMorran diet in family blends in order to make the diet more nutritious to budworms and I placed a 25% McMorran diet as control as well.

I used small cups (60 ml) as an assay unit. I weighed the diet before pouring it into cups and allowed the amended diet to solidify under a fume hood for 2 h at room temperature. I obtained live ESB larvae from the Great Lakes Forestry Centre, Canadian Forest Service (Sault Ste. Marie, ON, CAN). I reared the larvae in the McMorran diet till the 4^{th} instar, weighed them, and placed one 4^{th} instar ESB larva at the center of each cup with an amended diet. I measured the larval weight again at the 6^{th} instar, and then at the pupal stage. I also weighed the feces of the ESB larvae and the remaining diet in each cup. Overall, I obtained larval mortality (yes/no and noted time in hours), initial and final larval weight (mg), ingested food (g), feces excreted (g), total larval development time (4^{th} to 6^{th} instar), pupal mass (mg), and adult emergence of each larva at the end of the experiment.

2.2.6 Data analysis

I calculated descriptive statistics for ESB diet consumed, weight change, feces excretion, pupal mass, development time, and adult emergence. I checked data for the assumptions of homoscedasticity and normality by using Levene's and Shapiro–Wilk tests, respectively. Where necessary, I transformed (log+1) data prior to analysis. I tested the impact of cluster and family on ESB responses for statistical significance by ANOVA, followed by *post-hoc* pair-wise differences using Tukey's HSD test. I used one-way ANOVA for different white spruce family treatments tested against ESB responses. For adult emergence I used chi-square test to observe difference within families and clusters. In addition, I conducted a linear regression analysis and plotted it in order to explore and visualize relationships between total monoterpene concentrations and ESB larval mortality time. I also conducted logistic regression analysis for adult emergence. A gradient analysis with white spruce families with different monoterpenes and phenolics was conducted via the 2D non-metric multidimensional scaling (NMDS) to visualize

the spread and relationship among chemicals and responses of ESB. I considered significant differences at α=0.05. Statistical software R v3.4.4 (R Core Team 2018) was used for all statistical analyses.

2.3 Results

I compared the data from clusters and individual families with the 25% McMorran diet alone or with ethanol because the diet in the main treatments used contained only 25% McMorran diet. I also showed the budworm performance on the 100% McMorran diet with or without ethanol. These results indicate that ethanol had no effect on budworm feeding while the reduction in the McMorran in the media significantly reduced budworm feeding. Interestingly, adding host tissues to the diet improved budworm performance overall.

2.3.1 Monoterpene and phenolic profiles of white spruce

Overall, I identified 11 monoterpene and 19 phenolic compounds in all families of white spruce. Among monoterpenes, camphor was the most abundant (330 ng mg^{-1}) followed by bornyl acetate (238 ng mg^{-1}) and limonene (135 ng mg^{-1}). Among phenolics, catechin showed higher concentrations (3,561 ng mg^{-1}) followed by pungenol (414 ng mg^{-1}) and gallocatchin (254 ng mg^{-1}). Family 1917 had the highest concentration of monoterpenes (1,786 ng mg^{-1}) and family 195 had the highest concentration of phenolics (5,385 ng mg^{-1}) (Tables 2.2 & 2.3).

2.3.2 Effects of white spruce clusters and families on ESB larval weight change

Larval weight changes in diets representing both clusters 1 and 2 were significantly higher than either control treatments (25% McMorran diet alone or with ethanol) (Fig. 2.1a). However, both clusters showed further differences. Weight change was significantly less in diets based on

cluster 1 with monoterpenes plus phenolics (M+P hereafter) than in phenolics alone (P) treatment. In contrast, diets based on cluster 2, larval weight change did not differ between M+P and P treatments. Overall, cluster 1 is characterized by higher concentrations of monoterpenes as compared to cluster 2 (Table 2.2, Fig. S2.2).

Similarly, larval weight changes significantly varied between control treatments and families (Fig. 2.1b). The families 1580, 195, and 1917 in cluster 1 and 2106 in cluster 2 with M + P treatment significantly reduced larval weight change and caused 100% larval mortality. Furthermore, I observed that all the families in cluster 1 with P treatment significantly lowered the larval weight but did not cause larval mortality.

2.3.3 Effects of white spruce clusters and families on ESB larval development time

The larval development time from 4th instar to pupation varied between diets from the two clusters and control treatments as well between the two clusters (Fig. 2.2a). No pupation occurred in either control treatment, therefore no development time was recorded. Development time was significantly lower in the M+P amended diet in cluster 1 than either P amended diet in cluster 1 or both treatment diets of cluster 2.

Larval development time varied between clusters and among families (Fig. 2.2b). Larvae failed to reach pupation in either control treatments and in families 1580, 195, and 1917 with M+P treatment in cluster 1 and family 2106 with M+P treatment in cluster 2, and thus development time was not recorded for these treatments. Among the remaining treatments, the development time was higher in family 195 with P treatment in cluster 1 and family 178 with M+P treatment in cluster 2. Family 1978 with P treatment showed significantly lower development time as compared to all other families.

2.3.4 Effects of white spruce clusters and families on ESB adult emergence

The adult emergence varied between diets of the two clusters and control treatments (Fig. 2.3a). Cluster 1 with monoterpenes treatment showed lower adult emergence. In contrast, the adult emergence was similar in cluster 1 with M+P treatment. However, cluster 2 with both M+P and P treatments showed significantly higher adult emergence.

The families also showed differences in adult emergence (Fig. 2.3b). Both M+P and P treatments of family 2105 in cluster 1 and family 156 in cluster 2 showed 100% of adult emergence. In contrast, no adult emergence was observed in either control treatments and in M+P treatment in families 1580, 195, and 1917 in cluster 1 and 2106 in cluster 2. Among the remaining families, adult emergence was lower in M+P treatment in family 1978 and P treatment in family 1917 in cluster 1 as compared to other families in both clusters.

2.3.5 Effects of white spruce families on overall ESB performance

I conducted a gradient analysis to observe the relationship between families and budworm performance (Fig. 2.4a). This analysis indicated that all ten families spread widely in terms of the type of budworm performance measured. Overall, budworm performance was reduced in families (1917, 2106, 1580, 156, 195) with higher monoterpenes (limonene, total monoterpenes, α-pinene, β-pinene, myrcene, terpinolene, and camphene) and phenolics (piceol, apigenin, kampferol, and taxifolin) concentrations. In contrast, families (2105, 1978, 1924, and 178) with total phenolics, catechin, ferulic acid, naringenin, and gallocatchin improved budworm performance.

I conducted another gradient analysis to observe the association between families and budworm performance (Fig. 2.4b). This analysis revealed that budworm performance varied

among families. Family 195 with some higher phenolics showed increased development time. In contrast, families 178 and 1924 with phenolics (piceol and pungenol) increased the budworm weights.

2.3.6 Effects of white spruce families on ESB mortality

Regression analysis revealed a positive correlation between monoterpene concentrations and budworm mortality as the monoterpene concentration increased so did the budworm mortality (P<0.001, R^2=0.85; Fig. 2.5a). However, regression analysis for the total monoterpene plus the total phenolic concentrations did not show any relationship (P=0.965, R^2=0.05; Fig. 2.5b). Since the total monoterpenes were not correlated with the total phenolic concentrations (Fig. S4), this suggests that they had an independent effect on budworm performance. Interestingly, the family 1917 (cluster 1) with total monoterpene concentrations of 1800 ng mg^{-1} caused budworm mortality within 24 h (Table 2.4). Likewise, the family 1580 (cluster 1) with total monoterpene concentrations of 1,491 ng mg^{-1} caused 100% mortality within 48 h. However, the larval mortality in families (195 in cluster 1 and 2106 in cluster 2) with total monoterpene concentrations between 1,141 and 1,143 ng mg^{-1} was 100% but it took 72 h to reach that level of mortality. No mortality was observed in families having monoterpenes concentrations less than 1100 ng mg^{-1}.

2.3.7 Effects of total monoterpenes and total phenolics on ESB performance

Regression analysis showed a strong relationship between total monoterpene concentrations and budworm weight change as the total monoterpene concentrations increased the budworm weight decreased (P<0.001, R^2=0.77; Fig. 2.6a). Furthermore, increasing monoterpene plus phenolic concentrations also decreased the budworm weight change (P<0.001, R^2=0.34; Fig. 2.6b). There was no relationship between the total monoterpene concentrations and the budworm

development time (P=0.62, R^2=0.02; Fig. 2.6c). In contrast, I detected a positive relationship between monoterpene plus phenolic concentrations and budworm development time (P=0.05, R^2=0.19; Fig. 6d). Furthermore, logistic regression revealed that increasing monoterpene concentrations and monoterpene plus phenolic concentrations also reduced the adult emergence (P<0.001; Fig. 6e, P<0.001, Fig. 2.6f).

2.3.8 The proportion of monoterpenes and phenolics in each white spruce family

I used pie charts to show the proportion of monoterpenes and phenolics in each spruce family (Fig. 2.7). Overall, families 1580 and 1917 had a higher proportion of monoterpenes (0.60 and 0.55 respectively) and a lower proportion of phenolics (0.40 and 0.45, respectively). The remaining eight families contained higher proportions of phenolics, ranging from 0.57 to 0.77. Furthermore, the results of the Pearson correlation of individual monoterpenes and phenolics against each other indicated that some individual monoterpenes were correlated to each other (Fig. S4), supporting the results of regression analysis between total monoterpenes concentrations and budworm mortality timing. However, most of the individual phenolic compounds were not correlated with each other (Fig. S2.4).

2.3.9 Ranking suitability

Based on the results of the proportion of monoterpenes and phenolics and the larval performance bioassays, I ranked the suitability of white spruce families for budworm feeding development (Table 2.4). The proportion of monoterpenes ranged from 0.60 to 0.40 in the least suitable families (1580, 1917, 2106, 195) that caused 100% larval mortality; in the same families, the proportion of phenolics ranged from 0.40 to 0.60. In contrast, families with a relatively higher

proportion of phenolics (≥0.62) and a lower proportion of monoterpenes (≤0.40) were most suitable for budworms.

2.4 Discussion

Secondary metabolites are critical components of conifer defenses against insect defoliators. However, the proportions and concentrations of these metabolites vary within a tree species, resulting in phenotypic variations in tree resistance to defoliating insects in nature. Prior to the current study, the defensive role of monoterpenes and phenolics against ESB was demonstrated only for a few individual monoterpenes and phenolics. In contrast, I tested the suitability of selected families to ESB based on the complete monoterpene and phenolic profiles of mature white spruce trees. This is the first study that characterized the monoterpene and phenolic profiles of white spruce trees and discussed their relative contributions in spruce defenses to ESB. I identified 11 monoterpenes and 19 phenolic compounds. Major monoterpenes are α-pinene, β-pinene, limonene, bornylacetate, camphor, camphene, and myrcene, and major phenolics are catechin, pungenol, piceol, gallocatchin, kampferol, and taxifolin. The proportions of monoterpenes varied from 0.23 to 0.60 and the phenolics from 0.40 to 0.77. The concentrations of monoterpenes and phenolics varied from 419.00 to 1,796.00 ng mg^{-1}, and from 3,140.00 to 5,385.00 ng mg^{-1}, respectively. Overall, I found strong defense phenotype-budworm interactions. In particular, the budworms died or failed to complete their development in white spruce foliage that contained higher proportions and concentrations of monoterpenes. These results have not only improved our understanding of intraspecific variations in conifer defense responses to defoliators but also provided much-needed empirical evidence on the impact of monoterpenes and phenolics in spruce defenses on budworm performance.

Monoterpenes and phenolics differentially affected the budworm response depending on their proportions and concentrations in white spruce foliage. Particularly, the least suitable families that caused 100% budworm mortality had higher proportions (0.40–0.60) and concentrations (1,143–1,796) of monoterpenes. In contrast, the most suitable families of budworms had higher phenolic proportions (0.62–0.77) and concentrations (4,473–5,099) and relatively lower proportions (0.23–0.38) and concentrations (419–985) of monoterpenes. Altogether these results suggest that variations in monoterpene and phenolic profiles can influence white spruce resistance to ESB. In particular, the higher the proportions of monoterpenes in spruce foliage, the greater the toxicity to the budworm.

In general, monoterpenes tend to have a stronger insecticidal activity than phenolics whereas phenolics tend to have stronger fungicidal activity than monoterpenes (Cates *et al.*,1987; Zao & Cates, 1997; Abou-Zaid *et al.,* 2000; Witzell & Martín 2008, Daoust *et al.,* 2010; Delvas *et al.,* 2011; Cheynier *et al.,* 2013; Mageroy *et al.,* 2015). I showed that white spruce families containing higher concentrations of monoterpenes had a strong insecticidal impact on the budworms. For instance, the families 1917 and 1580 having total monoterpene concentrations of 1,796 and 1,491 respectively caused 100% mortality to the budworm in the first 24h to 48 h. The families 195 and 2106 having total monoterpene concentrations of 1,143 and 1,141 respectively caused 100% budworm mortality in 72 h. To the best of my knowledge, this is the first report on how monoterpene and phenolic defense phenotypes of white spruce foliage affect ESB performance. Earlier, Bauce *et al.* (1994) observed the higher spruce budworm mortality in *Abies balsamea* to the high concentrations of 3-carene, terpinolene, and bornyl acetate. Similarly, Bauce & Kumbasli (2007) reported that only resistant white spruce trees contained borneol and 3-carene. In the current study, even at the highest concentrations (3,140 to 5,385) phenolics did

not cause any budworm mortality but reduced adult emergence and increased larval development time. Earlier, Delvas *et al.* (2011) reported that two phenolic compounds individually and collectively reduced the performance of the budworms. Since the Pearson correlation analysis indicated that monoterpenes were not correlated with phenolics, my results indicate that monoterpene's impact on budworm was independent of phenolics.

In the current study, white spruce families showed substantial variation in their monoterpene and phenolic concentrations. The total monoterpenes ranged from 702 to 1,796. Camphor (106–705) and bornyl acetate (104–356) were the most abundant monoterpenes. The phenolic concentrations were higher than monoterpene concentrations and ranged from 3,140 to 5,385. Catechin (2,472–4,530) was the most abundant phenolic in white spruce foliage. Overall, monoterpene concentrations in the current study were higher and phenolic concentrations were lower as compared to white spruce populations in eastern Canada. For example, Despland *et al.* (2016) reported that the total monoterpene concentration in a 48-year-old white spruce population in Drummondville (Quebec) was 575 where camphene (120) followed by bornyl acetate (100) were the two most abundant monoterpenes. Similarly, Delvas *et al.* (2011) quantified phenolics in the 43-year-old white spruce population at the Sainte Cyrille de Wendover experimental forest near Drummondville and showed the amount of pungenol was 2,700 and piceol was 3,370. In contrast, in my study pungenol concentration ranged from 182 to 773 and piceol concentration ranged from 3.0 to 31.9.

There appear to be natural variations in monoterpene and phenolic composition of white spruce foliage among families in this study as well as in earlier studies. Such variations may be an adaptive evolutionary response to a large diversity of enemies and environments (Futuyma & Agrawal, 2009). Typically, having large variations in both proportions and concentrations of

different defense compounds likely limits herbivore damage, given that insect herbivores cannot overcome all defense chemicals they encounter, and assures the species' survival (Daoust *et al.,* 2010; Delvas *et al.,* 2011; Kumbasli & Bauce, 2013). Based on these results, I hypothesize that monoterpenes and phenolics play complementary roles against ESB depending on their proportions and concentrations. At high concentrations of monoterpenes (>1,141), budworms likely fail to complete their development due to the acute toxicity of monoterpenes. Here, phenolics likely play a minor role in causing direct budworm mortality. In contrast, at low concentrations of monoterpenes, phenolics play a major role in reducing budworm feeding, resulting in delayed development time; this likely increases the exposure of budworms to their natural enemies (i.e., indirect defenses) (Quayle *et al.,* 2003; Parent *et al.,* 2017).

The importance of the proportions and concentrations of monoterpenes and phenolics in determining host suitability indicates that ESB is sensitive to both the quantity and quality of these compounds. Based on my results, I developed susceptibility indices for the white spruce-ESB system. Similar indices were developed for *Pinus contorta* var. *latifolia* against *Dendroctonus ponderosae* (Ullah *et al.,* 2021). From an applied perspective, such indices can help tree breeders to screen resistant white spruce phenotypes (chemical fingerprinting); phenotypes screened for resistance can be used in roguing of orchards used for operational seedling production and future breeding for next-generation orchards.

In the current study, I conducted the laboratory feeding bioassays based on the result of well-replicated field surveys; however, I faced certain limitations in conducting the feeding bioassays. I conducted my bioassays by using the natural variations in the phenolic composition of white spruce foliage and manipulating the foliar monoterpenes using synthetic chemicals. However, this approach did not diminish the quality of my research because while freeze-drying

substantially removed monoterpenes from white spruce needles (Table S2.1) it had no or minimal effect on phenolics (Table 2.3), suggesting that my bioassay truly reflected the phenolic and monoterpene composition of white spruce foliage. Since I detected a range of variations from positive to negative impacts on ESB performance in response to the amendments, the only reasonable explanation for the observed results was the differences in the media content. Thus, the results of this study can help contribute to our understanding of the defense phenotypes of white spruce trees in nature.

In conclusion, in the current study, I highlighted the importance of defense chemistry in white spruce-ESB interactions. Earlier studies have suggested four factors in the same system (Pureswaran et *al*., 2016; Régnière *et al*., 2019; Berguet *et al*., 2021). Firstly, host phenology, particularly the timing of budburst and leaf flush, can be an important factor of spruce budworm outbreaks by affecting their survival and development (Pureswaran *et al*., 2015, 2019). Secondly, natural enemies of spruce budworms such as parasitoids, pathogens, and birds can affect the population dynamics of spruce budworms by asserting different levels of mortality at different life stages of budworms (Royama, 1984; Eveleigh *et al*., 2007; Venier & Holmes, 2010; Bouchard *et al*., 2018). Thirdly, the nutritional quality of host foliage can affect larval development as the budworm larvae are largely limited by nitrogen (Mattson *et al.*, 1991). Finally, forest stand structure and composition can influence the spruce budworm dispersal as well as the dispersal of natural enemies (Ludwig *et al*., 1978; MacLean & MacKinnon, 1997; Hennigar *et al*., 2008; Martin *et al*., 2019). Therefore, I conclude that in addition to the previously suggested four factors, white spruce chemical defenses can also be an important factor of the white spruce-spruce budworm interactions.

Table 2.1. Effects of *Picea glauca* monoterpenes and phenolics on different developmental stages of *Choristoneura fumife*

Budworm stages	**Phenolics**	**References**	**Monoterpenes**	**References**
Oviposition	Not Available	Not Available	Individual α-Pinene, β-Pinene, Limonene, and Camphene: Increased oviposition	Grant *et al*., 2007
			α-Pinene, β-Pinene, Limonene, and Myrcene: Combined with white spruce epicuticular waxes: Increased oviposition	Ennis *et al*., 2017
Larval survival	Combination of piceol and pungenol: Caused 64% mortality.	Delvas *et al.,* 2011	Individual α-Pinene and 3-Carene: Caused 22% larval mortality	Kumbasli & Bauce, 2013
			Combination of tricyclene, α-Pinene, Camphene, β-Pinene, Myrcene, 3-Carene, Limonene, δ-Phellandrene, Terpinolene, Borneol and Bornyl acetate: Caused 48% larval mortality	Kumbaşlı *et al*., 2011
Larval weight	Higher concentrations of total phenolics: Reduced larval weight.	**This study**	Individual α-Pinene, Bornyl Acetate, Camphene, 3-Carene, Terpinolene and Tricyclene: Reduced larval growth rate	Kumbasli & Bauce, 2013
Development time	Combination of piceol and pungenol: Retarded development time.	Delvas *et al.,* 2011	Not Available	Not Available
	Family 195 with higher concentration of phenolics 5,385 ng mg^{-1}: Increased larval development time.	**This study**		
Pupal mass	Combination of piceol and pungenol: Caused 50% loss of pupal mass.	Delvas *et al.,* 2011	Not Available	Not Available
Adult emergence	Higher concentration of total phenolics: Decreased adult emergence.	**This study**	Lower concentration of monoterpenes and phenolics: Increased adult emergence	**This study**

Table 2.2. The monoterpene concentration (ng mg^{-1}) in different *Picea glauca* families and clusters used for insect bioassays. The units are based on the fresh weight of foliage.

	Cluster 1					**Cluster 2**				
Family	**2105**	**1978**	**1580**	**195**	**1917**	**1951**	**156**	**1924**	**178**	**2106**
α-Pinene	47.9	41.9	90.8	73.2	99.1	25.0	38.3	26.6	45.8	75.8
Camphene	75.3	73.9	134.4	113.1	161.4	38.1	67.1	42.1	75.0	111.8
β-Pinene	4.8	4.5	11.0	7.2	13.9	3.1	3.7	3.1	5.6	11.8
Myrcene	66.2	105.7	216.4	52.3	172.5	73.9	49.2	92.3	46.6	87.1
Limonene	83.2	81.2	273.8	246.5	220.3	58.3	78.3	89.5	94.3	127.1
β-Phellandrene	0.0	9.2	4.6	4.2	14.4	0.0	2.2	0.0	0.0	1.8
Terpinolene	7.1	11.7	17.4	14.7	24.6	5.4	11.2	8.0	10.2	13.3
Camphor	252.1	377.5	353.2	382.3	705.0	105.5	191.1	266.1	285.8	378.0
Bornyl Acetate	171.0	265.6	356.4	226.2	350.6	104.2	211.0	165.0	224.9	303.0
α-Terpineol	0.0	3.1	6.4	3.6	15.5	0.0	4.8	1.2	5.0	13.9
Borneol	14.5	10.5	26.1	19.9	18.4	5.3	16.0	7.7	12.9	17.9
Total	*722.1*	*984.6*	*1490.5*	*1143.2*	*1795.7*	*418.8*	*672.9*	*701.7*	*806.1*	*1141.3*

Table 2.3. The phenolic concentrations (ng mg^{-1}) in different *Picea glauca* families and monoterpene clusters are shown in Table 1 above. The units are based on the dry weight.

Phenolic compounds	***Picea glauca* Families**									
	Cluster 1					**Cluster 2**				
	2105	**1978**	**1580**	**195**	**1917**	**1951**	**156**	**1924**	**178**	**2106**
Gallic acid	6.0	6.7	6.0	6.0	6.6	6.3	6.6	6.3	6.3	6.1
Gallocatchin	294.0	228.0	185.0	317.4	319.6	246.5	272.1	273.2	185.0	216.0
3,4-dihydroxybenzoic acid	4.6	13.6	5.9	6.5	10.9	8.2	11.6	7.9	6.7	5.4
Catechin	3869.0	3780.0	2472.0	4529.9	3361.5	3478.3	3280.9	3471.3	3415.0	3954.0
Protocatecuic aldehyde	0.4	0.5	0.4	0.4	0.5	0.5	0.5	0.4	0.4	0.4
Pungenol	255.0	773.0	182.0	210.3	727.1	297.7	635.3	416.9	357.0	284.0
Caffeic acid	2.0	12.4	5.5	9.8	17.8	23.3	16.8	12.3	5.4	19.7
Vanillic acid	0.0	0.0	10.7	0.0	9.1	12.8	6.9	18.9	6.6	0.0
Vanillin	6.0	0.0	2.1	2.2	5.4	2.2	0.0	3.7	3.3	2.3
Piceol	4.4	15.2	3.3	5.6	11.1	12.3	31.9	12.4	6.5	7.7
Taxifolin	24.8	37.6	34.1	112.0	21.9	93.6	58.7	56.3	108.0	67.6
p-coumaric acid	2.8	7.8	2.3	3.3	4.1	3.9	14.4	6.3	3.4	4.0
Ferulic acid	5.1	4.7	4.5	2.9	6.7	3.7	5.2	10.5	3.9	5.4
Myricetin	3.2	3.2	8.4	24.7	3.3	6.4	20.3	6.6	20.6	4.8
Quercetin	22.6	31.8	20.6	20.0	18.7	20.7	26.5	17.7	38.5	24.4
Naringenin	1.4	1.1	7.5	1.7	5.2	1.4	1.7	3.9	12.7	1.7
Kampferol	160.0	176.0	185.0	128.0	158.4	249.9	219.2	172.0	156.0	192.0
Apigenin	1.2	1.6	1.0	1.1	1.5	2.1	2.6	1.3	1.3	1.6
Isorhamnetin	3.4	6.2	4.5	2.8	4.4	3.4	5.1	2.9	3.1	7.5
Total	*4665.0*	*5099.0*	*3140.0*	*5384.8*	*4693.7*	*4472.9*	*4616.6*	*4501.1*	*4340.0*	*4805.0*

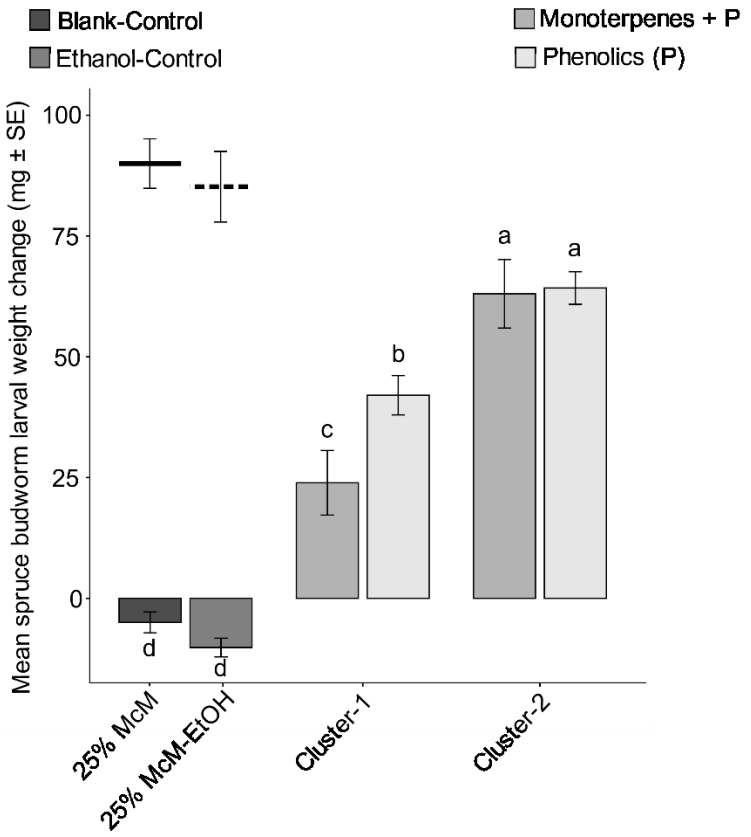
Table 2.4. Ranking suitability of *Picea glauca* to *Choristoneura fumiferana* based on % larval mortality, proportion and concentrations of monoterpenes and phenolics in each family

Suitability Ranking	Families	% larval mortality (h)	Total monoterpene concentrations (ng mg^{-1})	Proportion of total monoterpenes	Total phenolic concentrations (ng mg^{-1})	Proportion of total phenolics
Least suitable for larval feeding	1917	100% in 24 h	1796	≥ 0.55 ± 0.2	4694	< 0.45 ± 0.2
	1580	100% in 48 h	1491	≥ 0.60 ± 0.2	3140	< 0.40 ± 0.2
	2106	100% in 72 h	1141	≥ 0.43 ± 0.1	4805	< 0.57± 0.2
	195	100% in 72 h	1143	≥ 0.40 ± 0.1	5385	< 0.60± 0.3
Most suitable for larval feeding	1978	No mortality	985	< 0.38 ± 0.1	5099	≥ 0.62 ± 0.2
	178	No mortality	806	< 0.37 ± 0.1	4340	≥ 0.63 ± 0.3
	2105	No mortality	722	< 0.36 ± 1	4665	≥ 0.64 ± 0.3
	1924	No mortality	701	<0. 33 ± 0.1	4501	≥ 0.67 ± 0.3
	156	No mortality	673	< 0.32 ± 0.1	4617	≥ 0.68 ± 0.3
	1951	No mortality	419	< 0.23 ± 0.1	4473	≥ 0.77 ± 0.3

Table S2.1. Concentrations of individual monoterpenes (ng mg^{-1}) in *Picea glauca* foliage remaining after the freeze-dry treatment. The units are based on fresh weight. Concentrations without the freeze-dry treatment were reported on Table 2.2.

	Cluster 1					Cluster 2				
Family	**2105**	**1978**	**1580**	**195**	**1917**	**1951**	**156**	**1924**	**178**	**2106**
α-Pinene	0.07	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Camphene	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-Pinene	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Myrcene	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Limonene	0.2	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.1
β-Phellandrene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Terpinolene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Camphor	0.2	0.1	0.2	0.2	0.1	0.0	0.1	0.0	0.0	0.1
Bornyl Acetate	0.8	0.3	0.7	0.5	0.4	0.2	0.2	0.2	0.3	0.5
α-Terpineol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Borneol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	1.4	0.5	1.6	0.8	0.7	0.3	0.3	0.3	0.5	0.8

Figure 2.1 (a)


Blank-Control
Ethanol-Control
Monoterpenes + P
Phenolics (P)
Mean spruce budworm larval weight change (mg ± SE)
100
75
50
25
0
a
a
b
c
d
d
25% McM
25% McM-EtOH
Cluster-1
Cluster-2

(b)

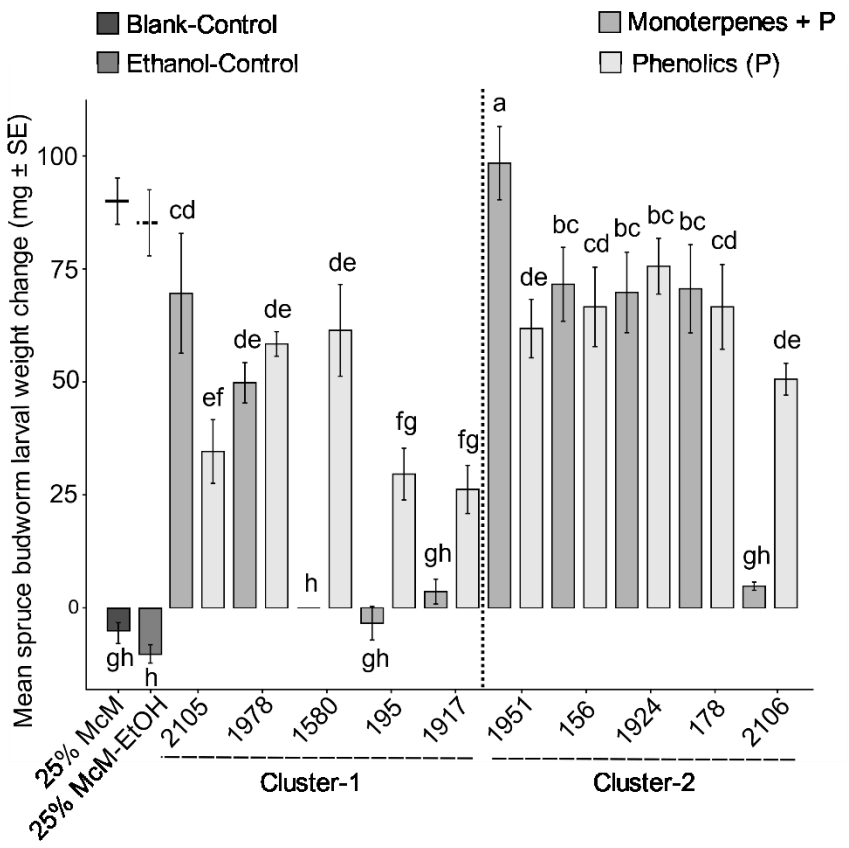


Figure 2.1. Mean (±SE) larval weight change (mg) of *Choristoneura fumiferana* in two *Picea glauca* clusters **(a)** and ten families **(b)**. Bars with different letters are statistically different (Tukey HSD tests, $P < 0.001$). Horizontal solid and dashed lines above the control treatment on the left of figure (a) and (b) represent the means (±SE) of 100% McMorran diet alone or with ethanol, respectively. A vertical dot line on (b) separates Clusters 1 and 2. McM refers to McMorran and EtOH refers to ethanol.

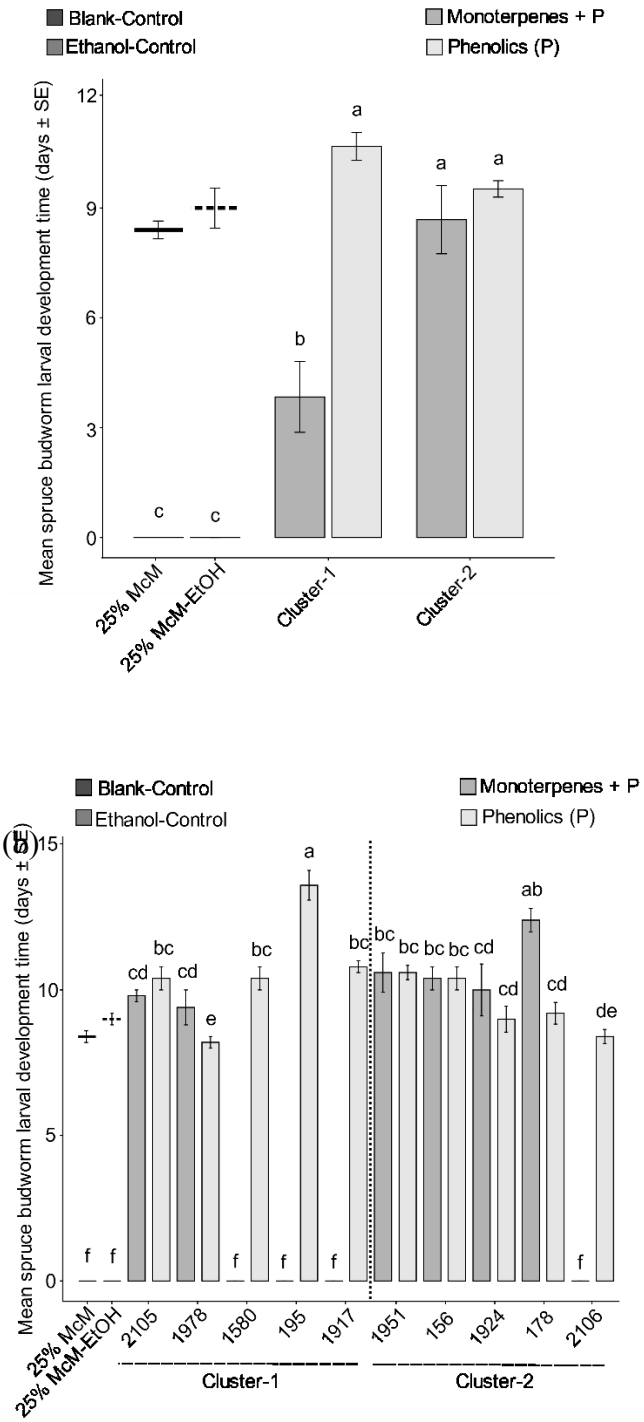
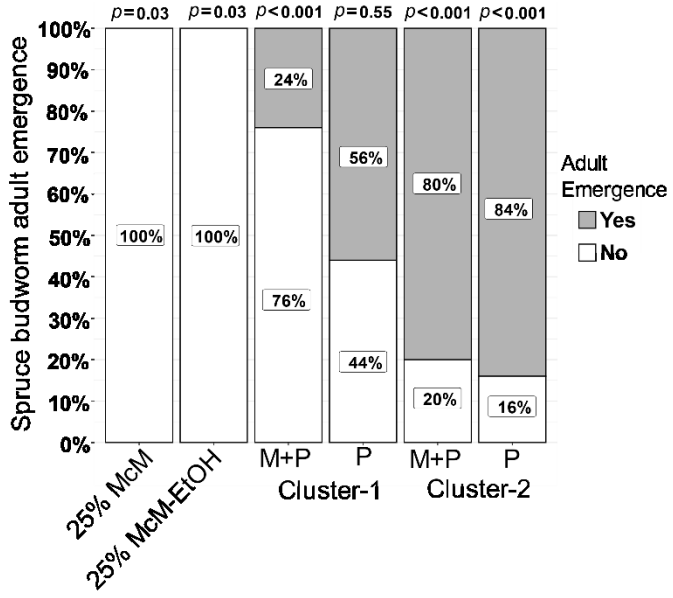


Figure 2.2. Mean (±SE) development time (days) of *Choristoneura fumiferana* in two *Picea glauca* clusters **(a)** and ten families **(b)**. Bars with different letters are statistically different (Tukey HSD tests, P <0.001). Horizontal solid and dashed lines above the control treatment on the left of figure (a) and (b) represent the means (±SE) of 100% McMorran diet alone or with ethanol, respectively. A vertical dot line on (b) separates Clusters 1 and 2. McM refers to McMorran and EtOH refers to ethanol.

(a)



(b)

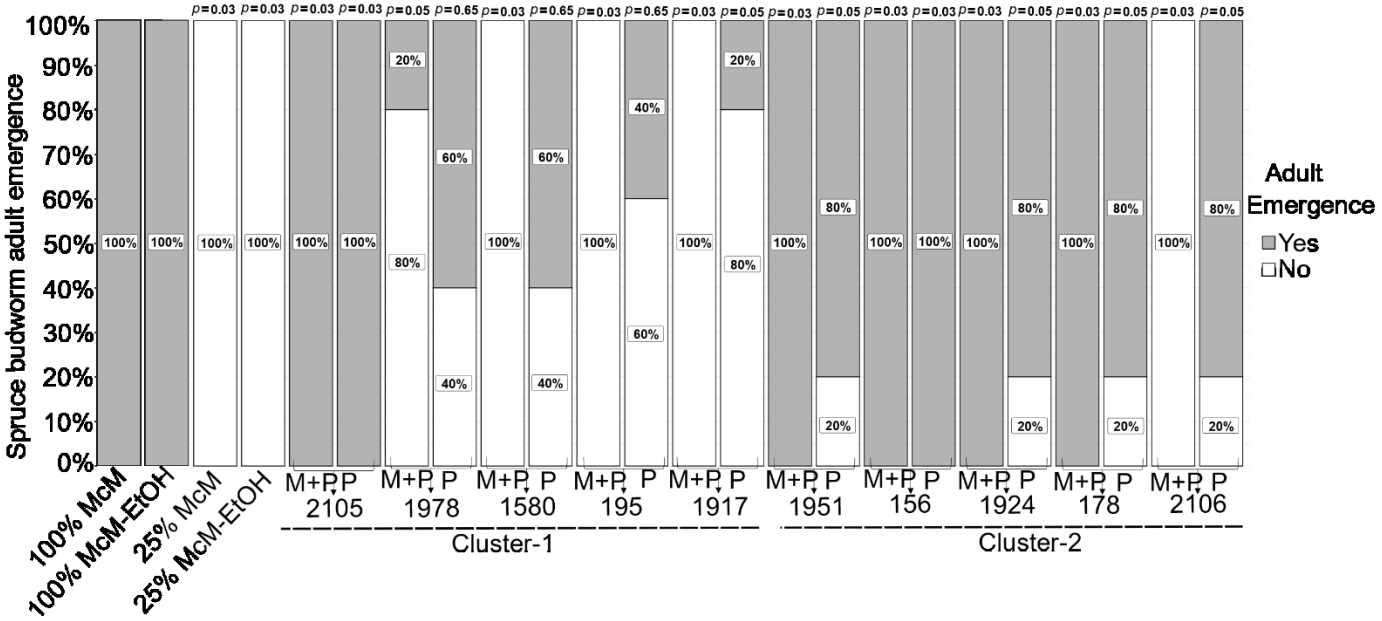
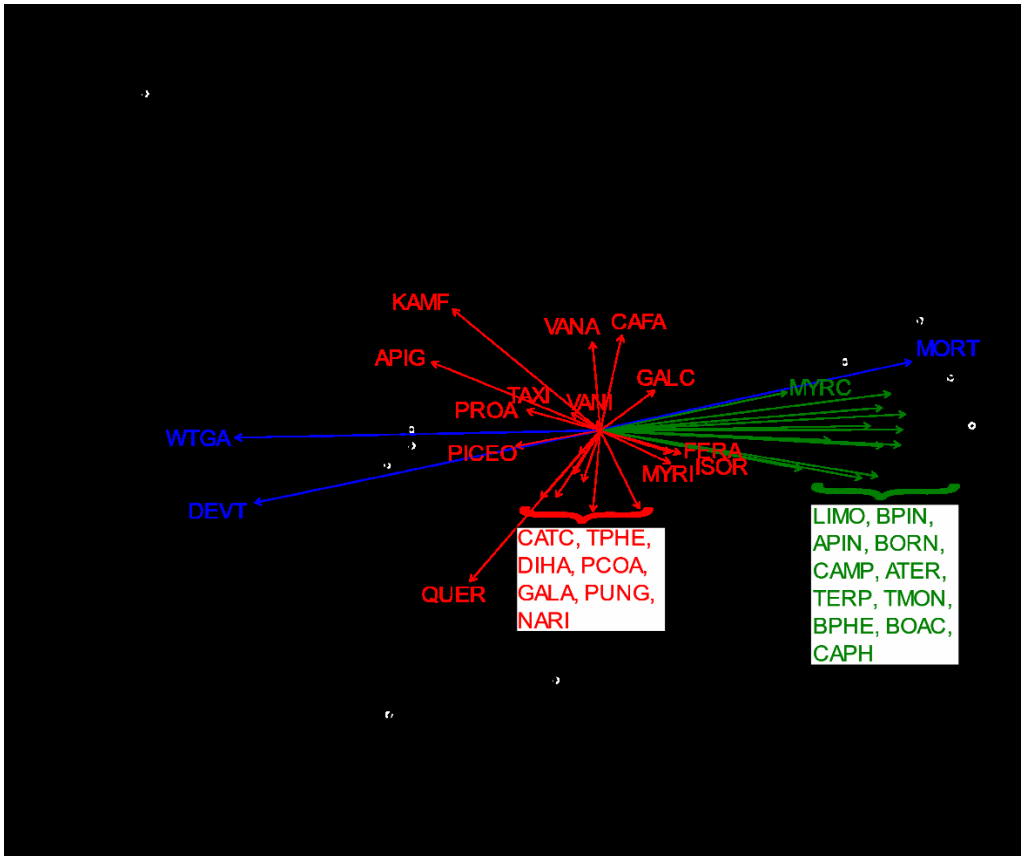


Figure 2.3. Adult emergence (percentage) of *Choristoneura fumiferana* in two *Picea glauca* clusters **(a)** and ten families **(b)**. Grey colours show successful (yes) and white colours show fail (no) adult emergence. M+P refers to monoterpenes plus phenolics and P refers to phenolics. McM refers to McMorran and EtOH refers to ethanol.


KAMF
VANA
CAFA
APIG
MORT
GALC
TAXI
VANI
MYRC
PROA
WTGA
PICEO
FERA
ISOR
MYRI
DEVT
CATC, TPHE, DIHA, PCOA, GALA, PUNG, NARI
QUER
LIMO, BPIN, APIN, BORN, CAMP, ATER, TERP, TMON, BPHE, BOAC, CAPH

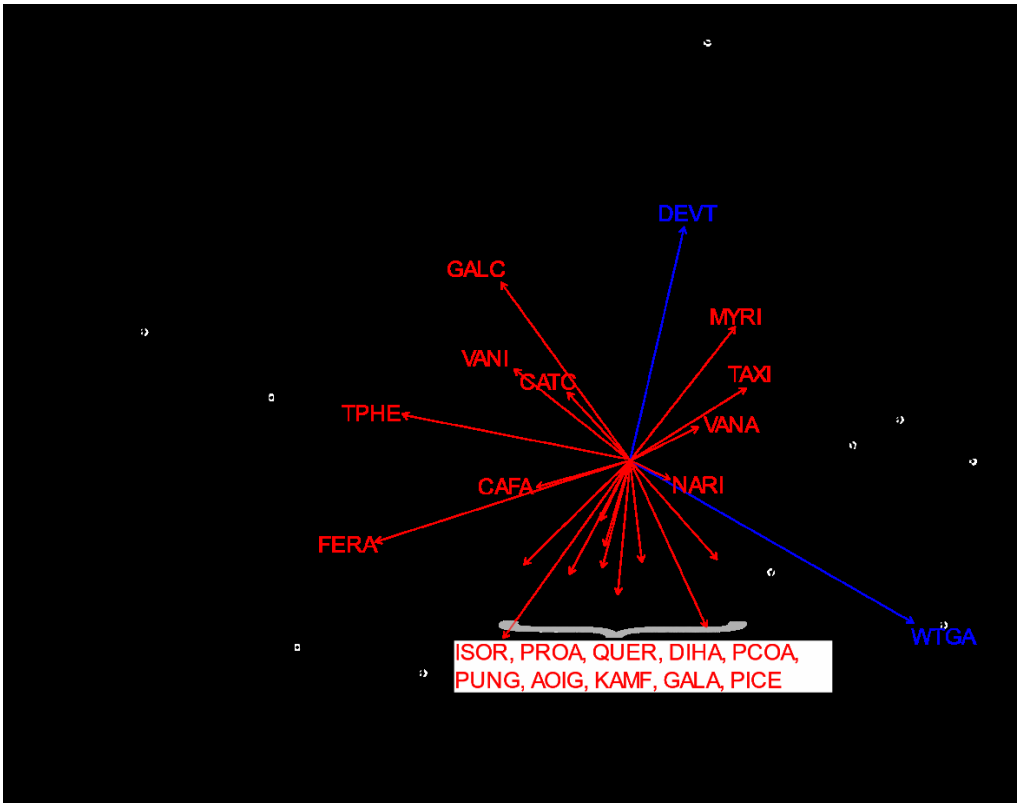

DEVT
GALC
MYRI
VANI
CATC
TAXI
TPHE
VANA
CAFA
NARI
FERA
WTGA
ISOR, PROA, QUER, DIHA, PCOA, PUNG, AOIG, KAMF, GALA, PICE

Figure 2.4. Non-metric multidimensional scaling plot of the effects of different *Picea glauca* families on the larval performance of *Choristoneura fumiferana*. Numbers represent the ten families tested in the bioassays. (a) Phenolics plus monoterpenes, (b) only phenolics. Green and red vectors represent individual monoterpene and phenolic compounds respectively and blue vectors show insect response. Individual concentrations of monoterpenes were shown in Table 2.2. Abbreviations for insect performance: MORT= mortality, WTGA = weight gain, DEVT = development time. Abbreviations for monoterpenes: BPIN = β-pinene, APIN= α-pinene, LIMO = limonene, MYRC= myrcene, CAMP = camphene, CAPH = camphor, BPHE = β-phellandrene, BORN = borneols, BOAC= bornyl acetate, TERP = terpinolene, and TMON = total monoterpenes. Abbreviations for phenolics: PICE = piceol, PUNG = pungenol, KAMF = kampferol, MYRI = myricetin, GALC = gallic acid, NARI = naringenin, FERA = ferulic acid, CATC = catechin, GALC = gallocatchin, ISOR = isorhamnetin, TAXI = taxifolin, APIG = apigenin, and TPHE = total phenolics. Since phenolics alone did not cause any larval mortality, (b) does not contain a mortality vector.

Figure 2.5

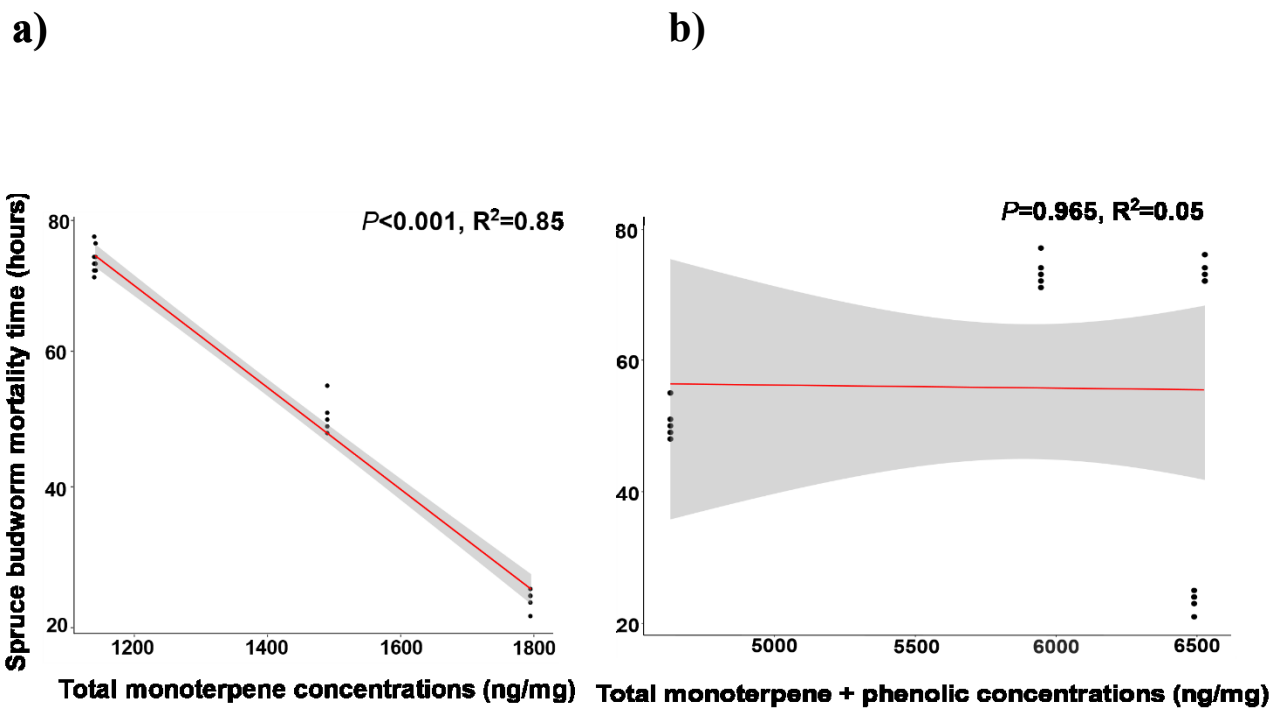


Figure 2.5. The relationship between total monoterpene concentrations (ng mg^{-1}; **a**), total monoterpene plus phenolic concentrations (ng mg^{-1}; **b**) of *Picea glauca* and time (hours) taken for *Choristoneura fumiferana* mortality. Line and shad denote regression line and 95% confidence interval, respectively.

Figure 2.6

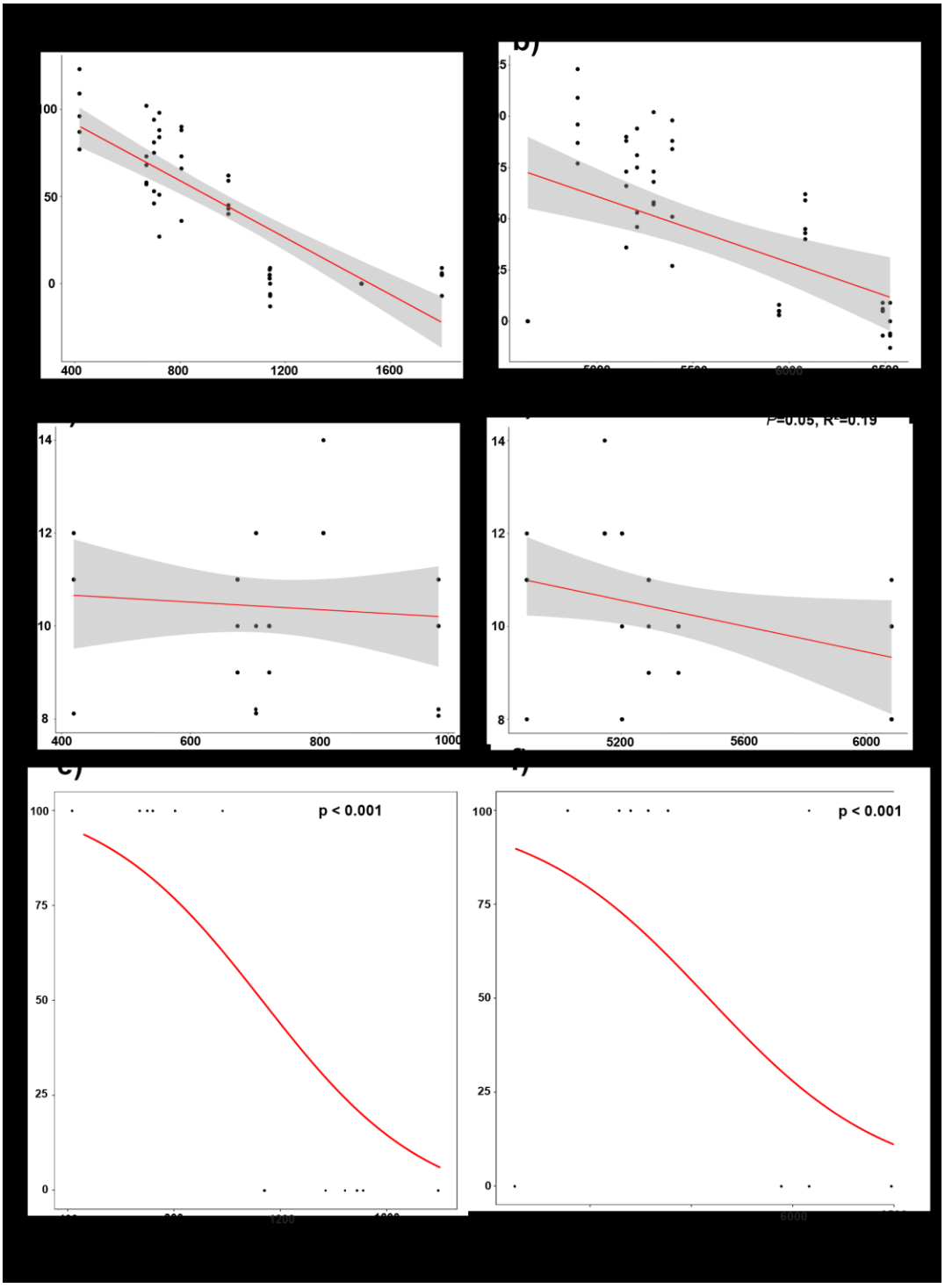


Figure 2.6. Relationship of monoterpene (left panel) and monoterpenes plus phenolic (right panel) concentrations (ng mg−1) of *Picea glauca* and *Choristoneura fumiferana* weight change (a,b), development time (c,d) and adult emergence (e,f). Solid red lines and shad denote the regression line and 95% confidence interval, respectively. Figure 6 (e,f) shows the logistic regression analysis and the red curve represent the logistic trend.

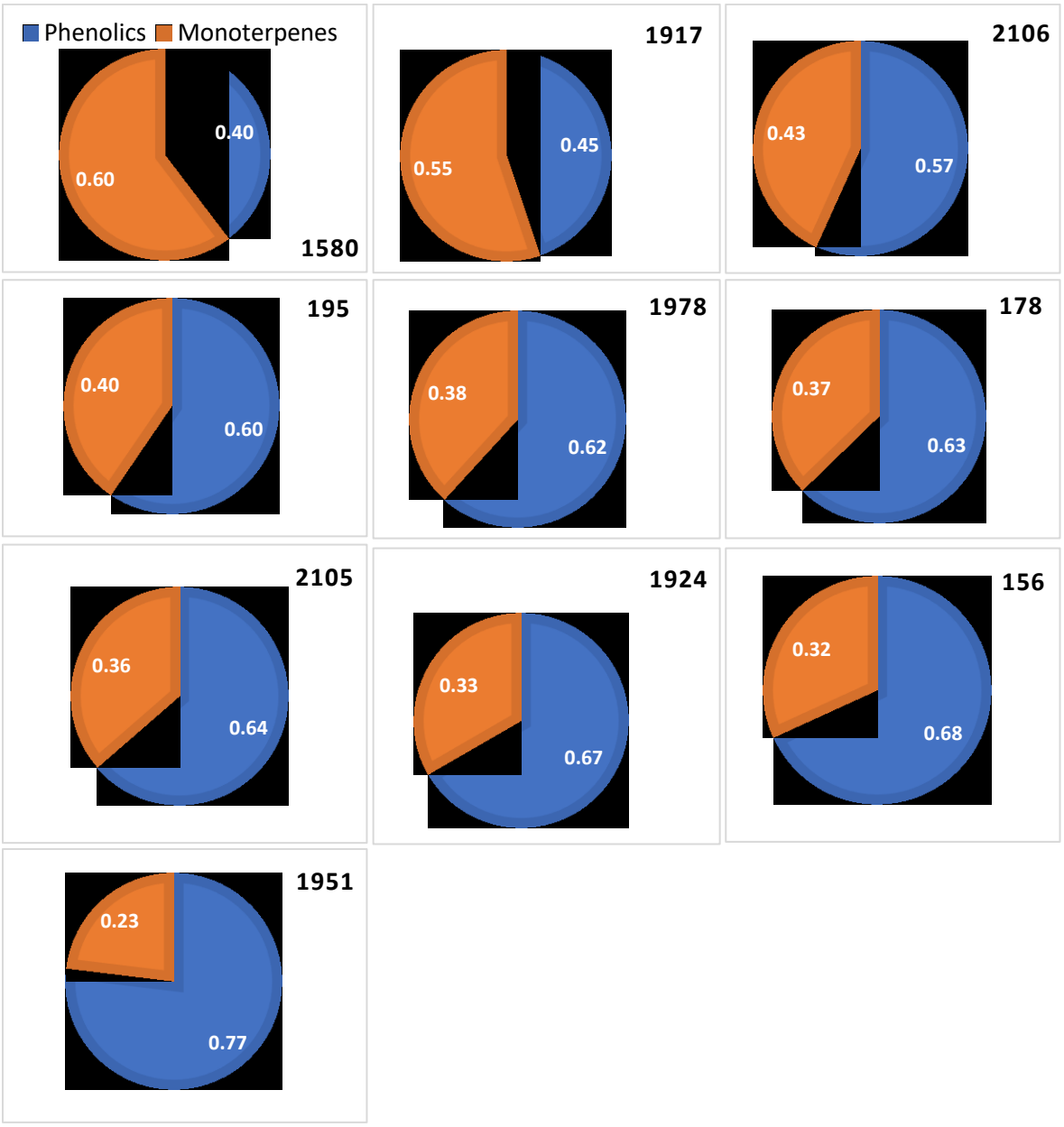


Figure 2.7. Pie charts showing the proportion of monoterpene and phenolic compounds in each of ten families of Picea glauca used in Choristoneura fumiferana bioassay.

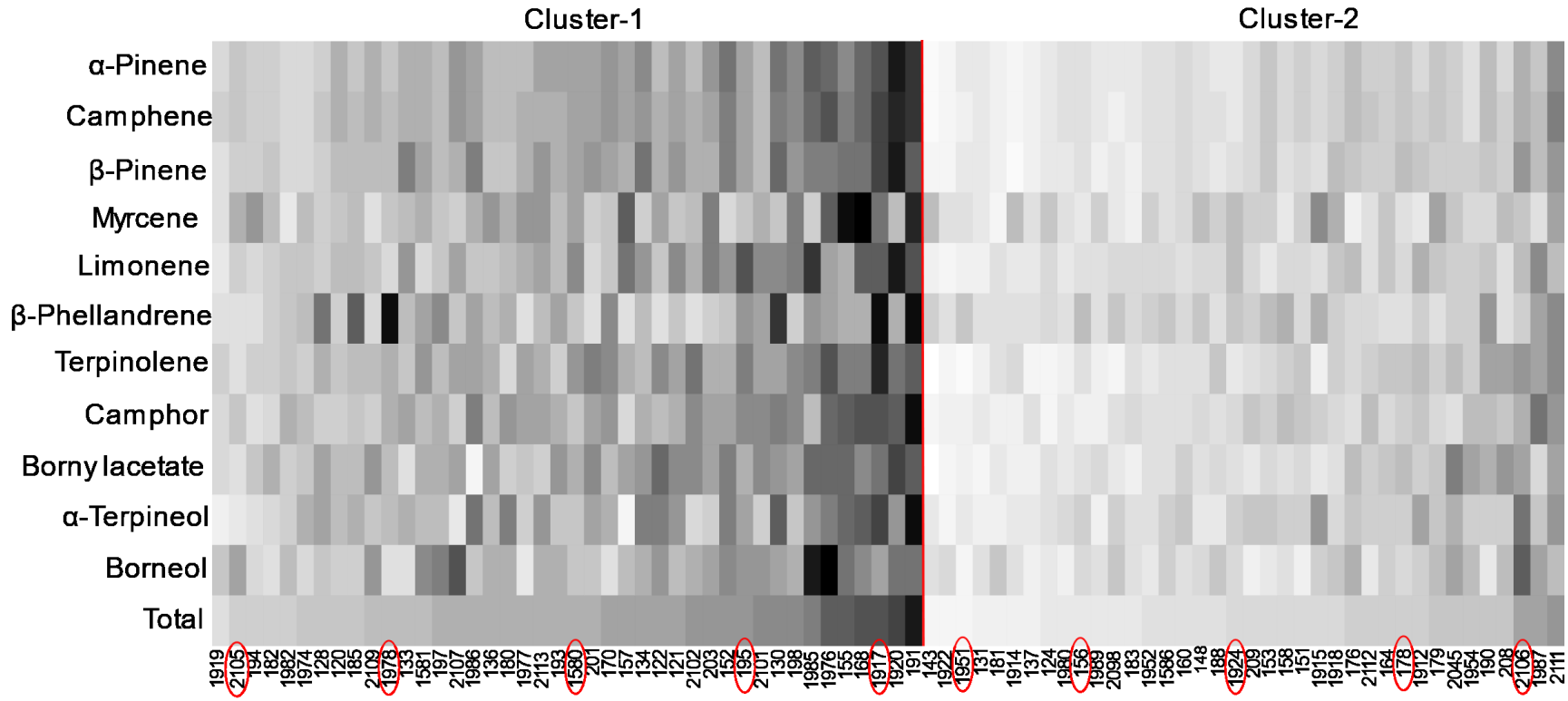


Figure S2.1. A heatmap showing the monoterpene profiles of 80 families of *Picea glauca* grouped in two different clusters. Concentrations (ng mg^{-1} Fresh Weight) from the lowest to the highest were demonstrated by light to dark colors, respectively. Concentration of individual compounds was compared among families. Red ovals show the families selected from each cluster for the laboratory bioassays. This heatmap was generated using the R ggplot2 package.

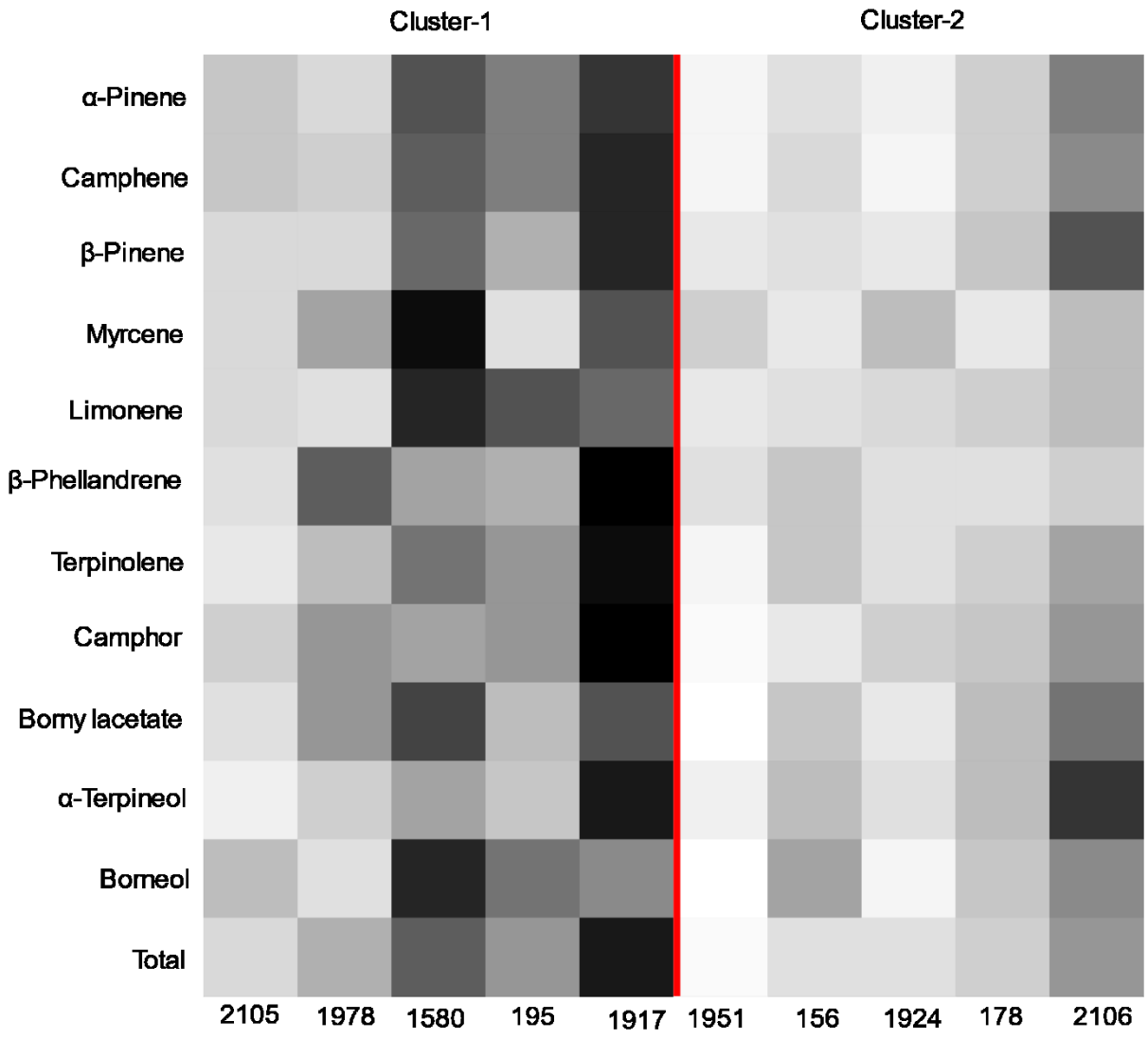


Figure S2.2. A heatmap showing the monoterpenes profiles of 10 families of *Picea glauca* that were used in the laboratory bioassays. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark colors, respectively. Concentration of individual compounds was compared across clusters and families.

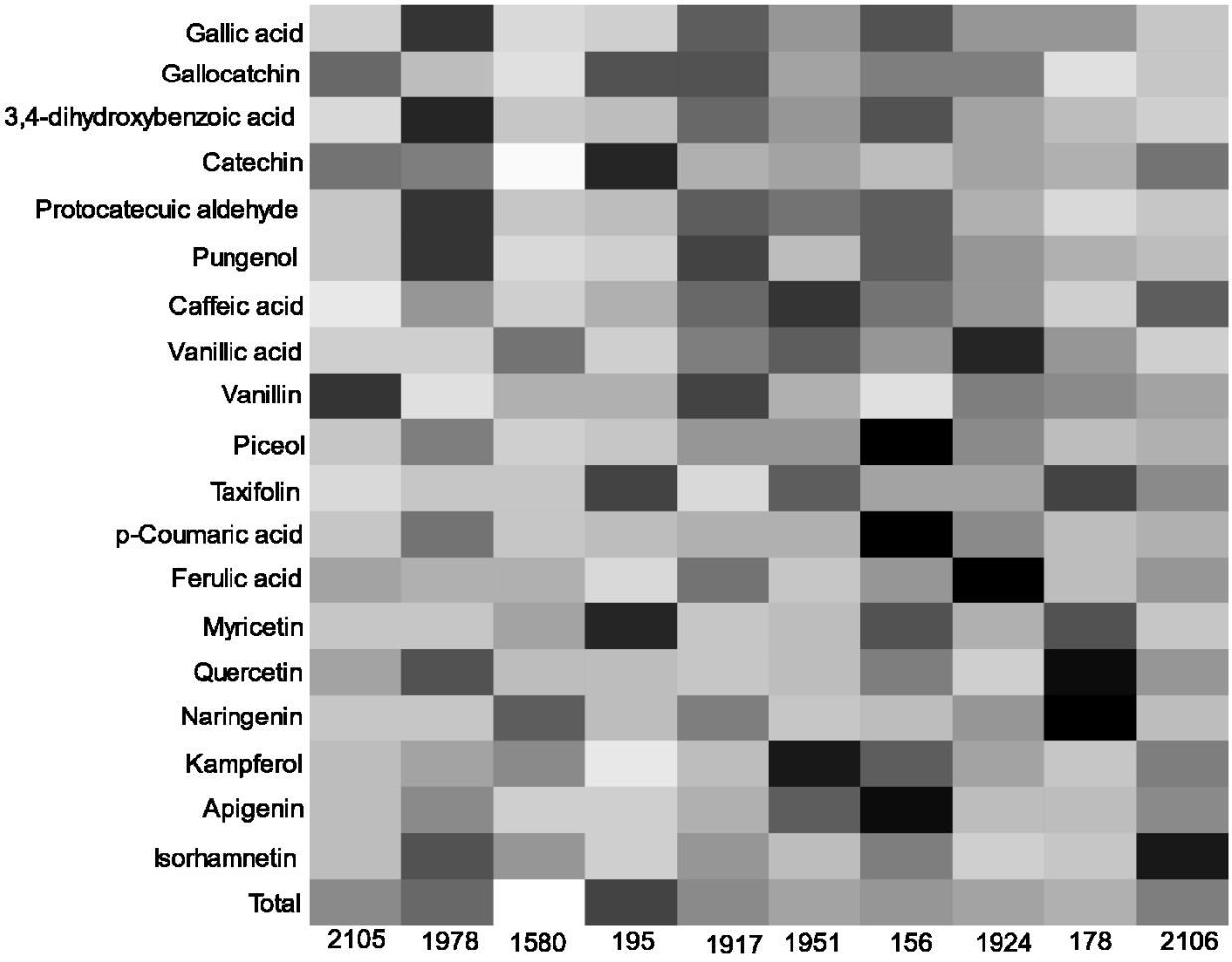


Figure S2.3. A heatmap showing the phenolics profiles of 10 families of *Picea glauca* that were used in the laboratory bioassays. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark colors, respectively. Concentration of individual compounds was compared across families.

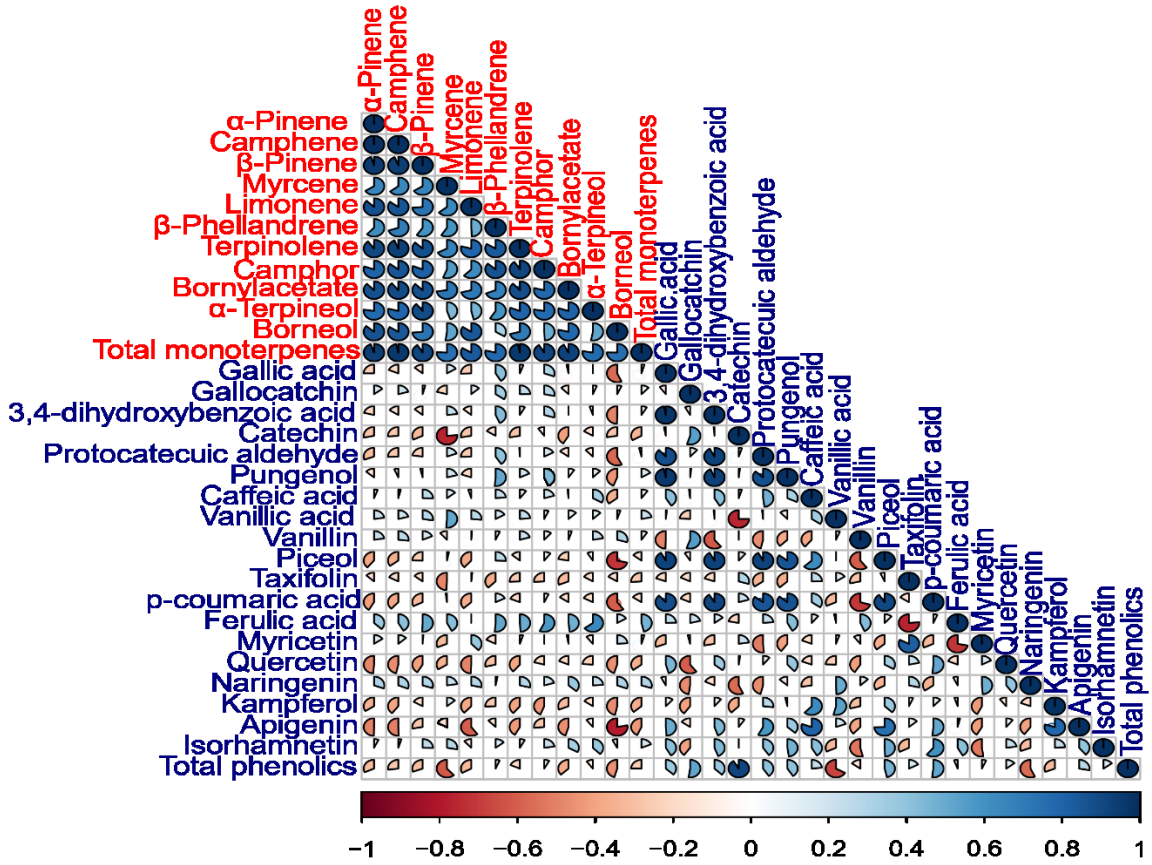


Figure. S2.4. Pearson correlation pie charts (r) for individual monoterpenes and phenolics concentration (ng mg^{-1} Fresh Weight) of *Picea glauca* used in the laboratory bioassays. Pie sizes are represented by values ranging from -1 to +1. The correlation pie charts with red texts are showing monoterpenes and blue texts showing phenolics. The darker the blue or red pie charts, the closer the r value is to either

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Chapter III

Foliar fungal endophytes alter tree defense metabolites and provide direct anti-herbivore resistance

3.1 Introduction

Plant defenses against insect defoliators depend heavily on defense metabolites. These metabolites often exhibit "inter" and "intra-specific" variations between and within species, respectively (Wittstock & Gershenzon, 2002; Mumm & Hiker, 2006; Moore *et al*., 2014; Moreira *et al*., 2014). The variation in defense metabolites within coniferous trees is often viewed as a result of coevolutionary feedback from their primary insect enemies (Moore *et al.*, 2014; Moreira *et al.*, 2014; Celedon & Bohlmann, 2019; Liu *et al.*, 2023). However, host plant-insect coevolution alone cannot fully explain the diverse composition and abundance of plant defense metabolites (Gatehouse, 2002; Müller & Hallaksela, 2000; Howe & Jander, 2008; Karst *et al.,* 2015; Chen *et al.,* 2019). It has been suggested that the coevolution of plants with their beneficial microbiome may be crucial in modulating plant biosynthetic machinery against pest insects (Stefani & Bérubé, 2006; Koukol *et al.,* 2012; Quiring *et al.,* 2019; Wang *et al.,* 2019; Patchett & Newman, 2021).

Many studies have already recognized the role of microbial symbionts in modulating host defense metabolites against insect herbivores (Sumarah *et al.,* 2008, 2009; Koricheva *et al*., 2009; Mithöfer & Boland, 2012; Karst *et al*., 2015; Wang *et al*., 2019). Fungal endophytes are heterotrophic organisms and use the same host substrate as insect herbivores, and thus they may both compete for the same plant resources. Consequently, many endophytes can produce bioactive compounds that are antagonistic to other plant consumers and may defend the host plants from herbivory damage. For instance, some endophytes can alter or enhance the

production of secondary metabolites against insect herbivores (Strobel, 2003; Zhang *et al.*, 2006; Howe & Jander, 2008). However, additional studies are needed to determine whether fungal endophytes modulate plant defenses directly through microbial secretions or indirectly by altering biosynthesis and accumulation of plant secondary metabolites.

Fungal endophytes are also known to release volatile organic compounds (VOCs). For example, the fungal endophyte *Cladosporium* sp. produced several VOCs including α-pinene, (-)-*trans*-caryophyllene, tetrahydro-2,2,5,5-tetramethylfuran, dehydroaromadendrene, and (+)-sativene (Paul & Park 2013). These VOCs may deter or attract insect herbivores (Davis *et al.,* 2013; Grunseich *et al.,* 2019). In Y-tube bioassay studies, for instance, Daisy *et al*. (2002) found that naphthalene, a VOC produced by endophytic fungus *Muscodor vitigenus*, repelled the adult wheat stem sawflies, *Cephus cinctus*. On the other hand, Steinebrunner *et al*. (2008) found that endophytic Epichloë fungus produced VOCs that attracted *Botanophila* flies under field conditions. Endophytic fungal VOCs can also alter the physiology of insect herbivores. Contreras-Cornejo *et al*. (2008) reported that 1-octen-3-ol and 6-pentyl-2H-pyran-2-one produced by the endophytic fungus *Trichoderma* spp. inhibited the growth of an insect herbivore *Spodoptera frugiperda.*

In conifers, fungal endophyte diversity and interactions with their hosts have only been reported for a few species (Barklund & Kowalski, 1996; Müller & Hallasksela, 2000). Therefore, fungal endophytes in this context represent substantially unknown biodiversity (Ganley *et al.,* 2004; Higgins *et al.,* 2007; Sokolski *et al.,* 2007). However, there is a growing interest in fungal endophytes for their contribution to the host tree resistance against insect herbivores (Saikkonen *et al.,* 1998; Miller, 2011, Speed *et al.,* 2015; Castaño *et al.,* 2020).

White spruce (*Picea glauca* (Moench) Voss) is one of the most widely distributed, ecologically and economically important conifer tree species in North America, ranging from Newfoundland and Labrador west across Canada along the northern limit of trees to Hudson Bay, Northwest Territories, and Yukon. It almost reaches the Arctic Ocean at latitude 69 °N. White spruce growth occurs from sea level to around an elevation of 1,524 meters (Sutton, 1969). It might be feasible for some individuals of this tree species to reach the age of 500, but this is extremely rare. The average lifespan is between 50 and 200 years (de Lafontaine *et al.*, 2010). Insects, however, represent a persistent risk to the survival of white spruce across its entire distribution in Canada (Royama *et al.,* 2017). Studies understanding the role of fungal endophytes could be crucial in combating major defoliating insect species including the eastern spruce budworms, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae).

Based on the limited studies conducted, we know that white spruce contains different species of fungal endophytes in its foliage, and they vary among white spruce populations (Stefani & Bérubé, 2006), while some being strongly specific to particular populations (Koukol *et al.,* 2012). Based on the outcomes of these studies, *Lophodermium piceae* is found to be the most abundant fungal endophyte, followed by *Mycosphaerella* spp*., Hypoxylon* spp*.,* and *Phomopsis* spp*.* Earlier studies in other systems have reported that the distribution of fungal endophytic communities in white spruce may also be influenced by the plant genotypes and environmental or climatic factors (Rodriguez *et al.,* 2008, 2009; Rajala *et al.* 2013, 2014; Menkis *et al.,* 2015; Millberg *et al.,* 2015; Eusemann *et al.,* 2016; Nguyen *et al.,* 2016; Moler & Aho, 2018). For instance, Rajala *et al*. (2013) reported that different genotypes of Norway spruce (*Picea abies*) have varying effects on the richness and their community structure of endophytic fungi.

The effects of specific endophytic fungus from white spruce on the eastern spruce budworm was reported for only two species of endophytes. Quiring *et al*. (2019) reported that the endophytic fungus, *Phialocephala scopiformis*, reduced the performance of the eastern spruce budworm. Furthermore, rugulosin, a secondary metabolite produced from white spruce foliar fungal endophyte *P. scopiformis*, inhibited the growth of the eastern spruce budworm (Sumarah & Miller, 2009). In addition, fungal endophytes such as members of the genus *Cladosporium* can release VOCs that may contribute to the cumulative secondary metabolite production of the holobiont (Jiang *et al.,* 2021). We need a comprehensive undertaking to determine the white spruce endophyte diversity and its impact on the eastern spruce budworm in western Canada.

My objective of this study was to determine whether foliar endophyte community and terpene defenses show variations among different plant genotypes and across two sampling locations in Alberta and whether white spruce foliar endophytic fungi synthesize terpenes and affect the performance (feeding and growth) of eastern spruce budworm. I explored whether endophytic fungi in the white spruce foliage can contribute to tree resistance to the eastern spruce budworm. I was interested in identifying the mechanisms underlying the effects of fungal endophytes on the budworm. Furthermore, I aimed to determine whether the endophytic fungal communities modulate host defense metabolites and provide direct anti-herbivore resistance. I characterized the compositions of monoterpenes, sesquiterpenes, and endophytic fungal communities of 30 white spruce genotypes planted at two progeny locations in Alberta, Canada. Since, fungal endophytes primarily produce low molecular weight terpenes (Kim *et al.,* 2014), I have specifically focused on the monoterpenes and sesquiterpenes as the lowest molecular weight and significant classes of terpenes. I isolated several endophytic fungal morphotypes from white spruce foliage and characterized their metabolite profiles. I further tested the effects

of these fungal morphotypes on the eastern spruce budworm performance either directly feeding the budworm on media amended with the fungal mycelium or testing the effects of fungal VOCs on the budworm feeding and attraction.

3.2 Materials and Methods

3.2.1 Field site and sample collection

In July 2020, I collected current-year and one-year old needles from 30 white spruce families (4 trees per family) in two progeny sites, Carson Lake (54°23'37.8"N 115°34'12.3"W; elevation 1,018 m) and Calling Lake (55°16'18.8"N, 113°09'54.6"W; elevation 639 m) (n = 240 samples per site) in Alberta. The 33 year old trees were spaced 2.5 m apart in a randomized complete block design organized into six trees per family per row with six replications in each row. Foliar samples were packed in Ziploc bags and transferred to a dry ice container in the field until stored at -40°C freezers. Since eastern spruce budworm primarily feeds on the current-year needles, subsequently moving to one-year and older needles, I sampled both types of needles in my study. These needles were used for DNA sequencing, terpene analyses, and fungal isolation.

3.2.2 DNA extraction and sequencing

Genomic DNA was extracted from the 50 mg of freeze-dried ground needles using EZNA® HP Fungal DNA Kit and quantified concentrations in an ND-1000 Nanodrop. The fungal ITS regions were amplified in a two-step PCR with the fITS7 (Ihrmark *et al.,* 2012) and ITS4 (White *et al.,* 1990) primers with Illumina overhang adapters to sequence on the Illumina MiSeq (Illumina Inc., San Diego, CA, USA) platform. The amplicaon size produced by primers was 459 bp. PCR reactions were prepared according to Platinum™ SuperFi™ Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) specifications and using 1µL of DNA extract and ran using a MyCycler™ Thermal Cycler (Eppendorf AG 22331 Hamburg, GER),

The first fITS7 PCR was performed with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 7 min. I used Mag-Bind® Total Pure NGS (Omega Bio-Tek) to purify the PCR products of each reaction before conducting an indexing PCR.

Following the manufacturer's instructions, I used 2.5 ul of each reaction for the indexing PCR to enable multiplexed sequencing. The Nextera XT Index Kit (Illumina) was combined with the same polymerase enzyme for this second PCR, which was conducted under the following circumstances: 95°C for 3 min followed by eight cycles at 95°C, 55°C and 72°C for 30 s each, and 72°C for 5 min. I once more purified PCR products in the manner previously described, and I utilized 5 ul of each sample pooled together for two different sequencing runs, each of which contained 240 samples. I randomly allocated samples from both locations in two separate runs since various sequencing runs might have an impact on the outcomes of sequencing. I examined the size and concentration of the pooled second PCR result using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). I divided the samples into two amplicon libraries, each with 240 samples, four positive controls, and four negative controls. Positive controls included one fungal species' DNA, whereas negative controls did not contain any fungal DNA in order to identify any contamination during the procedure.

I submitted the DNA to the Applied Genomics Core (TAGC) at the University of Alberta for sequencing in the Illumina MiSeq platform using 2 × 300 bp paired-end reads with v3 chemistry. Sequence data were deposited in the NCBI (BioProject ID: PRJNA952578).

3.2.3 Bioinformatic analysis

For bioinformatics I followed the methods used by Jean et al. (2021). The 'DADA2' algorithm in R software was used for bioinformatic analysis of Illumina paired-end sequences. Before loading demultiplexed sequences into "DADA2," FastQC was used to check them for non-biological DNA composition. Using Illumina adapters and primer complements, I used the "cutadapt" plugin (Martin, 2011) to preserve only biologically important sequences, and I used the "DADA2" plugin (Callahan *et al.*, 2016) to improve read quality, remove chimaeras, and resolve amplicon sequence variations (ASVs) of forward reads. Despite the fact that operational taxonomic units (OTUs) are widely used in fungus metabarcoding study, I selected ASVs due to their greater precision in identifying fungal communities and the uniformity in ecological findings obtained from the two methods (Glassman & Martiny, 2018; Pauvert *et al.*, 2019). In order to allocate taxonomy to ITS ASVs, I made use of the UNITE dynamic classifier, which analyzes individual branches and assigns taxonomy with a confidence level ranging from 97 to 98% (Abarenkov *et al.*, 2010). In order to conduct further statistical analysis, I rarefied the data by making use of species rarefaction curves. I made use of the FUNGuild database in order to categorize ASVs according to biological guilds (Nguyen *et al.*, 2016). Only species that were assigned to a single guild and received confidence ratings of "probable" or "high probable" from the endophytic fungus guilds were maintained in our collection.

3.2.4 Monoterpene and sesquiterpene analyses

To identify and quantify monoterpenes and sesquiterpenes, I extracted hexane-soluble compounds from 100 ± 5 mg (fresh weight) of frozen ground needles as described in Ullah *et al.* (2021). Briefly, I extracted the fresh needle tissue twice in 0.5 ml hexane with an internal standard of 0.004% pentadecane. Samples were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 0°C for 15 min. I combined the supernatant from each extraction and

identified monoterpene compounds in a sub-sample of extracts with a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) using authentic standards. To quantify monoterpene and sesquiterpenes in all the samples, I used a GC/Flame Ionization Detector (GC/FID, Agilent 7890B). The method used for GC/MS was as follows: a 1 µl of sample extract was injected with a split injection (10:1) into the GC equipped with 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). with helium carrier gas flow at 1.1 ml min^{-1}, and a temperature of 40 °C for 1 min, increased to 55 °C by 30 °C min^{-1}, and held for 0.5 min, increased to 122 °C by 8 °C min^{-1}, and held for 2 min, increased to 200 °C by 10 °C min^{-1}, and then to 260 °C by 20 °C min^{-1}, and held for 1 min. For monoterpenes analysis I used 11 authentic standards (mainly monoterpenes, plus bornyl acetate (acetate ester of borneol)) to identify and quantify the concentrations of individual compounds: borneol (chemical purity: 99%), α-terpineol (90%) (Sigma-Aldrich, St. Louis, MO, USA), terpinolene (90%), α-pinene (98%), β-pinene (98%), limonene (99%), myrcene (90%), camphene (90%), (+/-)-camphor (95%) (Fluka, Sigma-Aldrich, Buchs, CHE), bornyl acetate (97%), (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (99%) (TRC Toronto, Canada). Units for monoterpenes (ng mg^{-1}) were based on fresh weight (FW). For sesquiterpene analysis I used β-caryophyllene (80%), caryophyllene oxide (95%), and (+) aromadendrene (97%) (Sigma-Aldrich, St. Louis, MO, USA). Units for sesquiterpenes (ng mg^{-1}) were based on FW.

3.2.5 Fungal endophytes isolation

I surface sterilized the white spruce needles collected from both sites by shaking them for 1 min in 70% ethanol, followed by 4 min in 3% sodium hypochlorite, and a final 1 min rinsed in double distilled water (Arnold *et al.,* 2001). Surface sterilization aimed to remove microbes residing on

the needle surface. After sterilization, I placed the needles on the surface of potato dextrose agar media (PDA). The endophytic fungi started to grow after one week; I isolated the newly grown hyphae into other small media plates.

3.2.6 Sanger sequencing and bioinformatics

The Sanger sequencing method was used to establish the identity of endophytic fungi that colonize white spruce needles. Briefly, the DNA was extracted from individual endophytic fungal hyphae using Sigma Extraction Solution and Neutralization Solution B according to manufacturer guidelines protocols (Sigma-Aldrich).

The PCR amplification in 25 uL reactions was used to amplify the internal transcribed spacer (ITS) region of the ribosomal nuclear rDNA (ITS1 and ITS2; Yang *et al*., 2018). I carried out the amplification with the following conditions: 1.0 uL of DNA extract, 6.5 uL of autoclaved deionized water, 12.5 uL of PlatinumTM SuperFiTM Green PCR Master Mix (Invitrogen, Carlsbad, California, USA), 2.5 L of 10 M ITS1-F (Gardes & Bruns, 1993), and 2.5 L of 10 M ITS-4 (Gardes & Bruns, 1993).

The following thermal cycling parameters were used: a preliminary denaturation at 95°C for 5 min, 40 cycles of denaturation (95°C for 90 s, annealing at 55°C for 1 min, and extension at 72°C for 90 s), and final extension at 72°C for 10 min. Amplification was confirmed using 1.7% agarose gel electrophoresis. For subsequent investigation, only samples that generated distinct single bands were chosen. ExoSAP IT (New England Biolabs, Ipswich, Massachusetts, USA) was used to enzymatically clean and purify amplified products. Cycle sequencing was carried out in 10 uL reactions that each contained either the forward primer ITS1-F or the reverse primer ITS-4 at a concentration of 0.5 uM, 1 uL of cleaned PCR product, 0.5 uL of Big Dye Terminator v3.1 Ready Reaction Mix, and 1.5 uL of 5x Sequencing Buffer (Applied Biosystems, Foster

City, California, USA). The following were the conditions for thermal cycling that were applied to cycle sequencing reactions: initial denaturation at 96 degrees Celsius for one min, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 15 s, and extension at 60 °C for 2 min. Ethanol precipitation was used to clean the sequencing experiments, and then the reactions were conducted on an ABI 3730 DNA analyzer (Applied Biosystems).

In accordance with Karst *et al.'s* (2015) methodology, sequences were subjected to manual editing in Geneious prime (Biomatters Ltd., Auckland, New Zealand). The sequence ends were trimmed to a 3% error probability, and bases with phred scores of less than 20 were changed to the letter N. BioEdit 7.2.5 removed sequences above 2% Ns (Hall, 2005). In order to classify sequences into OTUs with a sequence identity of 97%, the program CAP3 (Huang and Madan, 1999) was used with the following non-default settings: clipping range = 6; overlap percentage identity threshold = 96; maximum overhang percentage length = 60; match score factor = 5. The nucleotide BLAST tool was used in order to do a comparison between the consensus sequences generated by each OTU and the sequences held in the GenBank database. The query dataset's BLAST results were evaluated for abnormalities, and probable assembly chimeras were excluded (Nilsson *et al.,* 2012). Since 97% sequence similarity is a solid approximation for fungi (Taylor *et al.,* 2014), I utilized it to establish taxonomy matches. Next, I used queries against the UNITE databases (Koljalg *et al.,* 2013) to confirm their existence. Sequence data from all OTUs of endophytic fungi will be deposited in GenBank (Table 3.1).

3.2.7 Fungal culture and extraction of fungal metabolites

To determine fungal metabolites, I grew individual fungal morphotypes in 200 ml flasks containing PDA broth (liquid media) approximately for 45 days; this yielded enough fungal mass for chemical analysis. The culture was filtered using Whatman No. 1 filter paper in a vacuum-

connected 25 mm Büchner funnel. The resulting layer of mycelium was transferred to a 10 ml glass tube (Kesell, Model No. BLP017). The mycelium was further dried in a freeze-dryer (Labronco Corp., Kansas City, MS, USA) for 72 h. I used 25 ± 5 mg (dry weight) of ground samples and extracted metabolites from them twice in 0.5 ml (50% ethyl acetate and 50% dichloromethane) with an internal standard of 0.004% pentadecane. Samples were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 0°C for 15 min. I combined the supernatant from each extraction and identified metabolites in a sub-sample of extracts with a GC/MS using authentic standards. The GC connected to a mass spectrometer equipped with a DB-5MS UI column was used to analyze the extracts (GC-MS; GC: 7890A, MS: 5062C, 30 m x 0.25 mm ID x 0.25 m film, product: 122-5532UI; Agilent Tech).

Helium was used as a carrier gas flowing at 1 mL min-1 with a temperature program beginning at 45°C to 50°C (held for 2 min), followed by an increase of 3°C min^{-1} to 70°C, then 5°C min^{-1} to 130°C, after that 12°C min^{-1} to 170°C, and finally the column temperature was brought to 300°C (held 2 min) at a rate of 30°C min^{-1}. A 1 µl sample injection volume was used, the injector temperature was 250°C, and samples were run in splitless mode. I used 7 standards to determine the concentrations of individual fungal metabolites including: ergosterol (chemical purity: 95%), farnesol (99%), β-caryophyllene (80%), dodecoinic acid (99%), methyl linoleate (99%), and methyl oleate (99%) (Sigma-Aldrich). Units were ng mg^{-1} dry weight (DW).

3.2.8 Morphotype culture

I grew isolated and identified endophytic fungal morphotypes on PDA for fungal VOC analysis. Briefly, I prepared the PDA agar by mixing 24g PDA and 15g agar (Difco BactoTM Agar) in 1 L of distilled water subjected to autoclave at Liquid20 autoclave cycle. After that, I poured the media into the small Petri dishes (60 mm diam. × 15 mm ht.). Media was left to solidify at 23 °C

for 24 h. I inoculated the 9-day-old morphotype culture from the advancing edge of an actively growing colony of each endophytic fungus into each Petri dish separately.

3.2.9 Fungal VOCs collection and extraction

To identify fungal VOCs, I followed a push-pull head space method reported by Cale *et al*. (2016). Briefly, two small Petri dishes with distinct morphotypes were placed inside a volatile collecting chamber, a 473 mL glass jar with Teflon tape on its threads and a metal cover. Each collection was 10 times per morphotype. I attached the jar with a vacuum pump (Cole-Parmer Canada Inc., Montreal, QC, CAN) and a flowmeter to maintain a constant airflow of 450 mL min^{-1} through the chamber lines. To filter and clean the air before it entered the collecting chamber, the intake channel was connected to a piece of Teflon tubing that was 30.5 cm long and filled halfway down with activated carbon (800 mg; 6–14 mesh; kept in place with glass wool) (air scrubber). The volatiles in the headspace were then collected for 12 h in a 7.5 cm plastic tube containing activated carbon (150 mg; 6−1 4 mesh, Fisher Scientific) and secured with glass wool at both ends. After the collecting time, pumps were turned off, trap tubes were removed and wrapped in labelled aluminum foil, and kept at −40°C until chemical extraction.

To extract the volatiles, 1 mL of dichloromethane containing tridecane as an internal standard (0.002%) was added to a microtube containing the activated carbon. Before collecting the extract and transferring it to a 2 ml glass GC vial, this combination was first vortexed for 30 sec, sonicated for 10 min, and then centrifuged at an acceleration of 18,213 g for 30 min.

3.2.10 Chemical analysis

To identify fungal VOCs, the extracts were examined by GC-MS using 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). Helium was employed as a carrier gas flowing at 1 mL min^{-1} with a temperature

program starting at 40°C (held for 1 min), 3 °C min^{-1} to 70 °C, 8 °C min^{-1} to 122 °C (held for 2 min), 10°C min^{-1} to 200 °C and 20°C min^{-1} to 325 °C and (held for 4 min). Samples were run splitless at 250 °C with a 1 ul injection volume. To identify the peaks that are particular to the medium, the control treatment peaks in the chromatograms of the fungal isolates were removed. The following standards were used to verify and quantify library matches for all of the observed fungal volatiles using the NIST/EPA/NIH Mass Spectral library version 2.0f: α-pinene (chemical purity: 98%), β-pinene (98%), p-cymene (99%), camphene (90%), 3-carene (95%), iso-butanol (>99%), β-caryophyllene (80%), 3-methyl−1-butanol, dodecoinic acid (99%), (>99%) (Fluka, Sigma-Aldrich, Buchs, CHE), and β-phellandrene (99%) (Toronto Research Chemicals).

3.2.11 Endophytic fungal amended diet preparation for eastern spruce budworm

To test the impact of endophytic fungal mycelium on budworms, I prepared three dosages of each morphotype. Briefly, I amended the 100 ml McMorran diet with an individual fungal morphotype separately by adding 50 mg, 100 mg, and 200 mg (n=10). I poured 10 ml diet from the stock a in dixie cup (60 ml) and placed one 4^{th} instar pre-weighed larva. I also prepare red McMorran diet without adding morphotypes to serve as controls. In total, I prepared 110 cups. I weighed the diet before it poured into the cups. I measured the larval weight again at the 6^{th} instar, and then at the pupal stage. Overall, I obtained larval mortality, initial and final larval weight (mg), ingested food (g), and total larval development time (4^{th} to 6^{th} instar) of each larva at the end of the experiment.

3.2.12 Testing the effects of fungal VOCs on eastern spruce budworm performance

To determine whether fungal VOCs affected the growth of budworms, I subjected budworm larvae to fungal VOCs. For this experiment, I grew the ten morphotypes in Petri dishes as described above. Immediately following fungal inoculation, two dishes without lids were placed

at the bottom of a glass jar. I hung a dixie cup containing the pure McMorran diet without any fungal endophytes and one 4th instar larva above the plates, allowing a 5 cm gap. I used budworm larvae obtained from the rearing facility of the Great Lakes Forestry Centre, Canadian Forest Service (Sault Ste. Marie, ON, CAN). I made 10 holes (2 mm each) on the lid and the bottom of the cup to provide space for fungal volatile transmission. Each morphotype had 10 replicates. The 10 jars with media plates excluding morphotypes and containing dixie cups with larvae were also prepared to serve as the control. In total 110 glass jars were prepared. I measured the larval weight before placing the larvae in cups and again at the 6th instar.

3.2.13 Two-choice olfactometer test on eastern spruce budworm larvae

In an olfactometer, the behavioural responses of budworm larvae to endophytic fungal VOCs were studied. I prepared a PDA media plate and inoculated morphotypes separately. The 10 morphotypes were grown on media for 4 days. I modified the olfactometer body with a 15-cm piece of flexible polyvinyl chloride (PVC) tube measuring 12 mm OD, 8 mm ID, 1 mm thickness; as the body of the olfactometer (Choe *et al.,* 2016). I used small endophytic fungal plugs because insect larvae have been shown to perceive tiny amounts of volatile organic compounds (Clavijo *et al.,* 2014). Briefly, I used three pieces of four-day-old individual fungal endophyte inoculum (4-mm) and three pieces of McMorran diet (4-mm), inserted in the Glass vial (2 ml, Agilent Tech.) and attached to one end of the tubing on the other end of the tubing Glass vial with only same size of PDA media and McMorran diet were attached and served as control. The single 4th instar budworm larva was introduced into the tubing by making a 2mm slit cut at the center of the tubing. After one hour I recorded the larval position either near treatment or control. The 10 larvae were examined for each morphotype treatment.

3.2.14 Data analyses

Before the main analyses, I tested whether terpene concentrations and endophytic fungal abundance varied by needle types (current-year vs. one-year and older) among 30 genotypes. The statistical analysis indicated that the needle type did not affect terpene concentrations or endophytic fungal abundance, and thus, I pooled data from both needle types in each genotype tested ($n = 4$) in each site.

Since, fungal read abundance can be used as quantitative measures within species (Amend *et al.,* 2010), therefore, I sued read abundance as a proxy to quantify the endophytic fungal abunacne in needle tissues. I calculated the proportion of fungal guilds and endophytic fungal genera from each site separately. I checked data for the assumptions of homoscedasticity and normality by using Levene's and Shapiro–Wilk tests, respectively. Where necessary, I transformed (log+1) data before analysis. I tested the effect of sites and spruce genotypes on the total terpenes and total endophytic fungal abundance for statistical significance by ANOVA and t-test, followed by *post-hoc* pair-wise differences using Tukey's HSD test. I used PERMANOVA (permutational multivariate ANOVA) to see the differences among individual terpenes and fungal genera in both sites and spruce genotypes.

I used Canonical Analysis of Principal Coordinates (CAP) and Non-metric Multidimensional Scaling (NMDS) based ordination techniques to visually explore the variables. I conducted a Pearson correlation analysis and plotted it in pie charts to explore and visualize the correlation between individual terpene concentrations and endophytic fungal genera abundance. I also conducted linear regression analysis for the total monoterpenes and total sesquiterpenes relationship. I generated heat maps to visualize the fungal metabolites and VOCs among different endophytic fungi. I conducted NMDS analysis to visualize the spread and relationship among fungal metabolites and VOCs on different endophytic fungi. I carried out one-way

ANOVA to determine budworm larval weight change in response to fungal assays (feeding) and VOCs as compared to the control. I used a two-sample t-test. Pearson correlation pie charts (r) were used to observe the correlation between (a) individual endophytic fungal metabolite, and (b) individual endophytic fungal volatile organic compounds. I considered significant differences at α=0.05. Statistical software R v3.4.4 (R Core Team 2018) was used for all statistical analyses.

3.3 Results

3.3.1 Foliage fungal community composition

I acquired a total of 18.3 million DNA reads from two Illumina MiSeq runs, an average of 38,936 per sample. After 'DADA2' quality control and filtering, 13.1 million DNA reads remained for downstream analysis, representing 12,681 ASVs. From these ASVs, I recorded a total of 1,198 taxa. Furthermore, based on species rarefaction curves, a total of 4,580 were randomly subsampled from each sample for comparison. Fungal guilds varied between the two sites (Fig. 3.1a). I identified 11 different fungal guilds in (*algal parasites, animal pathogens, bryophytes parasites, ectomycorrhizal, endophytes, epiphytes, fungal parasites, lichen parasites, lichenized fungi, plant pathogens, saprotrophs*) both sites. Carson Lake showed a higher proportion of endophytes than Calling Lake. Since my scope was to study the endophytic fungal diversity in spruce foliage, I did not include other fungal guilds in the remaining data analysis.

Carson Lake had 45 fungal genera while Calling Lake had 33 genera (Fig. 3.1b). The five most abundant genera were *Cladosporium*, *Tryblidiopsis*, *Venturia*, *Lophodermium*, and *Lirula* with an incidence of 95% in both sites. However, these five genera shared different proportions between sites. *Tryblidiopsis*, *Venturia*, and *Lophedermium* were the most abundant in Carson Lake whereas *Cladosporium* and *Lirula* were the most prevalent in Calling Lake. Sites also showed differences in

fungal abundance (# reads) which was significantly higher in Carson Lake (815 ±49) than in Calling Lake (606 ±31) (df=469, t=-3.512, P<0.001).

The endophytic fungal abundance (# reads) also varied among spruce genotypes in each site (Fig. 3.2). In Calling Lake, 12 families had a mean read abundance of 500 and lower (as low as 285) while the remaining families had a range of mean read abundance from 500 to 1,400. Families 1976 and 1951 had the highest abundance while families 178, 180, 195, 201, 203, 1982, and 1987 showed the lowest abundance (Fig. 3.2a). Six families in Carson Lake had a mean abundance of 500 or less (as low as 366), while the other families' means ranged from 500 to 1552 (Fig. 3.2b). Families 1580, 156, 180, 188, 190, 1976, and 1978 had the highest fungal abundance while families 143, 157, 158, 170, 176, 178, 1924, 1952, and 2106 had the lowest abundance.

3.3.2 Monoterpene and sesquiterpene composition

I identified and quantified 12 monoterpenes and 9 sesquiterpenes in both sites (Tables S3.1-S3.4, Fig. S3.1-S3.4). Among monoterpenes, in Calling Lake, camphor was the most abundant (990 ± 71 ng mg^{-1}) followed by bornyl acetate (421 ± 33 ng mg^{-1}) and camphene (275 ± 27 ng mg^{-1}) (Table S3.1). In Carson Lake, camphor (476 ± 71 ng mg^{-1}) was the most abundant but its concentration was much lower than that of Calling Lake (Table S3.2). The second most abundant monoterpene in Carson Lake was limonene (171 ± 22 ng mg^{-1}) followed by α-pinene (115 ± 15 ng mg^{-1}).

Among sesquiterpenes, in Calling Lake, germacrene d-4-ol had higher concentration (1,130 ± 97 ng mg^{-1}) followed by cubebol (162 ± 13 ng mg^{-1}) and Σ-cadinene (13 ± 1 ng mg^{-1}) (Table S3.3). In Carson Lake, germacrene d-4-ol was also most abundant (1,034 ± 138 ng mg^{-1}) followed by cubebol (159 ± 23 ng mg^{-1}) and Σ-cadinene (21 ± 2 ng mg^{-1}) (Table S3.4).

3.3.3 Monoterpene and sesquiterpene variations between sites and among spruce genotypes

Both total monoterpenes and total sesquiterpenes varied between sites. Overall, the total monoterpene concentration was higher in Calling Lake (2481 ± 125 ng mg^{-1}) than in Carson Lake (1448 ± 81 ng mg^{-1}) (df=469, t=6.841, P<0.001). In contrast, Carson Lake (1335 ± 67 ng mg^{-1}) had a higher total sesquiterpene concentration than Calling Lake (1784 ± 95 ng mg^{-1}) (df=469, t=-3.867, P<0.001).

The total monoterpenes and sesquiterpenes also varied among spruce genotypes in each site (Fig. 3.3ab). In Calling Lake, three families had the mean monoterpene and sesquiterpene concentrations ranging from 799 to 1058 ng mg^{-1} and 284 to 477 ng mg^{-1} while the remaining families had a mean range from 1058 to 4803 ng mg^{-1} and 477 to 2846 ng mg^{-1} respectively. The highest total monoterpene concentration was observed in family 188, followed by 190 and 180 (Fig. 3.3a, Fig. S3.1). Families 176, 156, and 170 had the highest total sesquiterpene concentrations (Fig. 3.3a; Fig. S3.3). In Carson Lake (Fig. 3.3b), the average monoterpene and sesquiterpene concentrations for 11 families ranged from 420 to 1010 ng mg^{-1} and from 117 to 721 ng mg^{-1}, respectively, while the concentrations for other families ranged from 1010 to 3276 ng mg^{-1} and from 117 to 4103 ng mg^{-1} respectively. The families 156, 176, and 133 showed higher concentrations of total sesquiterpenes (Fig. 3.3b Fig. S3.4). The total monoterpene concentrations were higher in the families 156, 1917, and 148 (Fig. S3.2).

I performed a linear regression analysis to study if total monoterpenes show any relationship with the total sesquiterpenes. These analyses revealed a positive relationship between these two classes of terpenes in both Calling Lake (P<0.001, R^2=0.2; Fig. 3.4a) and Carson Lake (P<0.001, R^2=0.85; Fig. 3.4b).

3.3.4 Correlations between individual terpenes and endophytic fungi

Between sites, the endophytic fungal read abundance was correlated with sesquiterpenes only in Carson Lake (Fig. 3.5). I further conducted the Pearson correlation analyses to determine if terpene concentrations are correlated with the fungal communities. The analyses of individual monoterpenes, sesquiterpenes, and the five most abundant endophytic fungal genera indicated strong correlations between some fungal genera and terpene in both sites (Figs. 3.6, 3.7). In Calling Lake, *Lirula* genera showed a positive correlation with all monoterpenes (Fig. 3.6). In contrast, there was no correlations with any of the fungal genera with any of the monoterpenes in Carson Lake (Fig. 3.7). Furthermore, five sesquiterpenes (α-cadinene, g-cadinene, γ-cadinene, α -cadinol, and tau.cadinoal) were positively correlated with *Venturia*. Three genera (*Cladosporium*, *Tryblidiopsis* and *Lophodermium*) were highly correlated with individual sesquiterpenes. I also detected that some monoterpenes and some sesquiterpenes were highly correlated with each other.

I further conducted NMDS ordination-based techniques followed by PERMANOVA to support the results of Pearson's correlation results (Fig. 3.8). In Calling Lake, monoterpenes are all correlated with one another and with *Lirula* genera (Fig. 3.8a) and sesquiterpenes were associated with one another and with *Venturia* genera. In Carson Lake, I observed strong correlations between sesquiterpenes and *Cladosporium*, *Lophedermium*, and *Tryblidiopsis*. None of the endophytic fungi showed any correlation with monoterpenes.

3.3.5 Metabolic profiles of endophytic ten morphotypes

I characterized the following eleven metabolites from fungal mycelium including one sterol (ergosterol), one oxygenated monoterpene (4-methyl-1,6-heptadien-4-ol), one sesquiterpene (farnesol), one triterpene (squalene), two fatty acids (9,12-octadecadienoic acid, n-hexadecanoic

acid), 3 fatty aldehydes (1-Octanol,2-butyl, 2-Dodecenal, 9-Octadecenal), and 2 glycols (oxalic acid_6-ethyloct-3-yl, 1-Decanol, 2-hexyl (Table 3.2). I generated a heat map based on the metabolite concentrations for each of the ten morphotypes (Fig. 3.9, Table S3.5). *Chalara*_1 had the highest total concentration (52,827 ng mg^{-1}), followed by *Geopyxis*_1 (32,183 ng mg^{-1}). The *Geopyxis*_1 also had higher concentrations of ergosterol (27,753 ng mg^{-1}), farnesol (87 ng mg^{-1}), and squalene (2772 ng mg^{-1}) than the remaining morphotypes. The *Dothideomycetes*_1 produced higher concentration of 4-Methyl-1,6-heptadien-4-ol. The *Cladosporium*_1 showed higher concentrations of n-hexadecanoic acid, dodecanal, and 9-octadecenal.

I further performed NMDS analysis combined with PERMANOVA to investigate metabolites produced by the same morphotypes (Fig. 3.9b). This analysis indicated that all morphotypes spread widely in terms of the type of metabolites they produced. Overall ergosterol, farnesol, and triterpene squalene were correlated with *Geopyxis*_1. In addition, the metabolite concentrations varied significantly across the ten morphotypes (R^2= 0.13, F9= 27.31, $p < 0.001$).

3.3.6 Fungal VOCs profiles of ten morphotypes

I characterized 13 fungal VOCs in five different classes including seven monoterpenes (α-pinene, β-pinene, camphene, 3-carene, β-phellandrene, p-cymene, 2,5-Dimethyl-1,5-hexadien-3-ol), two aldehydes (pentanal, butanal-3 methyl), two alkanes (2,4-Dimethyl-1-heptane, 2,4,6,8-Tetramethyl-1-undecane), one ether (hexyl octyl ether), and one ketone (2-cyclopenten-1-one) (Figure 3.10a; Table 3.3). The *Dothideomycetes*_1 had the highest total VOC concentrations (102 ng mg^{-1}), followed by *Geopyxis*_1 (86 ng mg^{-1}). Of the ten morphotypes, only three, *Dothideomycetes*_1, *Geopyxis*_1, and *Geopyxis*_4 produced monoterpenes. Interestingly, one oxygenated monoterpene 2,5-Dimethyl-1,5-hexadien-3-ol was also produced (Table S3.6).

I used NMDS analysis in conjunction with PERMANOVA to investigate fungal VOC profiles from the ten morphotypes (Fig. 3.10b). Overall, fungal VOCs were correlated with *Geopyxis*_1, *Geopyxis*_4, and *Dothideomycetes*_1. In addition, the quantities of fungal VOCs were substantially different among the morphotypes (R^2= 0.23, F_9= 17.44, $p < 0.001$).

3.3.7 Effects of endophytic fungal mycelia on eastern spruce budworm feeding

The fungal treatments significantly altered the larval weight depending on the fungal morphotype and the dose used (Fig. 3.11a). *Cladosporium*_1 at 200 mg caused 100% larval mortality; the same morphotype at 100 mg treatment reduced the larval weight without causing mortality. When *Cladosporium*_1 at 50 mg treatment was applied it did not impact larval weight. In contrast, *Clasosporium*_2 at 200 mg significantly increased the larval weight but did not have any impact at lower doses. *Didymella*_1, *Dothideomycetes*_1, and *Geopyxis*_1 at the highest dose also reduced the larval weight change relative to the control.

3.3.8 Effects of endophytic fungal VOCs on eastern spruce budworm feeding

The fungal VOCs differentially impacted larval weight (Figure 3.11b). Five morphotypes *Geopyxis*_1, *Geopyxis*_4, *Cladosporium*_1, *Cladosporium*_2, and *Dothideomycetes*_1 significantly reduced the larval weight, whereas *Geopyxis*_3 and *Chalara*_1 improved the larval weight relative to the control. The remaining three morphotypes did not have any effects on the larval feeding.

3.3.9 Effects of endophytic fungal VOCs on eastern spruce budworm attraction

I conducted two-choice olfactometer assays to determine if budworm larvae are attracted to the VOCs produced by endophytes. A two-sample t-test showed that there were significant

differences between the control group and the fungal treatments (Fig. 3.12). The *Geopyxis*_1 and *Dothideomycetes*_1 showed 100% repellency to the larvae. The *Geopyxis*_4, and *Cladosporium*_1, and *Cladosporium*_2 also exhibited 90%, 80%, and 70% repellency. However, all other five morphotypes were attractive to the budworm larvae.

3.3.10 Correlation between endophytic fungal metabolite and VOCs

The endophytic fungal metabolites showed a correlation with each other (Figure 3.13a). The ergosterol was correlated with all terpenoids (farnesol, squalene, and 4-methyl-1,6-heptadien-4-ol). Interestingly, these three terpenoids also exhibited a positive correlation with each other. Additionally, every metabolite in the same class showed a positive connection with one another. Furthermore, fungal VOCs also showed a correlation with each other (Figure 3.13b). Additionally, identical classes of fungal VOCs are correlated with one another. All the monoterpene VOCs, including α-pinene, camphene, p-cymene, β-pinene, 3-carene, and β-phellandrene, were shown to have a positive correlation with one another. Furthermore, ketone (2-cyclopentane-1-one) and total VOCs were positively correlated with all monoterpenes. Two aldehydes, pentanal and butanal-3 methyl, were shown to correlate with one another.

3.4 Discussion

This is the most comprehensive study undertaken to investigate the potential roles of endophytic fungi in tree resistance to insect herbivores in any conifer species. I found that the composition of terpenes and fungal endophyte communities varied between sites and among spruce genotypes sampled. Overall, I identified 45 distinct endophytic fungal genera from white spruce foliage. I also isolated ten endophytic fungal morphotypes and characterized metabolite compositions of their mycelia. Fungal mycelium extract contained 11 secondary metabolites and fungal

headspace contained 13 VOCs. Each morphotype had a unique metabolite profile. Although most fungi were harmful to the budworm (i.e., killed or reduced insect feeding), there were some fungi that have benefited budworm feeding depending on the fungal species and doses used in the behavioral bioassays. This is the first study on white spruce foliar sesquiterpene profiles, using next-generation sequencing to describe white spruce foliar endophytic fungal communities and their metabolites, and to investigate their effects on the eastern spruce budworm performance.

The current study provided two pieces of evidence that fungal endophytes can modulate the white spruce defense metabolites. First, I detected positive correlations between sesquiterpene concentrations and fungal abundance, suggesting the possible role of fungal endophytes in host tree defenses. White spruce genotypes sampled from Carson Lake contained substantially higher sesquiterpenes than those from Calling Lake; coincidentally, the endophytic fungal communities were more abundant in Carson Lake than in Calling Lake. In particular, the abundance of three endophytic fungal genera (*Cladosporium*, *Trybildiopsis,* and *Lophedermium*) was positively correlated with all sesquiterpenes identified. These results were further supported by Canonical analysis which showed that the total endophytic fungal communities were more closely aligned with and strongly correlated to sesquiterpenes in white spruce foliage in Carson Lake.

Secondly, the fungal endophytes produced an array of secondary metabolites including terpenes and some of which overlapped with those found in white spruce foliage. Earlier studies have also reported that fungal endophytes can produce terpenes such as helvolic acid (Zhao *et al.,* 2010), periconicins (Kim *et al.,* 2004), sordaricin and scoparasin-B (Pongcharoen et al., 2006, 2008), and cycloepoxylactone and cycloepoxytriol-B (Hussain *et al.,* 2009). With uncertainty, fungal endophytes also produced other classes of secondary metabolites that may

not be present in the white spruce foliage. Overall, these results suggest that fungal endophytes can contribute to the composition and richness of host tree metabolites, including those play direct role in host defenses against the eastern spruce budworm.

There are several ways in which fungal endophytes can influence the synthesis of terpenes by host plants. For instance, some fungal endophytes can produce enzymes that in turn change the host plant's terpene biosynthetic pathways, resulting in synthesis of new terpenes or altering the production of existing terpenes (Zhang *et al.,* 2006; Rudgers & Clay, 2007; Rodriguez *et al.,* 2009; Kusari & Spiteller, 2011; Jia *et al.,* 2016). In this study, I identified 11 metabolites from fungal mycelium, which included several terpenes. Furthermore, fungal endophytes can also synthesize several FVOCs (13 identified in this study); majority of which are terpenes along with other classes of chemicals. All these results suggest that fungal endophytes can either directly or indirectly influence the production of terpenes in host plants.

I identified two possible mechanisms explaining how endophytes can contribute to white spruce resistance to the eastern spruce budworm. Firstly, endophytic fungi can provide direct anti-herbivore resistance to the budworm. This was demonstrated through direct toxicity of the fungal mycelium or FVOCs to the budworm. The feeding bioassay clearly showed that fungal mycelia can cause up to 100% larval mortality at higher doses while at lower doses they can substantially reduce larval fitness, supporting the outcome of earlier studies in the same study system (Sumarah & Miller 2009; Quiring *et al.,* 2019). Of the ten morphotypes tested, *Cladosporium*_1 seems to be the most toxic at the highest dose, followed by *Didymella*_1, *Dothideomycetes*_1, and Geopysis_1. Interestingly some endophytes such as *Cladosporium*_2 at the highest dose benefit larva by increasing their body weight. Similarly, FVOCs of five of the ten morphotypes tested substantially reduced the budworm fitness by as much as 35% while

FVOCs of two morphotypes improved the fitness; the other three had no impact. Volatiles released by *Dothideomycetes*_1 were the most effective in reducing the budworm fitness. In a field study, Quiring *et al*. (2020) concluded that inoculating white spruce trees with the native endophytic fungus *Phialocephala scopiformis* DAOM 229536 Kowalski and Kehr (Helotiales, Ascomycota) reduced the amount of defoliation caused by eastern spruce budworm larvae and their survival by 27%.

Secondly, FVOCs can also contribute to white spruce resistance to the eastern spruce budworm by acting as a repellent to the budworm larvae. This phenomenon seems widespread in other host plant-endophyte pest insect systems. For example, *Muscodor vitigenus*, isolated from the liana *Paullina paullinioides*, produced naphthalene which repelled the wheat stem sawfly *Cephus cinctus* (Daisy *et al.,* 2002). Similarly, an unnamed endophyte (strain AP-796) obtained from green foxtail (*Setaria viridis*) made 3-(4-methylfuran-3-yl)-propane-1-ol, which acted as a deterrent to the white-spotted stink bug, *Ventral eysarcoris* (Nakajima *et al.,* 2010). In the current study, the results of olfactometer study suggested that budworm response was concentration dependent. The five fungi with high concentrations of FVOCs ranging from 33 to 102 ng mg^{-1} were strongly repellent to the budworm. In contrast, FVOC concentrations ranging from 21 to 26 ng mg^{-1} were either attractive to the budworm or showed no impact.

In addition, the endophytic fungal communities showed variation between sites. The abundance of endophytic genera was higher in Carson Lake than in Calling Lake. However, the five most abundant endophytic fungal genera were the same in both sites, *Cladosporium*, *Tryblidiopsis, Venturia, Lophodermium,* and *Lirula*. Some of these genera seem to be widespread in white spruce foliage or other conifer species. For instance, *Cladosporium* was observed in white spruce foliage from southern Québec; although it was considerably less abundant (Stefani

& Bérubé 2006). Likewise, Rim *et al.* (2021) cultured fungal endophytes from leaves, stems, and roots of four *Pinus* species (*P. thunbergia, P. rigida, P. densiflora,* and *P. koraiensis*) from 18 different sites in Korea and found *Cladosporium* was the most abundant.

Several studies reported that environmental variations such as climate and geography, and host characteristics like tree genotypes can influence the diversity of endophytic fungi (Arnold & Herre 2003; Wang & Guo 2007; Rivera *et al.,* 2011). The total endophytic abundance in the current study showed genotypic specificity as for instance, the overall fungal abundance ranged from 250 to 1500 sequence reads among white spruce genotypes in Calling Lake. Similar effects of Norway spruce genotypes on the endophytic fungal abundance have also been reported (Rajala *et al.*, 2013; 2014). All these eventually lead to an assumption that there is a close connection between plant genotypes and the endophytic fungal communities recruitment (Bálint *et al.*, 2013; Vandenkoornhuyse *et al*., 2015).

In addition to the fungal communities, sites and white spruce genotypes also influenced the white spruce foliar terpenes. I found positive associations between monoterpenes and sesquiterpenes in both sites; i.e., as monoterpene concentrations increased, so did the sesquiterpenes. Since both classes of terpenes are synthesized from farnesyl diphosphate biochemical pathway (Degenhardt *et al.,* 2009), such results are expected. Similarly, white spruce genotypes also influenced the concentrations of monoterpenes and sesquiterpenes.

In conclusion, endophytic fungal communities of white spruce foliage differ depending on the location and host plant genotypes. These fungal communities can play a significant role in defending white spruce trees against the eastern spruce budworm either through direct toxicity of fungi or repellency through FVOCs or indirectly affecting the host secondary metabolite profiles.

Both toxic mycelium or fungal VOCs have the potential to decrease larval viability or even kill the larvae. However, it appears that not all endophytic fungal contribute to the white spruce defenses as I found that some in fact improve the budworm fitness. The effect of fungi on insect herbivory is likely more complex than what I presented here. Due to toxicity of fungal secondary metabolites, feeding on plants containing fungal endophytes can not only reduce the insect herbivory (Jabel & Vidal, 2010; Paul & Park, 2013) but also lower the number of insect species feeding on the plants. Furthermore, fungal metabolites can reduce the insects' ability to obtain nutrition from plants (Contreras-Cornejo *et al.,* 2008). In addition, some studies reported that some endophytic fungi act as entomopathogens, killing insect directly (Mantzoukas & Eliopoulos, 2020; Wilberts *et al.,* 2022).

Table 3.1. Operational taxonomic units (OTUs) of endophytic fungus found on *Picea glauca* needles collected in Calling and Carson Lake, Alberta, Canada.

OTU	Best GenBank match	Best GenBank match accession number	% Identity	Query coverage (%)
***Chalara*_1**	*Chalara sp.* TMS-2011 voucher MS3p_50-44	HQ630988	100.0	100.0
***Cladosporium*_1**	*Cladosporium cladosporioides*	KU182497	100.0	100.0
***Cladosporium*_2**	*Cladosporium halotolerans*	NR_119605	100.0	100.0
***Didymella*_1**	*Didymella sp.* strain ICMP 12081	MZ098689	98.6	99.0
***Dothideomycetes*_1**	*Dothideomycetes sp.* 11143	GQ153116	96.9	99.2
***Geopyxis*_1**	*Geopyxis carbonaria* strain RAS132	MG663262	100.0	100.0
***Geopyxis*_2**	*Geopyxis carbonaria* voucher PRM149720	KU932495	98.2	100.0
***Geopyxis*_3**	*Geopyxis carbonaria* voucher DED 7357 (SFSU)	KU932486	99.1	99.1
***Geopyxis*_4**	*Geopyxis carbonaria* voucher K (M)181130	KU932489	97.0	98.0
***Pezizales*_1**	*Pezizales sp.* T4N26c(A)	AY465510	97.3	98.5

Table 3.2. The eleven secondary metabolite profiles from different classes identified in mycelium of 10 endophytic fungi of *Picea glauca* foliage.

No.	Secondary metabolites	Chemical formula	Class
1	Ergosterol	$C_{28}H_{44}O$	Sterol
2	Farnesol	$C_{15}H_{26}O$	Sesquiterpene
3	Squalene	$C_{30}H_{50}$	Triterpene
4	4-Methyl-1,6-heptadien-4-ol	$C_8H_{14}O$	Oxygenated monoterpene
5	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	Fatty acid
6	1-Octanol,2-butyl	$C_{12}H_{26}O$	Fatty aldehyde
7	oxalic acid_6-ethyloct-3-yl	$C_{19}H_{36}O_4$	Glycol
8	1-Decanol, 2-hexyl	$C_{16}H_{34}O$	Glycol
9	n-hexadecanoic acid	$C_{16}H_{32}O_2$	Fatty acid
10	2-Dodecenal	$C_{12}H_{22}O$	Fatty aldehyde
11	9-Octadecenal	$C_{18}H_{34}O$	Fatty aldehyde

Table 3.3. The 10 endophytic fungi of *Picea glauca* foliage produced 13 endophytic fungal

No.	Fungal VOCs	Chemical formula	Class
1	α-pinene	$C_{10}H_{16}$	Monoterpene
2	camphene	$C_{10}H_{16}$	Monoterpene
3	*p*-Cymene	$C_{10}H_{16}$	Monoterpene
4	β-pinene	$C_{10}H_{16}$	Monoterpene
5	3-carene	$C_{10}H_{16}$	Monoterpene
6	β-phellandrene	$C_{10}H_{16}$	Monoterpene
7	2,5-Dimethyl-1,5-hexadien-3-ol	$C_8H_{14}O$	Oxygenated monoterpene
8	2-cyclopenten-1-one	C_5H_6O	Ketone
9	2,4-Dimethyl-1-heptane	C_9H_{18}	Alkane
10	2,4,6,8-Tetramethyl-1-undecane	$C_{15}H_{30}$	Alkane
11	butanal-3 methyl	$C_5H_{10}O$	Aldehyde
12	Hexyl octyl ether	$C_{14}H_{30}O$	Ether
13	Pentanal	$C_5H_{10}O$	Aldehyde

Table S3.1: The individual monoterpenes (ng mg^{-1}) in 30 families/genotypes of *Picea glauca* foliage from Calling Lake, Alberta. The units are based on fresh weight.

Families	α-pinene	β-pinene	B-phellandrene	Bornyl acetate	Limonene	Tricyclene	Myrcene	Camphene	Terpinolene	Camphor	Borneol	α - terpineol	Total
128	87.7	15.9	17.9	195	67.3	5.3	48.9	24.6	10.3	312	9.4	4.9	799
133	95.2	15.6	47	78.3	86.6	4.9	84.1	25.7	9.4	377	5.08	4	833
143	230.6	37.1	161.9	522.4	286	11.4	144.5	64.4	35.5	1127.4	12.5	5.8	2639.7
148	241.6	39.1	123.2	354.8	252.2	12	108.6	66.7	26.9	1039.5	17.1	10.2	2292
156	235.4	44.9	153.3	663.5	357.6	12.9	82.4	69.1	37.9	1076	28.5	8.3	2770
157	296.8	51.48	145.4	535.9	421.8	17.96	113.9	109.3	40.12	1216	26.03	13.61	2988
158	93.8	16.3	54.3	250	94.7	15.7	33	148	14.2	506	6.2	7.9	1239
170	187.5	35.4	134.1	553.9	231.2	32.3	83.2	312.8	35.5	1146	11.1	13.3	2776
176	218	41.2	125	407	225	36.1	34.9	326	28	853	12.7	8.4	2315
178	155.7	27.1	87.3	356.7	158.3	29.3	71.7	251	18.1	628.9	17.2	6.3	1808
180	303	54.9	157	604	554	51.5	130	485	51.5	1697	24.8	24.4	4138
183	148.9	25.9	99.9	331.1	170.2	12.1	44	231.5	18.9	540.6	37.7	6.5	1667
188	414	75	168	860	505	19.9	249	512	63.7	1870	51.2	14.6	4804
190	367.2	74.1	182.4	622.8	478.7	16.9	234.9	548.7	53.8	1622	20.4	18	4239
195	191	34.3	138	257	311	9.3	69.5	293	26.8	900	25.1	7.9	2264
201	213.9	41.9	102.4	643.3	159.5	7.1	101.6	249.3	27.4	915.2	24.7	8.2	2494
203	247.6	41.8	133.1	431.5	255.5	11.8	109.8	366.2	29.8	1063	28.7	6.9	2726
1580	159.8	28.5	118.7	341	275.3	9.1	108.7	270.8	27	912.9	16.4	9.3	2278
1917	150	30.4	85.5	305.7	198.4	7.3	57.9	238.1	21	750	13.9	8.5	1867
1918	101.7	18.8	53.7	205.2	99.2	5	58.4	165.6	14.2	479.4	8.1	2.9	1212
1924	97.1	15.7	57.2	141.9	104.5	4.7	64.8	148	8.8	406.3	5.2	3.8	1058
1951	238.3	69.8	98.1	343.1	334.7	11	160.2	343.5	37.1	1140.9	20.4	31.5	2828.7
1952	214.2	35.3	136.8	533.6	252.2	10.2	151.2	336.9	27.9	1010	23.9	10.3	2743
1976	226.6	47.1	121.7	397.2	266.4	10.7	69.1	348.7	35.3	1019.1	12.3	11.9	2566.1
1978	245.9	40.9	157.8	485.8	283.9	11.7	77.1	381.3	30.1	1151	21.3	5.8	2892.5

1980	245.9	40.9	157.8	485.8	283.9	11.7	77.1	381.3	30.1	1151	21.3	5.8	2892.5
1987	188.3	32.8	158.9	341.8	286.8	9.2	60.9	304.6	30.7	883.1	14.9	5.1	2317.1
2105	257.5	49	140.5	365.4	292.5	14.2	127.9	389.7	26.7	1022	40	5.6	2732
2106	270.8	61.4	163.2	735.4	314.9	12.8	101.1	429.8	46.2	1545	43.6	16.4	3741
2113	298.3	51.6	141.4	285.9	337.3	13.7	206.6	441.8	37.3	1345	29.6	11.1	3200

Table S3.2: The individual monoterpenes (ng mg^{-1}) in 30 families/genotypes of *Picea glauca* foliage from Carson Lake, Alberta. The units are based on fresh weight.

Families	α-pinene	β-pinene	B-phellandrene	Bornyl acetate	Limonene	Tricyclene	Myrcene	Camphene	Terpinolene	Camphor	Borneol	α -terpineol	Total
128	84.9	16.5	38.9	264.1	51.4	5.8	50.7	133.1	10	306	5	4	970.7
133	102.3	18.1	80.5	234.8	144.1	6.8	38.7	141.1	14.5	561.7	12.7	8.8	1364.3
143	91.4	16	86.6	196.1	183.6	9.5	68.1	140.4	14	400.4	6.6	3.9	1216
148	168.1	29.9	112.5	423.5	257.2	12	159.3	277.9	24.9	793.9	13	6	2279
156	270.8	50.9	173.3	940.2	243.3	19.5	122.4	427.9	41.5	956.6	23.6	6.7	3277
157	88.8	17.4	68.5	274.3	208	6	24.7	136.8	10.7	317.1	1.9	3.5	1157.6
158	97.2	16.6	98.6	268.8	281.4	6.9	49.3	156.9	14.7	414.7	6	3.6	1415
170	72	11.8	45.9	167.1	231.2	5	31.3	109.9	8.6	290.2	1.3	2.5	976.9
176	131.9	26	110.6	536.1	242.3	8.6	32.6	201.2	18.3	543.2	13.6	4	1869
178	58.1	9.8	21.9	85.2	106.1	4.4	22.1	93.4	3.9	165.9	1.1	0.7	572.4
180	130.4	26.2	120.7	340.2	95.8	8.1	89	205.7	24.6	849.6	9.6	7.9	1908
183	37.7	6.8	27.9	107.7	49.7	3.2	10.4	61.1	4.3	127	1.6	1.3	438.8
188	68.5	11.7	45.8	150	217	6	42.4	108	9	311	3.2	2	976
190	165.7	29	148.7	500.5	265.5	4.4	115.2	270.7	23.8	746.8	15.4	4.8	2291
195	118	24.7	80.5	241	210	5.8	56	186	17.2	522	7.7	9.5	1480
201	76	15	25.8	269	67.9	2.8	50.7	118	10.7	360	9	5.2	1010
203	188.4	37.4	103.1	556.9	229.5	6.8	84.8	279.3	20.6	715.1	20.5	5.5	2248
1580	78	13	48.1	264.8	104.9	3.1	86	124.3	10.5	224.5	11.4	2.1	970.7
1917	179	33	153.6	647.3	358.3	6.9	114.8	290.8	30.5	664.2	15.2	9.4	2503
1918	142.2	31.8	113.6	512.9	244	5.4	147.2	234.6	28.7	759	18.2	12.3	2250
1924	116.7	21.5	74.4	248.3	159.7	8.2	131.8	173.2	13.2	500.9	5.7	5.2	1458.8
1951	50.3	9.7	20.7	127.2	46.3	1.8	17.9	77.5	4.7	165.6	2.9	3.9	528.5
1952	144.4	24.8	74	355.7	181.5	5.6	61.6	220	15.6	509.6	9.3	6	1608
1976	69.4	14	29.3	244.1	59.1	2.6	33.4	108.5	9.5	278.2	5.7	3.8	857.7
1978	129.5	20.7	60.8	341.7	132.8	4.4	75.6	186.6	13.3	380.6	4.5	2.4	1352.9
1980	55.5	10.3	25.3	206.5	52.4	2.3	29.3	66.3	5.9	151.7	6.4	2.1	613.9
1987	30.5	5.8	12.8	60.1	26.2	1.6	8.7	8.2	1.9	68.1	7.9	0.2	232.1

2105	163.1	28.5	104.7	269.7	264.7	9.5	117.8	49.9	22.7	766.5	18.8	5.2	1821.2
2106	221.6	49.7	131.3	464.4	282.8	10.9	85.4	61.1	31.6	920.3	19.5	9.5	2288
2113	107.2	23.5	71.8	209.4	135.6	6.4	50.7	30.3	16.4	509.2	20.4	4.8	1185.7

Table S3.3: The individual sesquiterpenes (ng mg^{-1}) in 30 families/genotypes of *Picea glauca* foliage from Calling Lake, Alberta. The units are based on fresh weight.

Family	α-Cadinene	α -Cadinol	Cubebol	Cubenene	Germacrene D-4-ol	Germacrene-D	γ-Cadinene	Σ-Cadinene	tau-cadinol	Total
128	0	0.2	42.8	0.3	274	0.3	2.3	3.9	1	325
133	0	0.9	117.7	2.9	634.1	0.4	9.1	13.4	5.9	784.4
143	0	0	91.5	0.9	439	0	6.4	8.8	5.1	551
148	0	5	210	6	1083	0.4	17.9	27.1	13.5	1363
156	0	9.136	323.6	7.36	2016	0.811	22.39	33.73	18.03	2431
157	0	2.4	126	2.57	783	0.3	9	14.1	6.6	944
158	0	0.8	171	1.5	944	2.8	0.9	3.2	2.4	1126
170	1.9	0	306.6	2.9	1947	6.3	3	9.2	2.4	2279
176	2.2	0	356	8.2	2447	10.1	4.4	13.1	5.1	2846
178	2.35	0	159	1.89	1056	3.9	3.9	9.5	2.1	1239
180	0.9	0	148	1.5	949	3	2.4	6.5	3.9	1115
183	0	0.4	93.2	0.7	643	1.9	1.8	3.5	0.8	745
188	0	2.4	207	0.5	1677	5.6	4.4	9.3	4.8	1911
190	0	0.8	164	0.3	1222	4.6	3.7	7.2	3.1	1406
195	1.5	1.1	168	0.5	1349	5.9	4.5	12.6	3.1	1546
201	0	2.1	183	0.3	1489	4.8	5.3	9.1	6.5	1701
203	0	1.8	215.7	0.6	1758	5.9	9.1	15.4	6.6	2013
1580	0	1.1	191	0.1	1637	4.8	8.8	12.7	7.8	1863
1917	1.5	0.7	176.1	0.1	1380	5.2	10.9	14	9.4	1598
1918	2.8	0.4	93.6	0.1	576.2	2.1	7.8	7.9	5.6	696.4
1924	0.7	0.4	62.9	0	394.9	2.2	5.1	8.4	2.4	477
1951	1.9	1.3	108.3	0.6	569.2	3	10.4	12.9	5.9	713.6
1952	1.6	3.2	213.9	0.1	1389	6	21	28.2	17.2	1681
1976	0	0.2	99.9	0.1	661.7	1.9	8	9.7	5.6	787.2

1978	0	0.9	139.6	0.7	1042	4.3	9.7	16.9	7.2	1221
1980	0	0.9	139.6	0.7	1042	4.3	9.7	16.9	7.2	1221
1987	0	1.6	150.8	0.4	1100.8	6.3	8.8	12.7	5.3	1286.6
2105	1.2	0.7	93.7	0	712.1	2.8	5.6	12	2.3	830.5
2106	1	1.4	190.1	0	1598.4	7.6	8.7	14.9	6.8	1828.9
2113	0	2.1	131.7	0.3	1081.8	6.1	8.3	16.4	6.4	1253.1

Table S3.4: The individual sesquiterpenes (ng mg^{-1}) in 30 families/genotypes of *Picea glauca* foliage from Carson Lake, Alberta. The units are based on fresh weight.

Family	α-Cadinene	α -Cadinol	Cubebol	Cubenene	Germacrene D-4-ol	Germacrene-D	γ-Cadinene	Σ-Cadinene	tau-cadinol	Total
128	11	0.5	192.2	1.8	834.3	3	6.4	9	3.4	1061.6
133	29.5	3.6	315	7.6	1996.5	8.4	21.4	32.6	15.3	2429.9
143	3.1	0.8	84.6	1.7	562.8	2.5	4.9	10	3.2	673.6
148	22.1	1.3	173.7	5	821.3	5.9	14.8	26	9.2	1079.2
156	79.7	11	665.5	15.7	3200.5	17	38.6	47.4	28.4	4103.7
157	11.8	0.9	94.1	2	532.5	2.8	7.4	10.8	3.9	666
158	17.5	2.2	164.7	3.6	1034.1	4.4	10.3	15.4	5.3	1257.6
170	15.7	1.4.0	93.2	3	579.4	2.9	8.5	11.3	5.8	721.3
176	64.4	5.6	357.1	12.1	2648.3	14.8	34.3	44.9	26.9	3208.4
178	0.8	0	16.1	0	95.5	0.1	1.6	2.7	0.4	117.3
180	20	2.6	124.5	5.3	886.3	5.4	12.1	15.8	8.8	1080.8
183	14	2.6	68.6	3.7	439.6	4.8	5.4	10.5	4.7	553.9
188	38.9	5	81.9	9.5	532.7	7.4	19.1	34.8	4.1	733.3
190	38.7	4.2	71.4	8.5	483.2	11.5	30.5	46.1	3.8	698
195	33.9	4.7	153.3	15.3	1202.5	18.7	20.7	38.8	9.5	1497.4
201	20.5	2.9	142.7	4.8	1073.6	5.8	12.6	19.4	14.3	1296.5
203	35.5	3.4	249.3	8.8	1869.5	9.9	21.5	36.2	24.5	2258.6
1580	25.5	0.9	154.6	5.2	1234.9	5.5	15.4	20.2	15.6	1477.9
1917	35.4	3	215.3	7.2	1651.3	9.5	20	29.1	26	1996.8
1918	10	1.5	79.3	2.2	629.9	4	6.6	10.4	6.8	750.8
1924	6.4	0.3	57.2	0.7	419.2	2.4	4.7	7.4	2.9	501.3
1951	5.9	0	48.9	0.8	295.5	2	4.1	6.3	2.5	366.1
1952	35	4.3	188.5	8.3	976.5	4.5	19	30.5	19.2	1285.8
1976	6.9	0	70.6	1	430.3	3.3	5.4	8.6	1.9	528.1
1978	21.3	2	181.8	4.6	1298.7	7.3	14.2	23	13.2	1566.2

1980	6.1	0.9	70.8	2.1	442	2	5.7	8.8	4.9	543.2
1987	0	0	31.2	0.8	196.2	0	2.2	3.1	0.8	234.4
2105	0	1.7	158.3	3.3	1190.1	0.1	10.9	17.4	9.2	1391.1
2106	1	3.8	310.8	8.3	2433.3	1.2	20.8	30.5	16.7	2826.4
2113	0	2.1	148.2	2.4	1023.9	0.4	7.8	11.9	8.2	1204.8

Table S3.5. Concentrations of individual mycelium metabolite (ng mg^{-}1) in 10 endophytic fungi of *Picea glauca* foliage. The units are based on dry weight.

Endophytic fungi	Ergosterol	Farnesol	Squalene	4-Methyl-1,6-heptadien-4-ol	9,12-Octadecadienoic acid	1-Octanol, 2-butyl	oxalic acid_6-ethyloct-3-yl	1-Decanol,2-hexyl	n-hexadecanoic acid	Dodecenal	9-Octadecenal	Total
*Chalara*_1	23889.5	25.1	49.8	14.6	20729.9	754.4	58.2	6.6	6880.0	1	27.3	52436.5
*Cladosporium*_2	7987.1	16.0	136.7	28.4	5348.2	1039.0	56.5	11.2	12624.0	93.1	123.4	27463.2
*Cladosporium*_2	10777.2	17.3	164.3	18.6	5234.5	52.1	90.5	15.6	4415.5	1	92.7	20879.4
*Didymella*_1	18178.6	22.9	29.6	16.1	241.3	955.2	28.5	28.7	64.8	11.8	20.9	19598.4
*Dothideomycetes*_1	11185.0	14.2	50.5	56.8	6842.7	299.4	73.4	22.6	2045.1	12.7	43.6	20645.9
*Chalara*_1	23889.5	25.1	49.8	14.6	20729.9	754.4	58.2	6.6	6880.0	1	27.3	52436.5
*Geopyxis*_2	5150.9	6.3	20.3	5.4	8545.9	278.2	7.9	5.6	2682.7	14.4	0.0	16718.6
*Geopyxis*_3	6343.3	14.9	373.8	25.9	327.7	883.2	66.8	10.9	516.4	41.4	17.5	8621.8
*Geopyxis*_4	21639.4	22.8	1143.0	25.3	1318.8	1170.0	53.3	14.9	232.2	10.4	83.8	25714.0
*Pezizales*_1	11503.4	21.5	303.4	37.9	125.2	525.0	52.4	21.6	77.4	28.9	47.1	12743.9

Table S3.6. Concentrations of individual volatile organic compounds (ng mg^{-1}) in 10 endophytic fungi of *Picea glauca* foliage. The units are based on dry weight.

Family	α-Pinene	Camphene	p-Cymene	β-Pinene	3-Carene	β-Phellandrene	2,4-Dimethyl-1-heptane	2,5-Dimethyl-1,5-hexadien-3-ol	2-cyclopenten-1-one	2,4,6,8-Tetramethyl-1-undecane	butanal-3 methyl	Hexyl octyl ether	Pentanal	Total
*Geopyxis*_1	8.1	1.2	1.4	17.6	10.4	18.5	5.3	6.1	3.8	0.5	2.2	10.5	0.4	85.8
Chalara sp.	0	0	0	0	0	0	6.4	5.9	0	0.7	2.8	9.0	0.6	25.4
*Cladosporium*_1	0	0	0	0	0	0	6.4	8.4	0	3.7	3.2	11.5	0.6	33.6
*Geopyxis*_2	0	0	0	0	0	0	5.3	5.9	0	0.5	1.4	7.4	0.5	21.2
*Dothideomycetes*_1	7.8	1.1	15.8	13.6	21.8	7.3	5.8	6.2	7.7	0.5	2.7	11.7	0.4	102.4
Pezizales 1	0	0	0	0	0	0	5.4	7.1	0.0	0.5	2.3	10.6	0.5	26.4
Cladosporium 2	0	0	0	0	0	0	5.6	9.9	0	1.5	3.4	12.4	0.5	33.3
*Geopyxis*_4	4.4	0.54	1.4	4.2	5.8	9.6	4.2	3.4	4.6	5.3	1.0	14.8	0.3	59.5
*Geopyxis*_3	0	0	0	0	0	0	3.2	3.4	0	6.8	1.3	10.7	0.3	25.7
*Didymella*_1	0	0	0	0	0	0	3.3	3.3	0	8.1	1.1	10.0	0.2	25.9

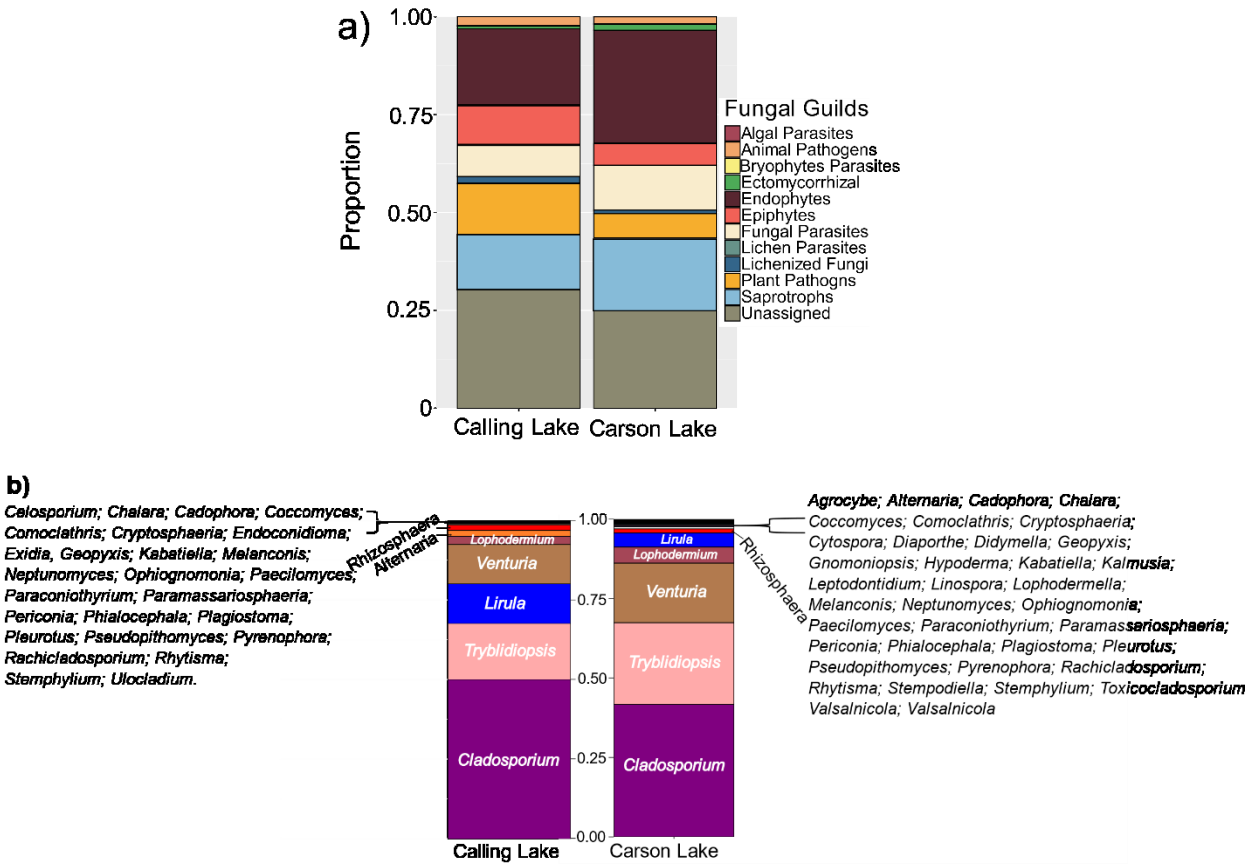

Figure 3.1. Proportions of (a) fungal guilds and (b) endophytic fungal genera identified in *Picea glauca* families from Calling Lake and Carson Lake, Alberta based on FUNGuild database (Nguyen *et al.,* 2016).

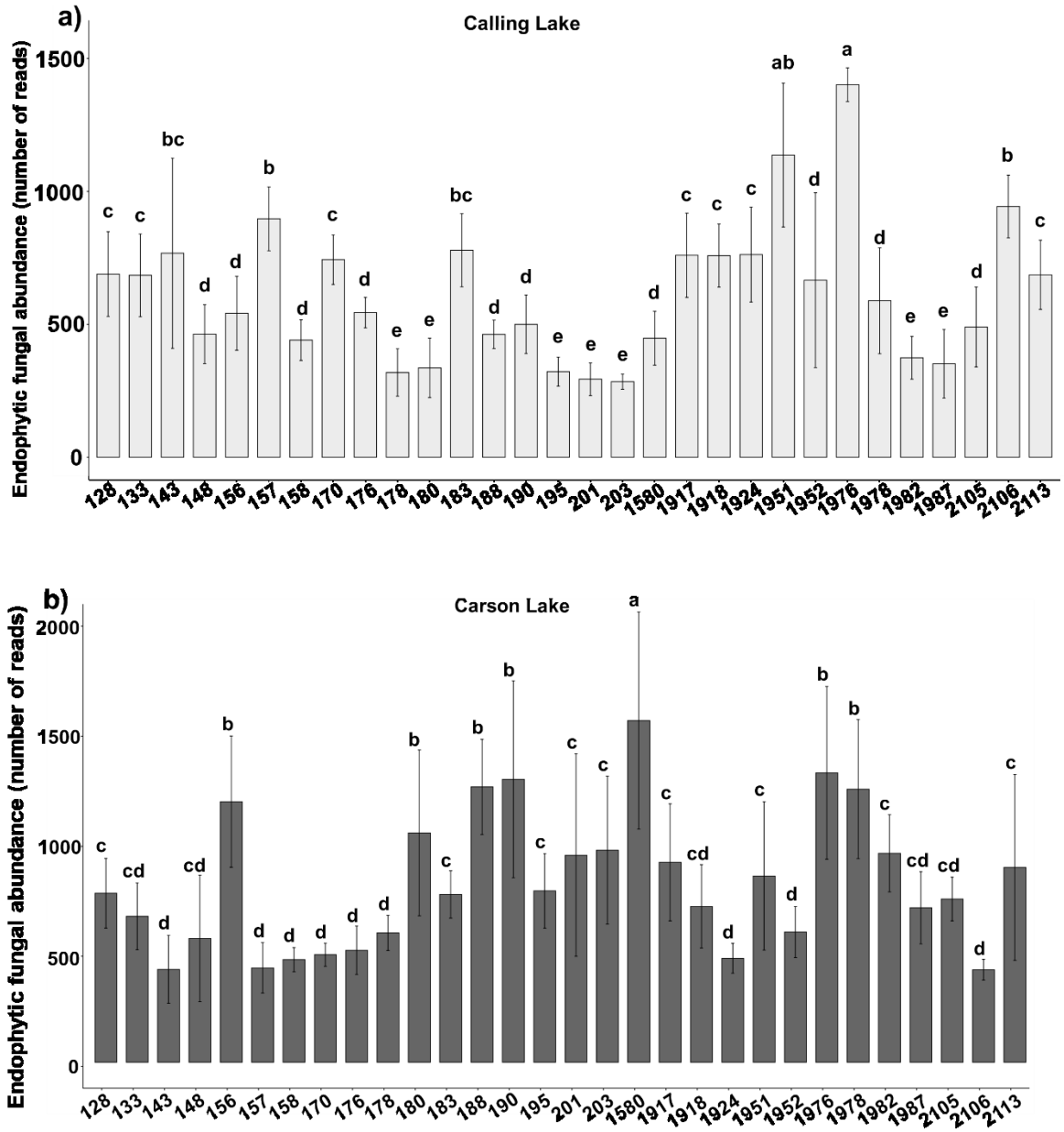

Figure 3.2. Mean (±SE) endophytic fungal abundance (number of reads) in 30 different phenotypes of *Picea glauca* in Calling Lake (a) and Carson Lake (b), Alberta. Bars with different letters are statistically different as indicated by Tukey HSD tests. *P* value indicates results of one-way ANOVA. Calling Lake (a): $F_{29,210}$=2.95, *P* <0.001, n=4/family; Carson Lake (b): $F_{29,197}$=1.99, *P* <0.001, n=4/family

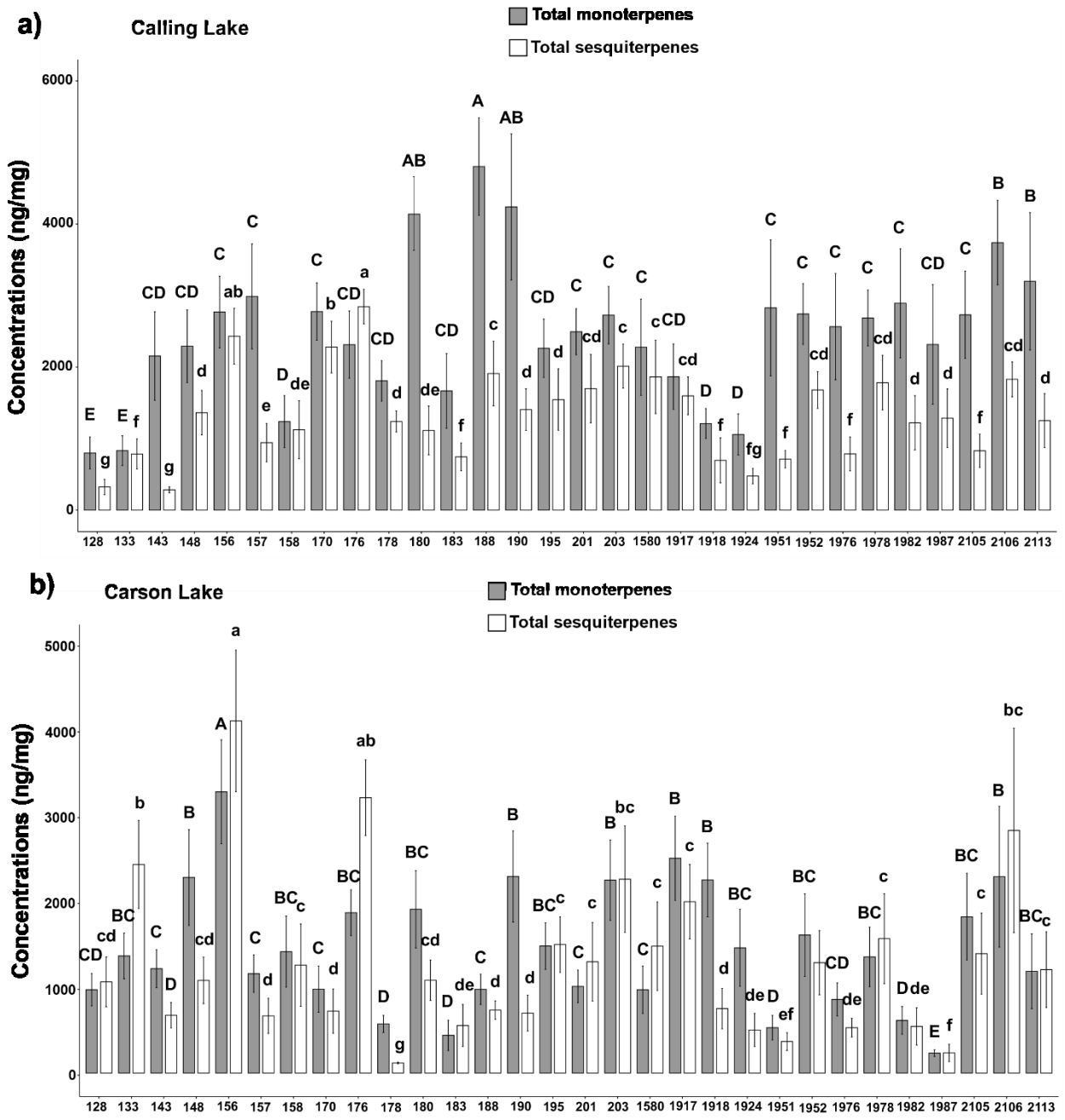

Figure 3.3. Mean concentrations (±SE) of total monoterpenes (grey bars) and total sesquiterpenes (white bars) from 30 different of *Picea glauca* phenotypes in **a)** Calling Lake and **b)** Carson Lake, Alberta. Bars with different capital letters representing total monoterpenes and small letter representing total sesquiterpenes are statistically different as indicated by Tukey HSD tests. *P* values indicate results of one-way ANOVA. Calling Lake: Total monoterpenes: $F_{29,210}$=2.3, P<0.001; Total sesquiterpenes: $F_{29,210}$=3.94, P<0.001. Carson Lake: Total monoterpenes: $F_{29,210}$=3.99, P<0.001; Total sesquiterpenes: $F_{29,210}$=4.63, P<0.001.

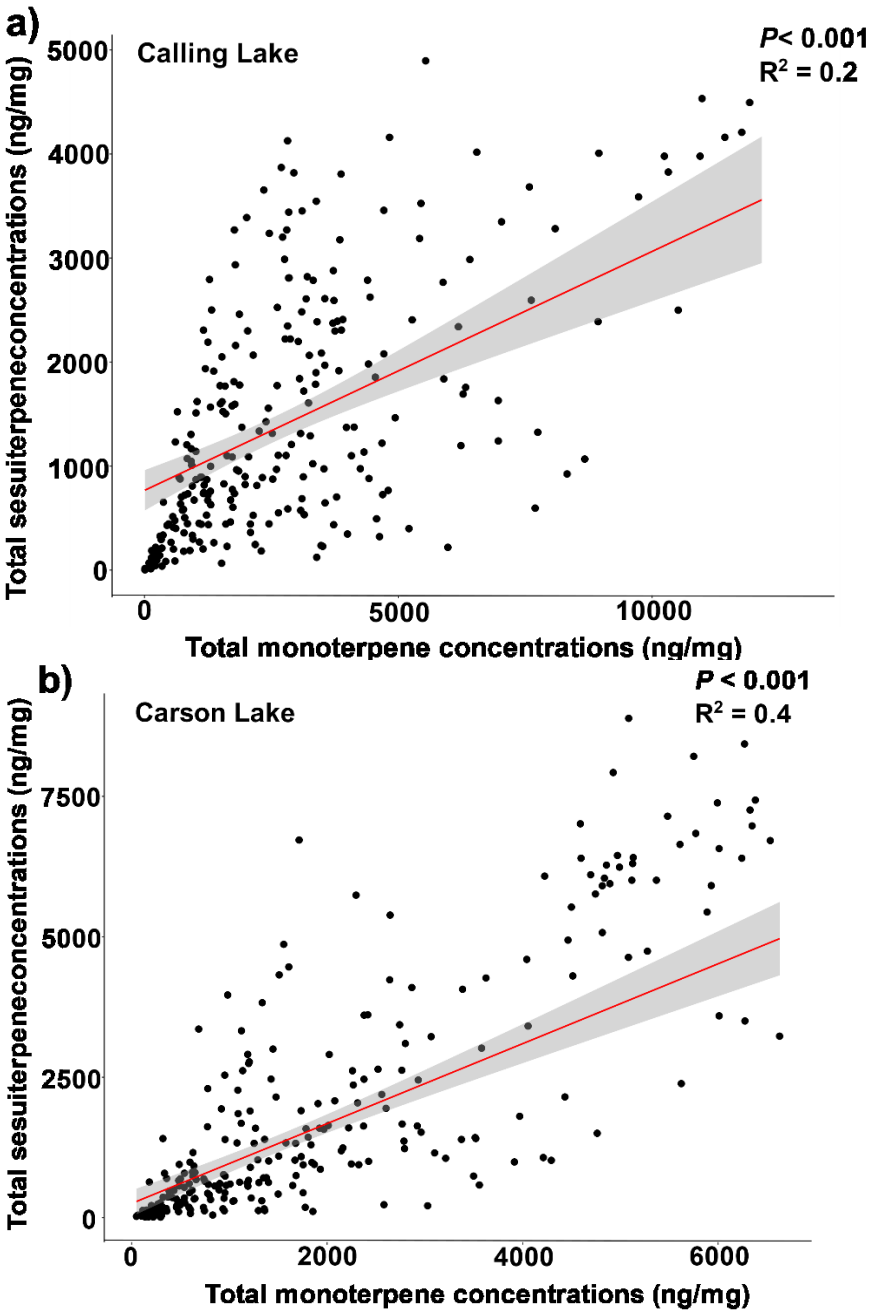

Figure 3.4. The relationship between *total monoterpene and total sesquiterpene concentrations* (ng mg^{-1}) of *Picea glauca* from **a)** Calling Lake and **b)** Carson Lake, Alberta. Line and shade denote regression line and 95% confidence interval, respectively.

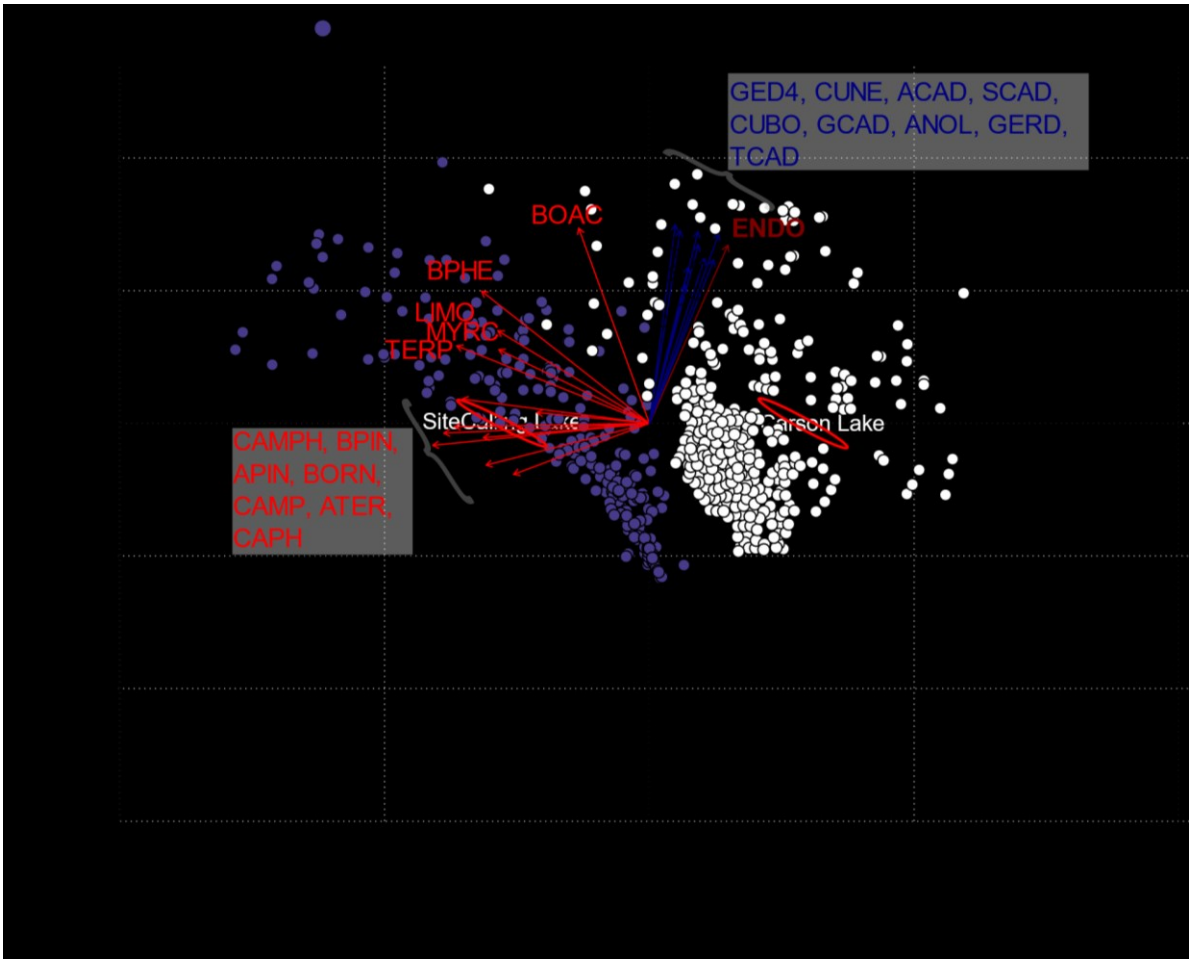

Figure 3.5. Canonical Analysis of Principal Coordinates (CAP) plot the distribution of individual monoterpenes, sesquiterpenes, and total endophytic fungal abundance in *Picea glauca* from Calling Lake and Carson Lake, Alberta. Red vectors represent individual monoterpenes, blue vectors showing individual sesquiterpenes and brown vector showing fungal endophytic abundance. Abbreviations for monoterpenes: BPIN = β-pinene, APIN= α-pinene, LIMO = limonene, MYRC= myrcene, CAMP = camphene, CAPH = camphor, BPHE = β-phellandrene, BORN = borneols, BOAC= bornyl acetate, TERP = terpinolene, and ATER = α-terpineol. Abbreviations for sesquiterpenes: GED4 = germacrene-D-4ol, CUNE = cubenene, ACAD = α-cadinol, SCAD = Σ -cadinene, CUBO = cubebol, GCAD = γ-Cadinene, ANOL = α-cadinol, GERD = Germacrene.D, TCAD = tau.cadinol. ENDO refers to total endophytic fungal abundance.

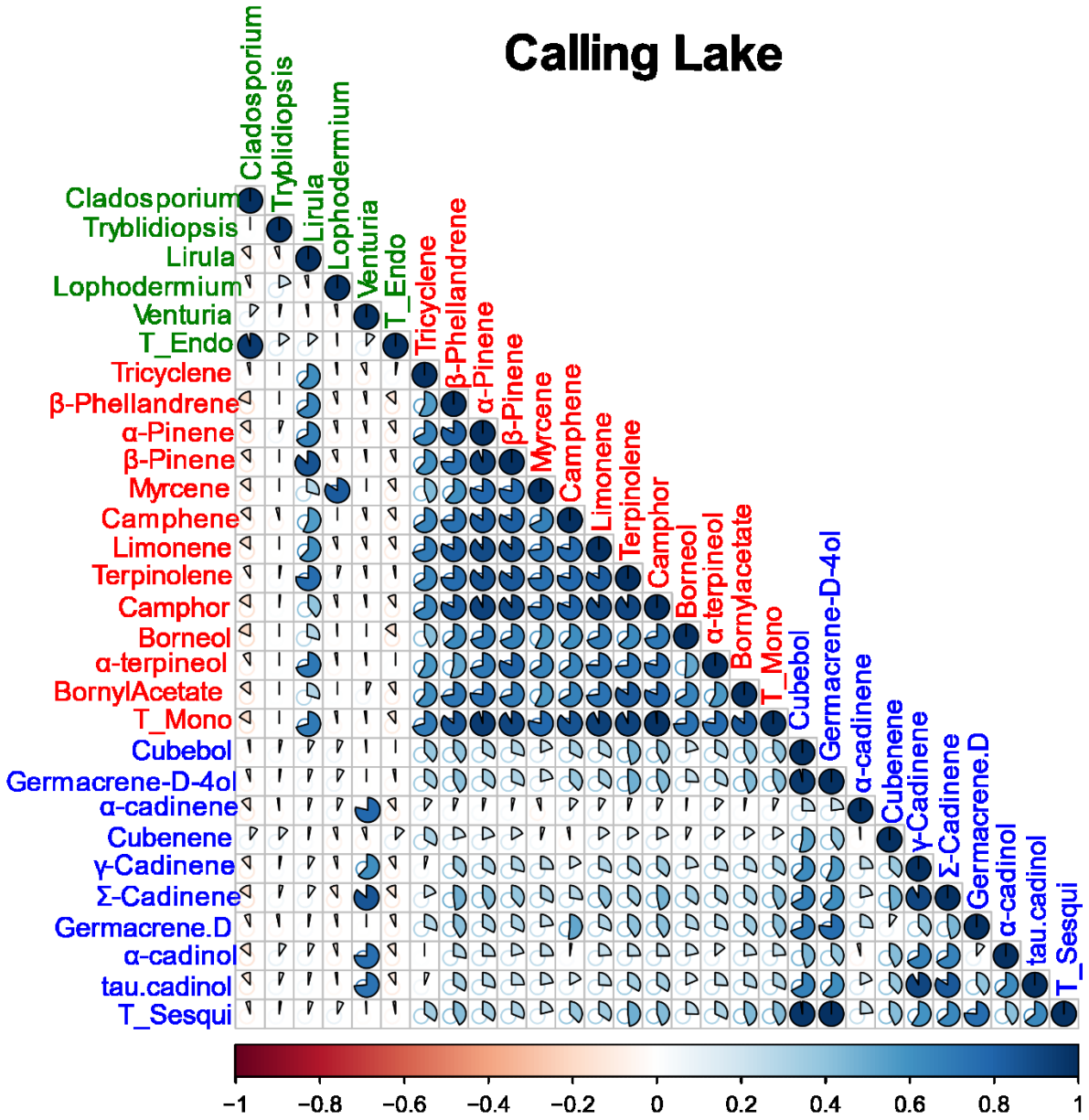

Figure 3.6. Pearson correlation pie charts (r) for individual monoterpenes (red text), individual sesquiterpenes (blue text), (ng mg^{-1} Fresh Weight) and endophytic fungal genera abundance (green text) of *Picea glauca* obtained from Calling Lake, Alberta. Pie sizes are represented by values ranging from -1 to +1. The darker the blue or red pie charts, the closer the r value is to either 1 or - 1.

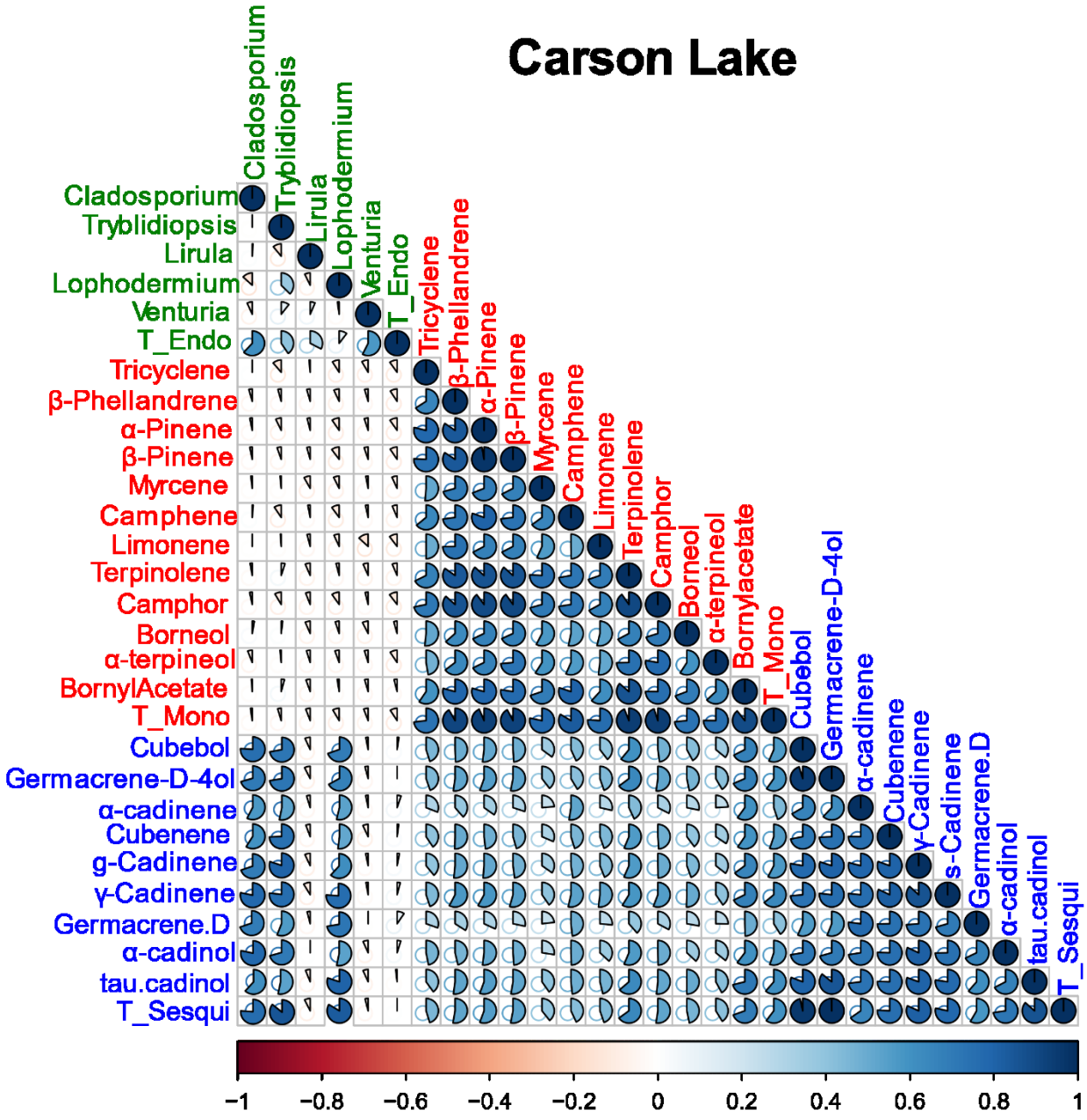

Figure 3.7. Pearson correlation pie charts (r) for individual monoterpenes (red text), individual sesquiterpenes (blue text), (ng mg^{-1} Fresh Weight) and endophytic fungal genera abundance (green text) of *Picea glauca* obtained from Carson Lake, Alberta. Pie sizes are represented by values ranging from -1 to +1. The darker the blue or red pie charts, the closer the r value is to either 1 or - 1.

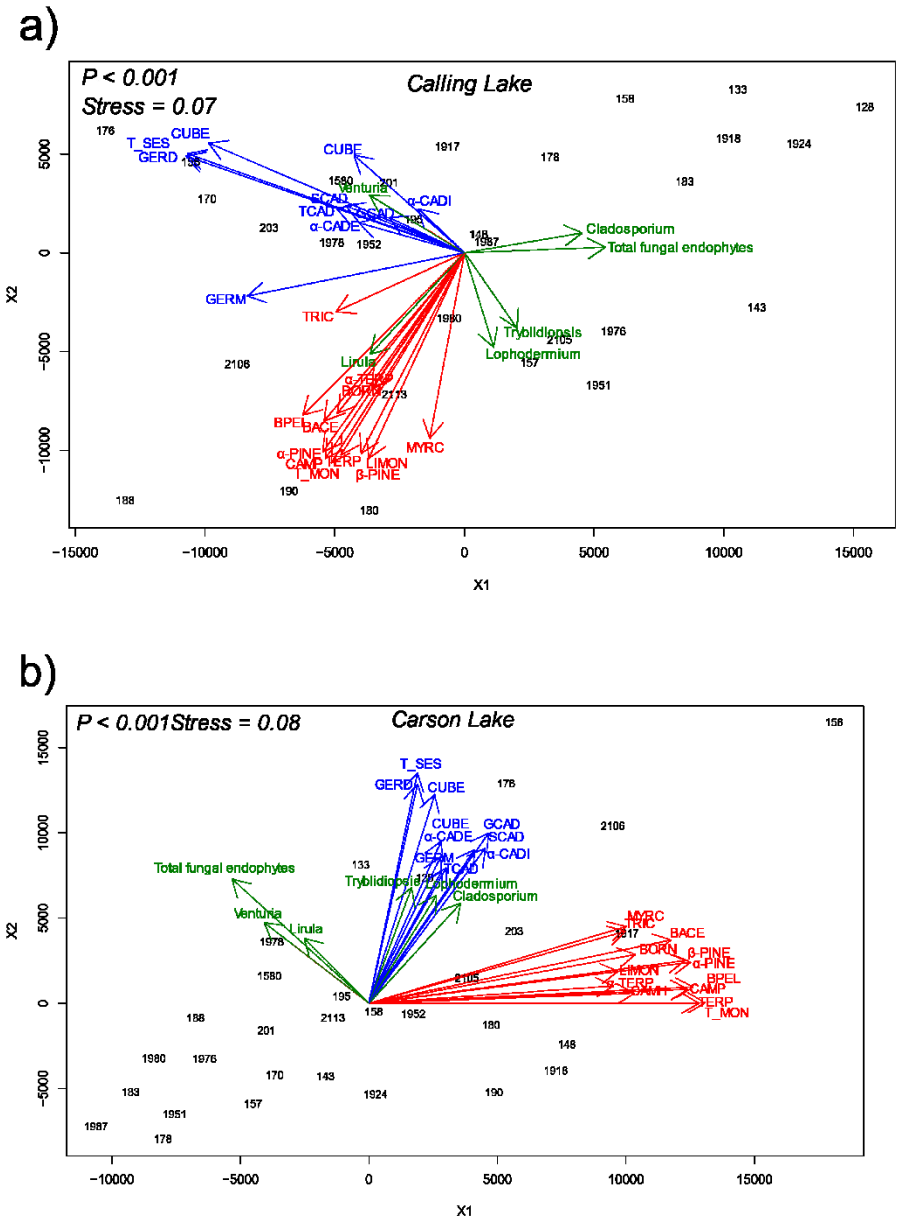

Figure 3.8: NMDS plot the distribution of individual monoterpenes, sesquiterpenes, and total endophytic fungal abundance in different *Picea glauca* phenotypes **a**) Calling Lake and **b**) Carson Lake. Red vectors represent individual monoterpenes, blue vectors individual sesquiterpenes, green vector fungal endophytic abundance. Abbreviations for monoterpenes: BPIN = β-pinene, APIN= α-pinene, LIMO = limonene, MYRC= myrcene, CAMP = camphene, CAPH = camphor, BPHE = β-phellandrene, BORN = borneols, BOAC= bornyl acetate, TERP = terpinolene, and ATER = α-terpineol. Abbreviations for sesquiterpenes: GED4 = germacrene-D-4ol, CUNE = cubenene, ACAD = α-cadinol, SCAD = Σ -cadinene, CUBO = cubebol, GCAD = γ-Cadinene, ANOL = α-cadinol, GERD = Germacrene.D, TCAD = tau.cadinol. Significance difference between phenotypes was determined by PERMANOVA at α = 0.05.

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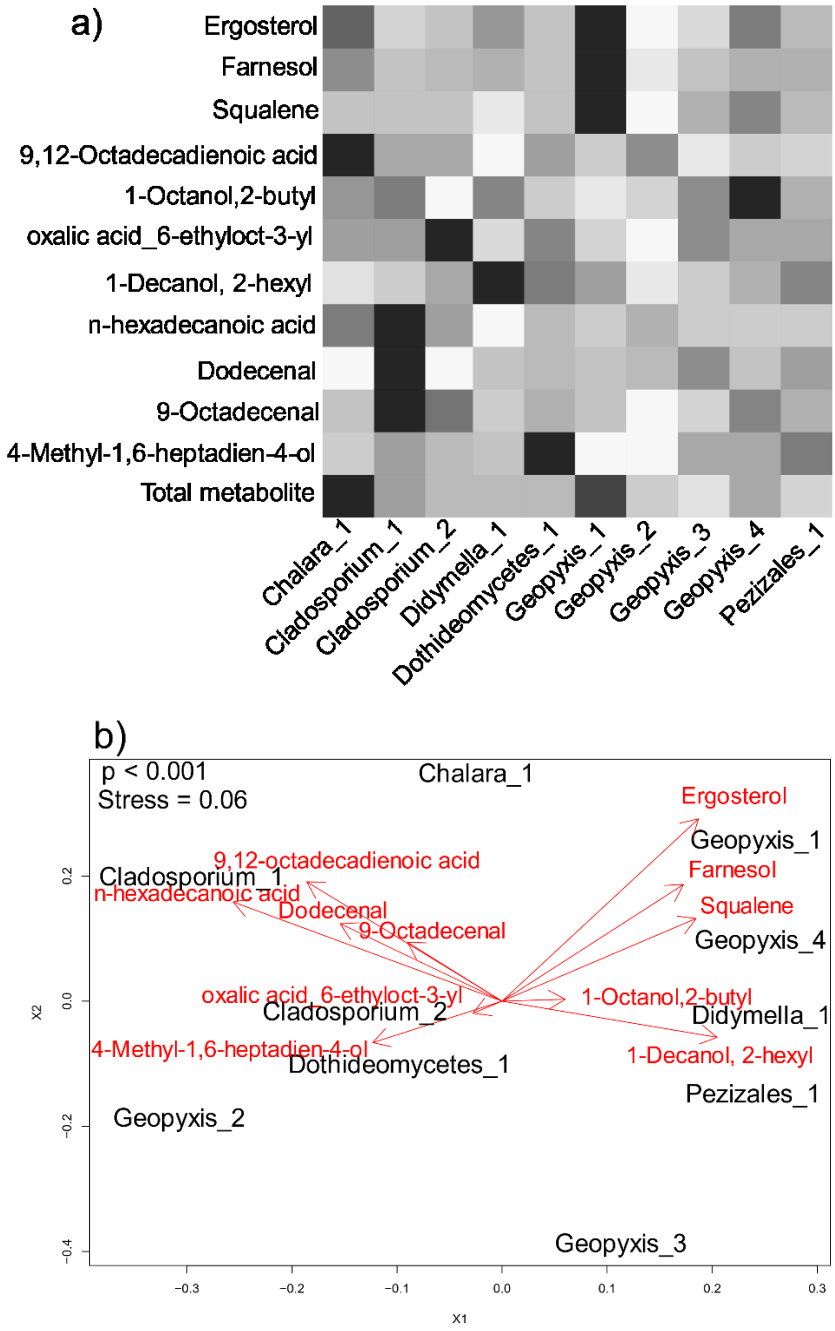

Figure 3.9. a) A heatmap showing the metabolite profiles of ten endophytic fungi. Lowest to highest concentrations (ng mg-1 dry weight) were demonstrated by light to dark colors, respectively. Concentration of individual compounds was compared across endophytic fungi. **b)** NMDS plot the distribution of individual endophytic fungal metabolites (red vectors) abundance in different endophytic fungi (black text).

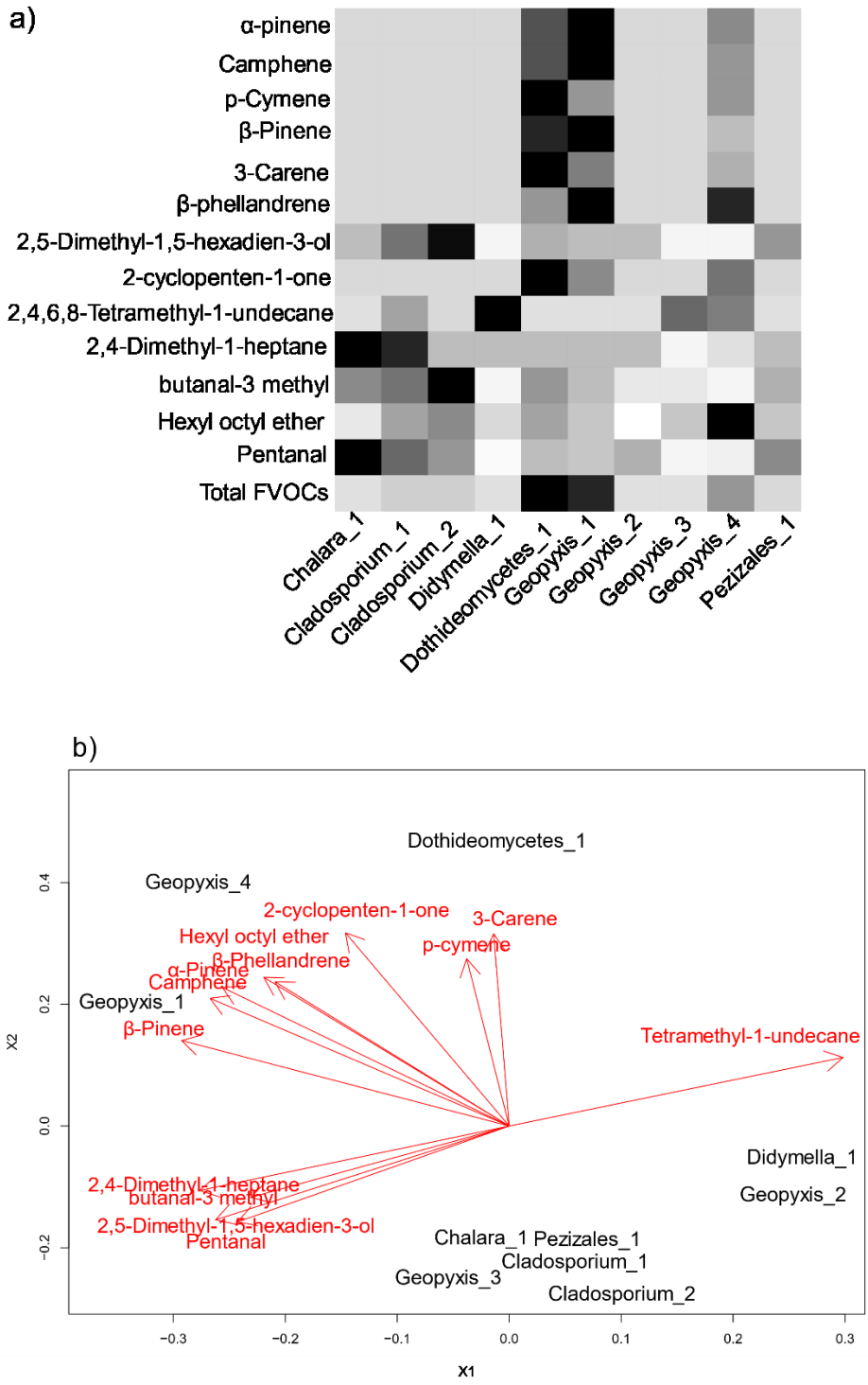

Figure 3.10. a) A heatmap showing the volatile organic compounds profiles of ten endophytic fungi. Lowest to highest concentrations (ng mg^{-1}) were demonstrated by light to dark colors, respectively. Concentration of individual compounds was compared across endophytic fungi. **b)** NMDS plot the distribution of individual endophytic fungal volatile organic compounds (red vectors) abundance in different endophytic fungi (black text).

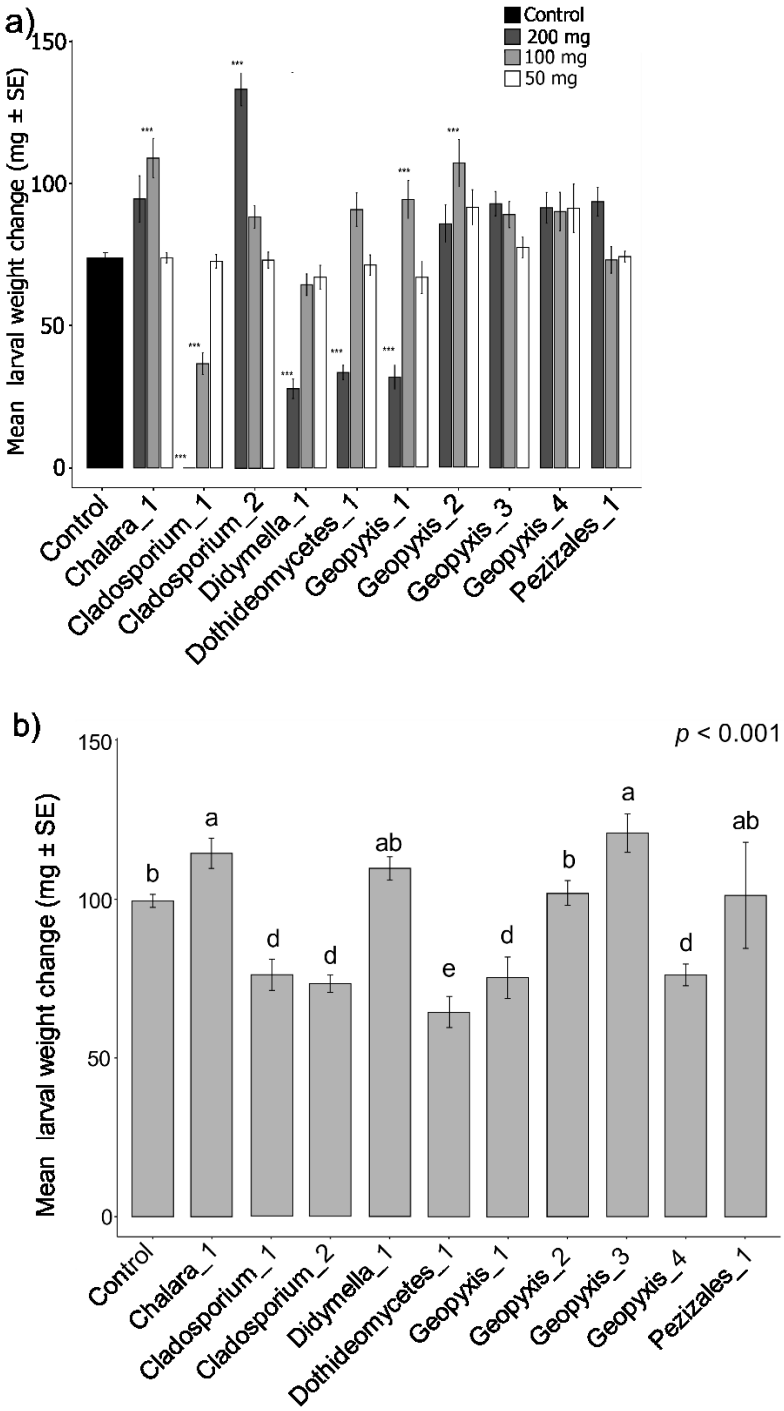

Figure 3.11. Mean (±SE) larval weight change (mg) of *Choristoneura fumiferana* on **(a)** 10 endophytic fungi with 3 different dosage and **(b)** endophytic fungal volatile organic compounds. (a) The one-way ANOVA was conducted separately by comparing control vs each endophytic fungus with 3 dosages separately. The asterisks show significant differences from control. (b) One way ANOVA was conducted among different endophytic fungi and control treatment. Bars with different letters are statistically different (Tukey HSD tests, P <0.001).

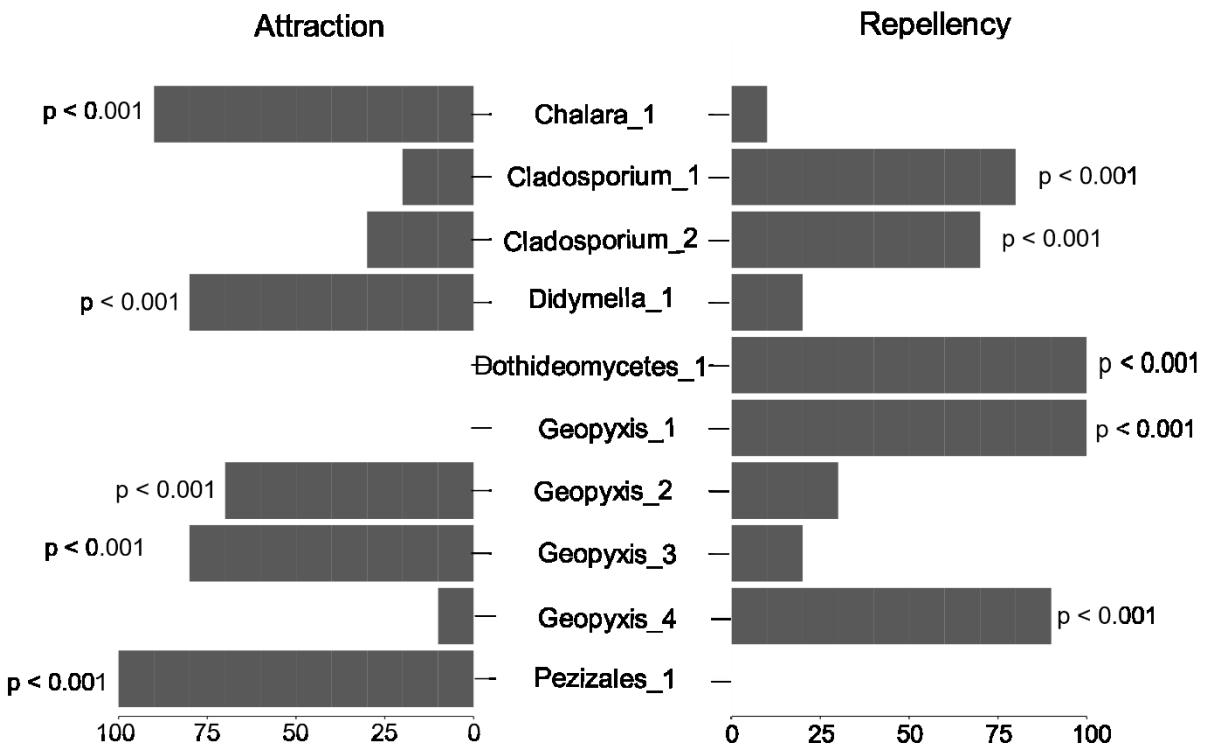

Figure 3.12. Percentage of behavioral responses of spruce budworm larvae to endophytic fungi. The larvae's reaction to the fungal treatments is shown in the right bar graph, whereas the control group's response is depicted in the left bar graph. Two-sample t-test results demonstrate a statistically significant difference at the 0.05 level of probability.

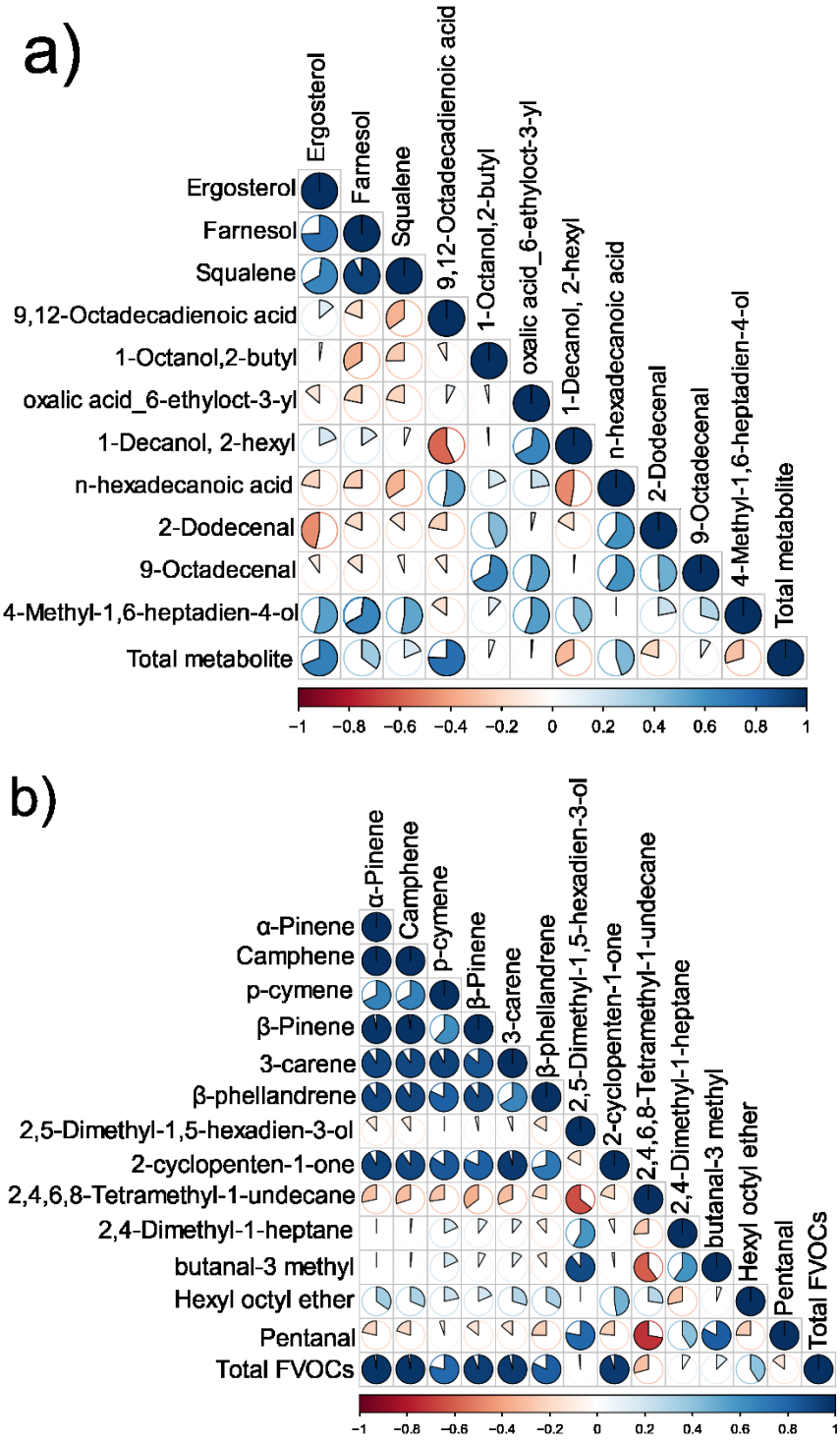

Figure 3.13. Pearson correlation pie charts (r) for **(a)** individual endophytic fungal metabolite, **(b)** individual endophytic fungal volatile organic compounds. The darker the blue or red pie charts, the closer the r value is to either 1 or - 1.

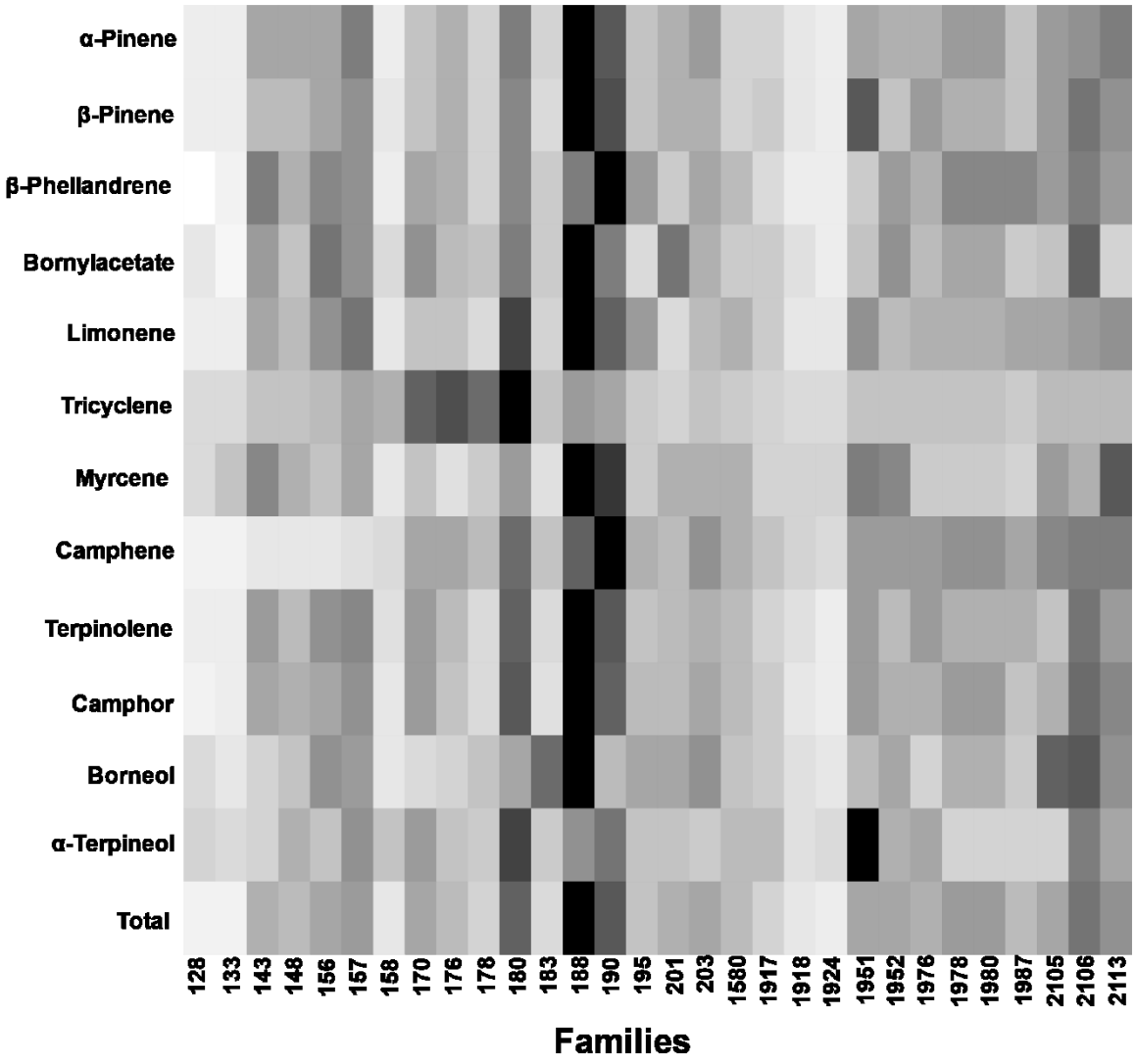

Figure S3.1. A heatmap showing the monoterpenes profiles of 30 families of *Picea glauca* that were sampled from Calling Lake, Alberta. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark shades, respectively. Concentrations of individual compounds were compared across families.

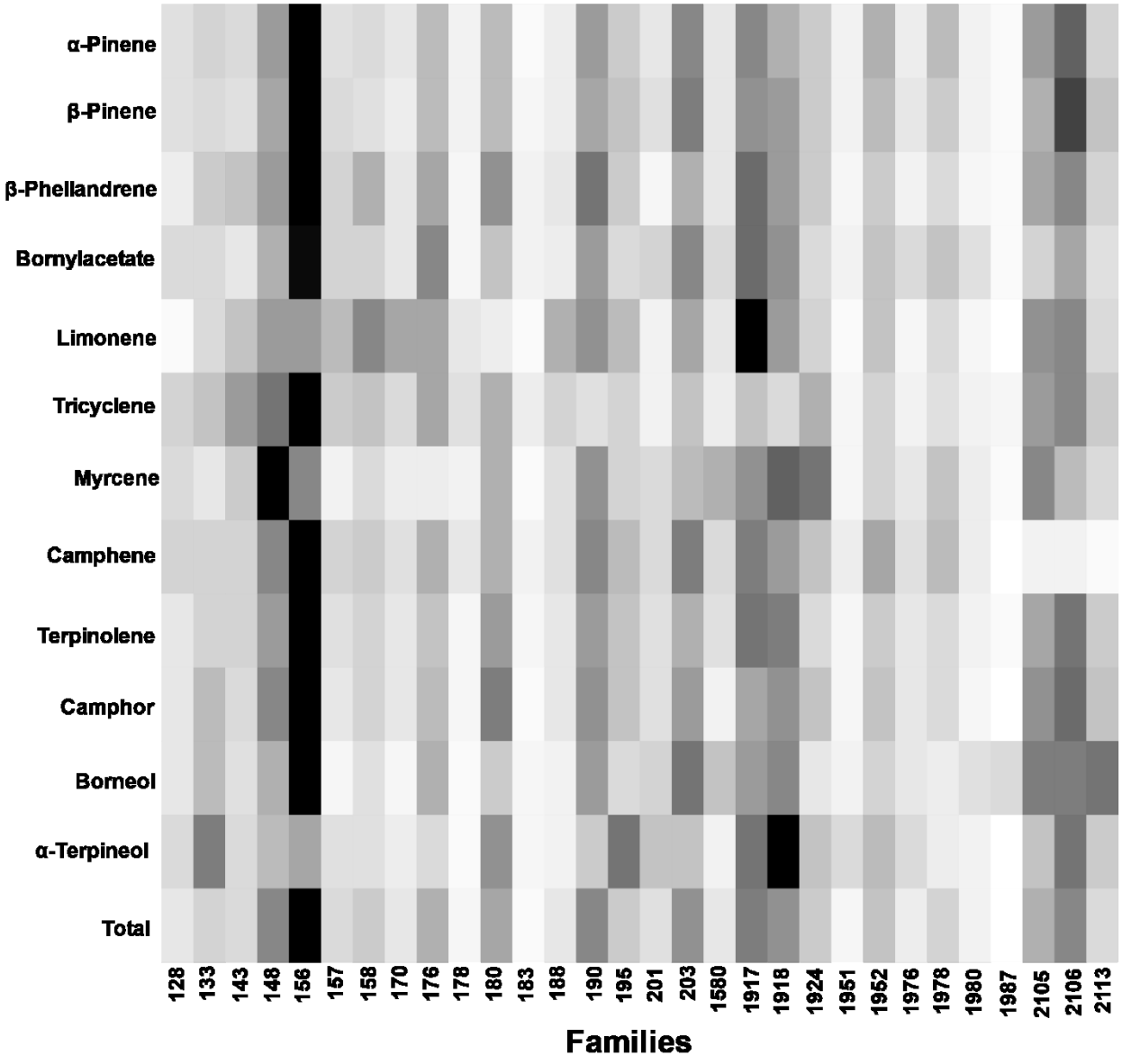

Figure S3.2. A heatmap showing the monoterpenes profiles of 30 families of *Picea glauca* that were sampled from Carson Lake, Alberta. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark shades, respectively. Concentrations of individual compounds were compared across families.

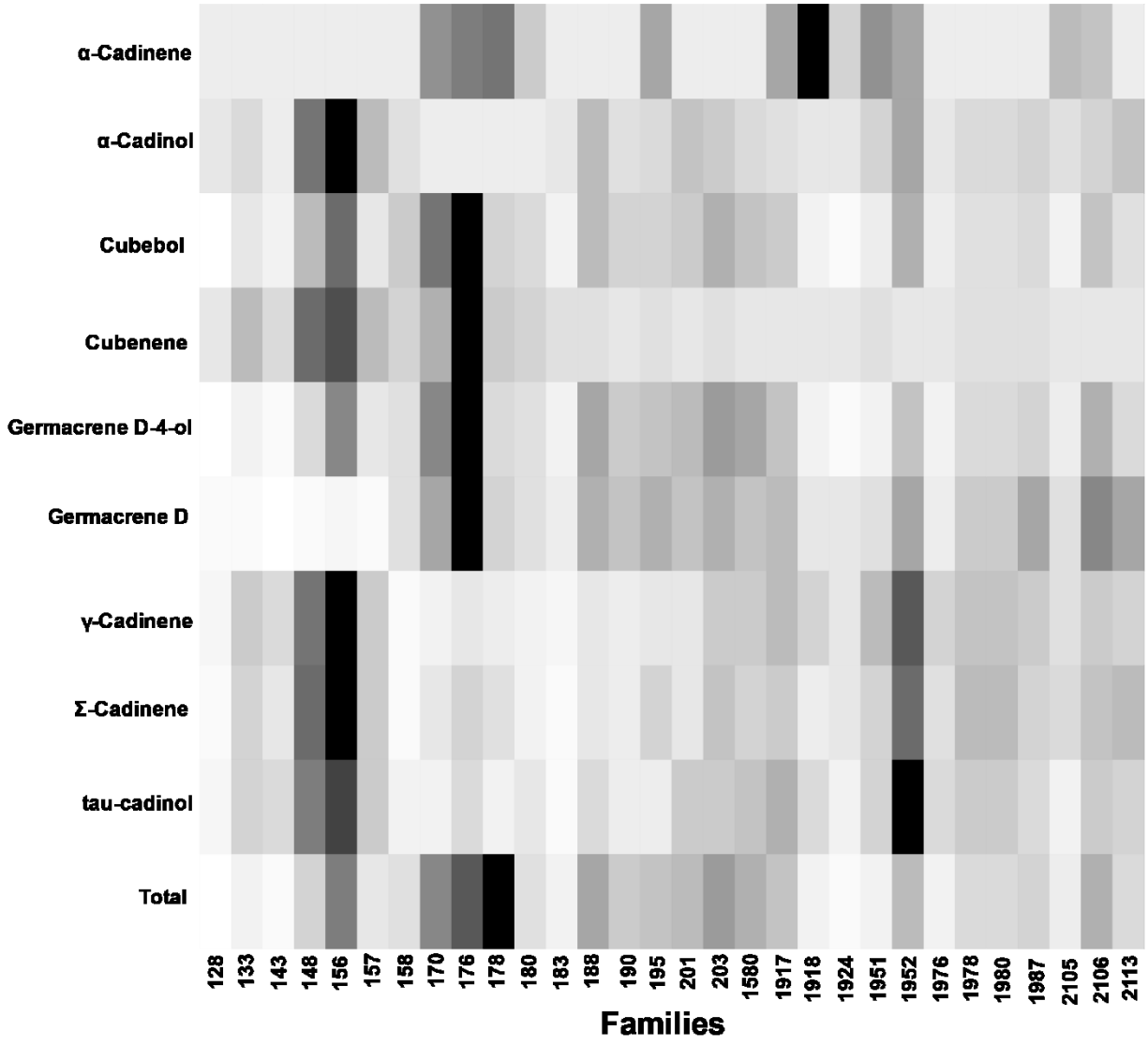

Figure S3.3. A heatmap showing the sesquiterpenes profiles of 30 families of *Picea glauca* that were sampled from Calling Lake, Alberta. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark shades, respectively. Concentrations of individual compounds were compared across families.

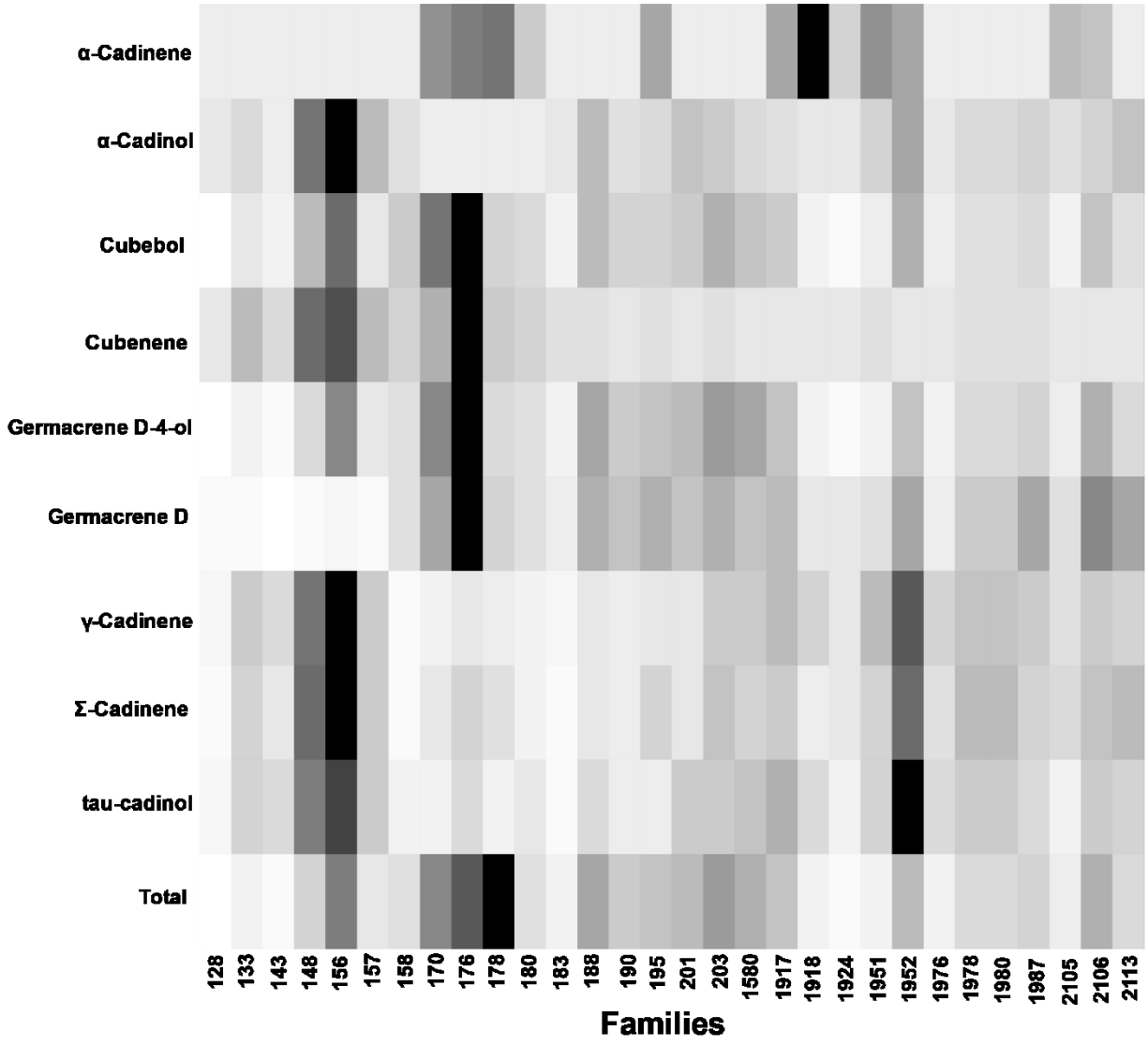

Figure S3.4. A heatmap showing the sesquiterpenes profiles of 30 families of *Picea glauca* that were sampled from Carson Lake Site. Lowest to highest concentrations (ng mg^{-1} Fresh Weight) were demonstrated by light to dark shades, respectively. Concentrations of individual compounds

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Chapter IV

Fungal endophytes of white spruce modulate tree defenses

4.1 Introduction

Fungal endophytes are a diverse and fascinating class of fungi that have gained much attention in recent years due to their unique relationship with host plants (Strobel & Daisy, 2003; Rodriguez *et al.*, 2009). Fungal endophytes colonize plant tissues including foliage, roots, bark, and phloem without causing any visible symptoms or harm to the host plants, but rather establish mutualistic relationships with them (Saikkonen *et al.*, 2004; Aly *et al.*, 2011). Fungal endophytes can be divided into two main groups based on their host specificity and transmittance: clavicipitaceous and non-clavicipitaceous (Rodriguez *et al.*, 2009). Clavicipitaceous fungal endophytes belong to the Epichloe and Neotyphodium genera and have a limited range of hosts, primarily grasses (Kuldau & Bacon, 2008). Non-clavicipitaceous fungal endophytes, on the other hand, establish symbiotic relationships with a wide range of hosts and are transmitted vertically and horizontally (Partida-Martínez & Heil, 2011). The diversity of the fungal endophytes and their host specificity is not fully understood, and various factors such as geography, environment, and host genotype may contribute to the variation in diversity and specialization among fungal endophytes communities (Stefani & Bérubé, 2006a; Bálint *et al.*, 2013). Furthermore, although fungal endophytes are well-known for regulating their plant growth and development, we currently lack experimental demonstration of whether fungal endophytes can also alter plant secondary or defense compounds.

In conifers, each tree species is associated with a distinct community of endophytes (Stefani & Bérubé, 2006b; Millberg *et al.*, 2015). In addition, there appears to be an ontogenetic effect on

the fungal assemblages as older trees tend to have a more diverse fungal communities than younger trees (Ganley & Newcombe, 2006; Bowman & Arnold, 2021; Neeraja *et al.*, 2021). Several endophytic fungal genera are commonly isolated from conifer species, including *Cladosporium halotolerans, Cladosporium cladosporioides, Chalara* sp.*, Geopyxis* sp.*,* and *Didymella* sp. *Dothideomycetes* sp., and *Pezizales* (Chapter III), *Trichoderma, Penicillium, Aspergillus, Mucor, Alternaria, Sphaeropsis, Fusarium,* and *Chaetomium* (Fu-qiang *et al.*, 2004; Stefani & Bérubé, 2006b). These genera are not just limited to conifers and can be found in a wide range of non-conifer species, highlighting the broad host range that non-clavicipitaceous fungal endophytes can establish symbiotic relationships with (Petrini, 1991; Larran *et al.*, 2007; Liu *et al.*, 2018).

White spruce (*Picea glauca* (Moench) Voss), is widely distributed conifer species in North America and has a significant ecological and commercial value (Lu *et al.*, 2014). White spruce foliage contains a diversity of endophytic fungi. In eastern Canada the most abundant foliar fungal endophyte is *Lophodermium piceae*, followed by *Mycosphaerella* spp., *Hypoxylon* spp., and *Phomopsis* spp. (Stefani & Bérubé, 2006a). However, when surveyed in two locations in Alberta, Canada the most abundant foliar fungal endophyte is *Cladosporium* spp., followed by Trybliodiopsis spp., and *Venturia* spp. (Chapter III of this thesis). Since most fungal endophytes produce several classes of secondary metabolites such terpenoids which can have a wide range of antimicrobial and insecticidal properties (Sumarah *et al.*, 2005b; Alurappa *et al.*, 2018; Fadiji & Babalola, 2020; Choudhary *et al.*, 2021), there is a growing research interest about understanding the possible contribution of fungal endophytes in white spruce resistance to its primary defoliator, the eastern spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) (Sumarah *et al.*, 2005b; Frasz *et al.*, 2014; Quiring *et al.*, 2019b,a).

Recent research has investigated the potential application of fungal endophytes for enhancing plant growth and defenses against pests and diseases. These studies have found that in general, application of fungal endophytes has improved host plant biomass and physiology including primary and secondary metabolites, food absorption and respiration (Gupta *et al.*, 2021). For instance, when the fungal endophyte 5WS22E1 (DAOM 229536) was inoculated into nine half-sib families of white spruce seedlings, the inoculated seedlings produced a much greater quantity of the secondary metabolite rugulosin (Sumarah *et al.*, 2005b). Follow-up studies showed that the rugulosin inhibited the growth of the eastern spruce budworm (Sumarah & Miller, 2009). Another study reported that endophytes isolated from *Pinus halepensis* produced a range of secondary metabolites that had antifungal activity against plant pathogens such as *Fusarium oxysporum* and *Botrytis cinerea* (Rodriguez *et al.*, 2009).

All these studies together suggest that continued research on fungal endophytes is essential to understand their role in plant ecology and to harness their potential for practical applications in pest resistance. For instance, understanding the relationship between white spruce trees and their fungal endophytes can help in developing more sustainable and effective forest pest management practices. However, such understanding requires additional research to determine the mechanisms underlying the interactions between fungal endophytes and their host plants, as well as the potential applications of fungal endophytes in improving host tree resistance and overall health in the face of changing climatic conditions.

The objective of this study was to determine whether the changes in foliar fungal endophyte communities alter the terpene profiles and biomass of white spruce seedlings. Investigating the contributions of fungal endophytes on plant defense metabolites can deepen our understanding of the ecological and co-evolutionary interactions between plants and fungal endophytes.

Furthermore, the outcome of this study could potentially result in the application of fungal endophytes to improve seedling establishment and growth.

4.2 Materials and Methods

4.2.1 Seed stock preparation

I obtained seeds from the same 30 half-sib white spruce families from Smokey Lake Tree Nursery (Smokey Lake, Alberta) as were used in chapters II and III of this thesis. I stratified the seeds for 30 days prior to sowing. Briefly, 5% bleach was used to surface sterilized the seeds for 15 min, rinsed in distilled water, after that soaked in distilled water for 24 h. Seeds were dried and placed in cold storage (4° C) for 28 days.

4.2.2 Seedling fertilization and dormancy protocol

Beginning five weeks post germination, seedlings were fertilized with 300 ppm phosphorus using a 10-52-10 (N:P:K) fertilizer mix and 18 ppm iron chelate every four weeks. Fertilizer was applied to avoid phosphorus and iron deficiencies that could be identified by the reddening and yellowing of seedling needles, respectively. The seedling dormancy protocol was adapted from Kanekar *et al.* (2018). Seedlings were gradually acclimated to cold storage for two weeks; first stored at 15℃ during the day and 10℃ at night for the first week and then 6℃ for the second week. After seedlings were acclimated to the cold, they were stored at 4℃ for five weeks and then the temperature in cold storage was gradually increased 3℃ every day for a week until the temperature in the growth chamber reached 23℃. While dormancy was being simulated in the growth chamber, seedlings were watered weekly and kept under a 12h:12h (light: dark) light regime. No fertilization application took place during dormancy.

4.2.3 Fungal endophyte spore preparation

I prepared potato dextrose agar (PDA, 24g potato dextrose broth (DifcoTM Potato Dextrose Broth), 15g agar (Difco BactoTM Agar), and 1 L distilled water) and then autoclaved it at $Liquid_{20}$ autoclave cycle. It was then cooled down at room temperature. After that, I poured the media into the Petri dishes (100 mm diameter × 15 mm height). The media was allowed to set for 24 h at 23 °C. These dishes were ready for fungal inoculation after 24 h. I inoculated the nine-day-old culture from the advancing edge of an actively growing colony of endophytic fungus; one fungal species inoculated per dish. Multiple dishes were inoculated with individual fungal endophytes in order to obtain a large number of spores. I harvested the fungal spores after 10-14 days when the fungal culture produced significant amounts of spores on Petri dishes.

4.2.4 Fungal application on seedlings

I grew 20 seedlings from each white spruce family in a growth chamber under controlled conditions for until they were 10-month-old (total 600). I randomly divided seedlings from each family into two groups (n=10 per treatment per family). One group was inoculated with a mixture of five species of fungal endophytes by leaf-spraying and the other group was used as controls (no fungal inoculations). I prepared the spore stock suspension for each fungus by using an established protocol (Raghavendra & Newcombe, 2013). Briefly, spore suspensions were prepared using endophytic fungal cultures varying in age from 10 to 14 days. Multiple culture plates from each endophyte species were used to make a spore suspension, which was subsequently homogenized and suspended in sterile distilled water. The spore concentrations were set at 1×10^8 conidia ml^{-1}.

For the purpose of inoculation, equal quantities of five different endophytic fungal spore suspensions were mixed together along with Tween 20 (1 ml/L). Each seedling received 10 ml of a mixture of endophytic fungal solution *via* sterilized hand-sprayer. After inoculation the seedlings were covered with a plastic bag for a period of 24 h in order to maintain high humidity inside the bags to accelerate fungal infection. The control seedlings were only sprayed with Tween 20 suspension. After two months of fungal inoculation, I collected the needles from each seedling and stored then at –40 °C until processing. I also calculated the fresh biomass of needles and branches separately.

4.2.5 Foliage sterilization and molecular analysis

Two months following inoculation, I collected foliage from all seedlings. The surface of foliage was sterilized by first washing it for 4 min in a solution that contained 3% sodium hypochlorite (NaOCl), and then rinsing it twice for 1 min in sterile distilled water. I freeze-dried the spruce foliage for a total of three days. After that, I used a TissueLyser II (Qiagen, Montreal, QC, CAN) to grind the freeze-dried foliage.

Using the EZNA® HP Fungus DNA Kit, genomic DNA was isolated from 50 mg of freeze-dried powdered foliage and measured in an ND-1000 Nanodrop. The two-step polymerase chain reaction (PCR) was used to amplify the eukaryotic ribosomal internal transcribed spacer region (ITS1) of the nucleus ribosomal DNA. In order to sequence the Illumina MiSeq (Illumina Inc., San Diego, CA, USA), the fungus ITS regions were amplified in a two-step PCR using the fITS7 (Ihrmark *et al.,* 2012) and ITS4 (White *et al.,* 1990) primers with Illumina overhang adapters. The PCR reactions were produced in accordance with the specifications of the PlatinumTM SuperFiTM Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA), and 1 uL of

DNA extract was used in each one. The reactions were then run on a MyCyclerTM Thermal Cycler (Eppendorf AG 22331 Hamburg, GER). PCR results were verified using 2% agarose gel electrophoresis with SYBR™ Safe DNA Gel Dye (Invitrogen) 100V, 0.5 hr. and Gene Genius Bio imaging system (Syngene, Frederick, MD, USA).

PCR amplicons were cleaned using Mag-Bind TotalPure NGS Kit (Omega BioTek, Norcross, GA, USA) to remove primers and primer dimers, then tested using UV light and gel electrophoresis. The first fITS7 PCR was done with a 2-min starting denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 1 min, with a 7-min final extension at 68°C. Prior to performing a reference PCR, I used Mag-Bind® Complete Pure NGS to filter the PCR results of each reaction. The second PCR reaction used primers from the Nextera XT Index Kit v2 to encode products from the first PCR reaction (Illumina Inc.). The PlatinumTM SuperFiTM Green PCR Master Mix 17.5 ul, 2.5 ul of Illumina Nextera forward index primer, 2.5 ul of Illumina Nextera reverse index primer, and 2.5 ul of DNA sample were used in this process. As negative controls for both PCR cycles, samples containing DES water (Invitrogen) instead of DNA template were used.

For this second PCR, the Nextera XT Index Kit (Illumina Inc.) was coupled with the same polymerase enzyme, which was performed under the following conditions: 95°C for 3 min, then eight rounds of 30 s at 95°C, 55°C, and 72°C, followed by 5 min at 72°C. I filtered PCR products in the same way as before and used 5 ul of each sample combined together for two separate sequencing runs, each containing 211 samples. I randomly allocated samples in two separate runs since various sequencing runs might have an impact on the outcomes of sequencing. Using an Agilent 2100 Bioanalyzer, I measured the amount and quantity of the combined second PCR result (Santa Clara, CA, USA). I split the into two PCR libraries of 211

samples each, with four negative controls. The negative controls were used to identify contamination throughout the procedure. I sent the DNA to the University of Alberta's Applied Genomics Centre (TAGC) for sequencing on the Illumina MiSeq technology with 2 x 300 bp paired-end reads and v3 chemicals. The sequences are available on NCBI (BioProject ID: PRJNA954773).

4.2.6 Bioinformatic analysis

For bioinformatics I followed the methods used by Jean et al. (2021). The 'DADA2' algorithm in R software was used for bioinformatic analysis of Illumina paired-end sequences. Before loading demultiplexed sequences into "DADA2," FastQC was used to check them for non-biological DNA composition. Using Illumina adapters and primer complements, I used the "cutadapt" plugin (Martin, 2011) to preserve only biologically important sequences, and I used the "DADA2" plugin (Callahan *et al.*, 2016) to improve read quality, remove chimaeras, and resolve amplicon sequence variations (ASVs) of forward reads. Despite the fact that operational taxonomic units (OTUs) are widely used in fungus metabarcoding study, I selected ASVs due to their greater precision in identifying fungal communities and the uniformity in ecological findings obtained from the two methods (Glassman & Martiny, 2018; Pauvert *et al.*, 2019). In order to allocate taxonomy to ITS ASVs, I made use of the UNITE dynamic classifier, which analyzes individual branches and assigns taxonomy with a confidence level ranging from 97 to 98% (Abarenkov *et al.*, 2010). In order to conduct further statistical analysis, I rarefied the data by making use of species rarefaction curves. I made use of the FUNGuild database in order to categorize ASVs according to biological guilds (Nguyen *et al.*, 2016). Only species that were assigned to a single guild and received confidence ratings of "probable" or "high probable" from the endophytic fungus guilds were maintained in our collection.

4.2.7 Monoterpenes and sesquiterpenes analysis

I extracted hexane-soluble chemicals from 100±5 mg (fresh weight) of frozen powdered needles following the procedure described in Ullah *et al.* (2021). Briefly, I extracted the fresh needle tissue twice in 0.5 ml of hexane while using 0.004% pentadecane as an internal reference. Samples were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 0°C for 15 min. I combined the supernatant from each extraction and identified monoterpene compounds in a sub-sample of extracts with a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) using authentic standards. The method used for GC/MS was as follows: a 1 µl of sample extract was injected with a split injection (10:1) into the GC equipped with an 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.). with helium carrier gas flow at 1.1 ml min^{-1}, and a temperature of 40 °C for 1 min, increased to 55 °C by 30 °C min^{-1}, and held for 0.5 min, increased to 122 °C by 8 °C min^{-1}, and held for 2 min, increased to 200 °C by 10 °C min^{-1}, and then to 260 °C by 20 °C min^{-1}, and held for 1 min.

For monoterpenes analysis, I used 11 authentic standards (mainly monoterpenes, plus bornyl acetate (acetate ester of borneol)) to identify and quantify the concentrations of individual compounds: borneol (chemical purity: 99%), α-terpineol (90%) (Sigma-Aldrich, St. Louis, MO, USA), terpinolene (90%), α-pinene (98%), β-pinene (98%), limonene (99%), myrcene (90%), camphene (90%), (+/-)-camphor (95%) (Fluka, Sigma-Aldrich, Buchs, CHE), bornyl acetate (97%), (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (99%) (TRC Toronto, ON, CAN). Units for monoterpenes (ng mg^{-1}) were based on fresh weight (FW). For sesquiterpenes analysis I used β-caryophyllene (80%), caryophyllene oxide (95%), and (+)

aromadendrene (97%) (Sigma-Aldrich). Units for sesquiterpenes (ng mg^{-1}) were based on fresh weight.

4.2.8 Data analyses

I calculated the relative abundance of fungal guilds from inoculated and control spruce families separately. I checked data for the assumptions of homoscedasticity and normality by using Levene's and Shapiro–Wilk tests, respectively. Where necessary, I transformed (log+1) data prior to analysis. I tested the impact of fungal inoculations on total monoterpenes, total sesquiterpenes, and total endophytic fungal abundance for statistical significance by t-test. I used PERMANOVA (permutational multivariate ANOVA) to determine the differences between individual terpenes and endophytic fungal abundance in both inoculated and control treatments. Furthermore, I used Canonical Analysis of Principal Coordinates (CAP) ordination techniques to visually explore the variables. In addition, I conducted a regression analysis in order to determine the correlation between total terpenes, total sesquiterpenes, foliage biomass, and endophytic fungal abundance. I further conducted the Pearson correlation analyses to determine if terpene concentrations are correlated with the fungal endophyte communities. I considered significant differences at $\alpha=0.05$. Statistical software R v3.4.4 (R Core Team 2018) was used for all statistical analyses.

4.3 Results

4.3.1 Foliage fungal community composition

I acquired a total of 1.59 million DNA sequences through the use of two Illumina MiSeq runs, with an average of 41,763 reads per sample. Following the completion of DADA2's quality control and filtering procedures, a total of 0.9 million DNA sequences were made accessible for the sequential processing steps. These reads represented 990 ASVs. On the basis of these ASVs,

I determined that there was a total of 545 distinct biological species. For comparison, 1,850 randomly picked subsamples from each sample were chosen using species rarefaction curves.

Relative abundance of different guilds in the inoculated seedlings varied from those in the control seedling (Fig. 4.1a). Both seedling groups contained a total of nine distinct fungal guilds, including lichenized fungi, ectomycorrhizal fungi, epiphytes, lichen parasites, plant pathogens, fungal parasites, animal pathogens, wood saprotrophs, and endophytes. However, inoculation of seedings resulted in almost 3-fold increase in the relative abundance of endophytes in the white spruce seedlings when compared to the control seedlings. But such inoculation also reduced the relative abundance of the remaining eight non-endophytic fungal guilds. For the rest of the chapter, I just focused on the fungal endophytes in my data analyses.

The white spruce seedlings inoculated with the mixture of five endophytic fungi (*C. halotolerans, C. cladosporioides, Chalara* sp.*, Geopyxis* sp.*,* and *Didymella* sp.) showed overall ~0.90 proportion of incidence as compared to control, i.e., incidence of finding any of the inoculated fungi increased 90%. *Cladosporium cladosporioides* showed higher proportion followed by *Geopyxis* sp.*, Didymella* sp., and *Chalara* sp.*; C. halotolerans* had the lowest proportion (Fig. 4.1b). The control seedlings only had three endophytic genera (*C. cladosporioides, Geopyxis* sp.*,* and *Didymella* sp.) with an overall proportion of ~0.10 as compared to the inoculated seedlings. The other two fungi, *C. halotolerans,* and *Chalara* sp. were absent in the control seedlings. These results show that our inoculation was successful.

Further analysis with the Constrained Analysis of Principal Coordinates (CAP) and the Permutational Analysis of Variance (PerMANOVA) showed that the abundance of five fungal endophytes was substantially greater on the inoculated seedlings (Fig. 4.2). While the abundance

of *C. halotolerans, Didymella* sp.*, Geopyxis* sp., and *Chalara* sp. was clustered together, *C. cladosporioides* was separated from the others.

Furthermore, I compared the abundance of individual fungal species between inoculated and control seedlings (Fig. 4.3). All three fungi, *C. cladosporioides*, *Didymella* sp., and *Geopyxis* sp. were significantly higher in the inoculated seedlings than the non-inoculated control seedling (Fig. 4.3 a,b,c). Since *C. halotolerans* and *Chalara sp.* were absent in the control seedlings, I did not perform t-tests for these two fungal groups (Fig. 4.3 d,e).

4.3.2 Endophytic fungal abundance in 30 white spruce families

I also compared the abundance of each of the five fungi between inoculated and control seedlings by using t-test in each of the 30 spruce families (Fig. 4.4). In comparison to the control, *C. cladosporioides* was more abundant in 28 families examined (Fig. 4.4a). The range of the mean read abundance of *C. cladosporioides* in the inoculated families was from 80 to 530, while the mean read abundance in the control families was consistently below 200. Among inoculated seedlings, the most abundance was found in family 203, while the least abundance was found in families 133, 156, and 157.

The *Didymella* sp. was also significantly higher in all the inoculated families as compared to control (Fig. 4.4b). The mean read abundance in inoculated families ranged from 125–800, while control families showed below 100. The five control families did not have *Didymella* sp. Among inoculated families, Family 148 had the highest abundance of *Didymella* sp.*,* while families 128, 157, and 158 showed the lowest abundance.

All but one of the families inoculated (133) had substantially greater abundance of *Geopyxis* sp. when compared to the control (Fig. 4.4c). The range of the mean read abundance in

inoculated families was 40–370, whereas the mean read abundance in control families was below 100. Six control families did have any *Geopyxis* sp. Family 148 had the greatest abundance of *Geopyxis* sp. among the inoculated families, whereas families 128 and 133 exhibited the lowest abundance of the species.

Cladosporium halotolerans and *Chalara* sp. were not detected in any of the non-inoculated control families (Fig. 4.4 d;e). However, among inoculated families the *C. halotolerans* mean read abundance ranged from 80–580. A wide variety of read abundance of *Chalara* sp., from 18–510, was observed in inoculated families.

4.3.4 Monoterpene and sesquiterpene profiles

I identified 25 monoterpenes and six sesquiterpenes in both inoculated and non-inoculated white spruce seedlings (Tables 4.1& 4.2). In inoculated families, the total monoterpene concentrations varied from 4,116 to 11,314 ng/mg, while in the control families, the concentrations were ranged from 1,267 ng/mg to 9,123 ng/mg (Table 4.1). The sesquiterpene concentrations in the inoculated families varied from 106 to 418 ng/mg, while the concentrations in the control families ranged from 66 to 328 ng/mg (Table 4.2).

4.3.5 Monoterpene and sesquiterpene variation within and between inoculated and control spruce seedlings

Both total monoterpenes and sesquiterpenes were 42% and 41% significantly higher in the inoculated seedlings as compared to control (non-inoculated) seedlings (Fig. 4.5). The CAP analysis showed skewed distribution of monoterpenes towards the inoculated seedlings (Fig. 4.6a). Furthermore, PerMANOVA results revealed that individual monoterpenes were significantly higher in inoculated seedlings than control. All monoterpenes were clustered on

inoculated seedlings except geranyl acetate that found on the control seedlings. In addition, CAP analysis revealed that sesquiterpenes were clustered in the inoculated seedlings (Fig. 4.6b). According to PerMANOVA test, the concentrations of all individual sesquiterpenes were statistically higher in the inoculated seedlings than in the control seedlings.

The total monoterpenes and sesquiterpenes also varied between control and inoculated white spruce families (Fig. 4.7). Overall, 23 inoculated spruce families showed higher concentrations of total monoterpenes, ranging from 4,850 to 11,850 ng/mg. The remaining seven families were similar between inoculated and control treatments (Fig. 4.7a). Furthermore, 17 inoculated spruce families showed higher concentrations of total sesquiterpenes, ranging from 120 to 420 ng/mg. When compared to the inoculated group, the control group of one family 1982 displayed greater concentrations of total sesquiterpenes. The total sesquiterpene concentration in remaining 12 inoculated families were not different from control (Fig. 4.7b).

4.3.6 Relationship between total endophytic fungal abundance and total terpenes

To determine if total endophytic abundance correlates with total monoterpenes and sesquiterpenes, I used a linear regression analysis. Families of white spruce that varied in the amount of endophytic fungi present exhibited a favorable relationship with the overall amount of monoterpenes and sesquiterpenes (Figure 4.8a;b). An increase in the total endophytic abundance follows the increase in the concentrations of monoterpenes or sesquiterpenes.

4.3.7 Seedling leaf fresh biomass variance between inoculated and control families

The seedling foliage fresh biomass significantly varied between inoculated and control families (Fig. 4.9). With the exception of five families, all other families inoculated had significantly higher foliage biomass, ranging from 4 to 9 g. However, five families showed no difference

between control and inoculated treatments. I further conducted a regression analysis to determine if the total endophytic abundance correlates with the foliage biomass (Fig. 4.10). The regression analysis revealed that endophytic fungal abundance and foliage biomass are positively correlated.

4.3.8 Correlations between individual terpenes and endophytic fungi

The Pearson correlation analysis shows that fungal endophytes correlate to individual terpene concentrations (Fig. 4.11). Except for *C. halotolerans,* all four fungal endophytes were correlated with most of the individual and total monoterpenes. *Didymella* sp., *C. cladosporioides*, and *C. halotolerans* were the only three fungal endophytes that showed a positive correlation between individual and total sesquiterpenes. I also detected that some monoterpenes and some sesquiterpenes were highly correlated with each other.

4.4 Discussion

Here, I examined the influence of foliar fungal endophytes on the metabolic profiles and biomass of white spruce seedlings. Inoculation of seedlings with a bouquet of five fungal endophytes resulted in changes in their foliar fungal endophytes, which in turn increased plant biomass as well as increased the concentrations of two defense terpenes, monoterpenes and sesquiterpenes. I identified 25 monoterpenes and six sesquiterpenes from the white spruce seedling foliage. This is the first study providing direct evidence that the changes in foliar fungal endophytes can lead to the changes in secondary chemistry of any conifer species. Furthermore, I found a significant effect of white spruce family on their responses to the fungal inoculations. Overall, these results shed light on the potential application of fungal endophytes for enhancing plant growth and defenses against insect herbivores. Furthermore, the current research contributes to a deeper

understanding of the role that fungal endophytes play in spruce defenses and extends our knowledge of the ways in which host genotypes and fungal endophytes interact.

Changes in the foliar endophytic fungal communities altered the monoterpene and sesquiterpene profiles of white spruce seedlings. Here, I demonstrated that the fungal endophytes and terpene concentrations showed a positive linear relationship. Interestingly, the inoculated seedlings had higher concentrations of both monoterpenes and sesquiterpenes as compared to the non-inoculated control seedlings. Although I did not investigate potential mechanisms in this study, there are several distinct pathways *via* which fungal endophytes might exert their effects on the production of terpenes in their host plants. The fact that both endophytes and host tree share the same biosynthetic pathway and genes encoding enzymes catalyzing different stages supports the idea that the fungal endophytes have likely acquired the ability to manufacture these metabolites from their host plants because of their long evolutionary interactions (Chandra, 2012). Furthermore, the same homologous genes that are involved in the production of secondary metabolites in their host plants are present and active in the endophytic fungi (Jennewein *et al.*, 2001). For example, the fungal endophyte taxol producer *Paraconiothyrium* sp. stimulated the expression of the taxol biosynthetic gene in the *Taxus* sp. plant, which confers increased resistance to certain plant diseases. Non-taxol-producing endophytes such as *Alternaria* sp. and *Phomopsis* from the same host plant "Taxus" stimulated (eightfold) taxol-biosynthetic genes in *Paraconiothyrium* sp. (Soliman & Raizada, 2013).

According to the long co-evolutionary interactions between plants and endophytes, it is feasible for endophytic fungi to defend their host plants by the production of additional secondary metabolites (Mattoo & Nonzom, 2021). In a recent study, Ullah *et al*. (Chapter III of this thesis) showed that fungal endophytes used to inoculate seedlings in the current study

produced five different classes of secondary metabolites including monoterpenes (α-pinene, β-pinene, camphene, 3-carene, β-phellandrene, p-cymene, 2,5-Dimethyl-1,5-hexadien-3-ol), aldehydes (pentanal, butanal-3 methyl), alkanes (2,4-Dimethyl-1-heptane, 2,4,6,8-Tetramethyl-1-undecane), ether (hexyl octyl ether), and ketone (2-cyclopenten-1-one). Earlier studies also reported an increase in the concentrations of secondary metabolite rugulosin in several half-sib families of white spruce seedlings in response to inoculation with the fungal endophyte 5WS22E1 (DAOM 229536) (Sumarah *et al.*, 2005a). The current study clearly shows that fungal endophytes have the ability to alter the terpenoid compositions of white spruce seedlings. In general, these findings lend support to the hypothesis that fungal endophytes can make contributie to the metabolite profile of their host trees, including those compounds that are relevant to the host tree defenses.

I found that fungal inoculation significantly increased the biomass of white spruce seedling foliage relative to the control treatment. Similarly, diazotrophic endophyte inoculated on Douglas-fir (*Pseudotsuga menziesii*) seedlings substantially increased the seedling biomass when compared to the non-inoculated control seedlings (Khan *et al.*, 2015). Although I did not investigate the underlying mechanisms, such increase in the plant foliar biomass is likely explained by the production of plant hormones governing plant growth such as auxins by the fungal endophytes as reported in earlier studies (Contreras-Cornejo *et al.*, 2009; Gundel *et al.*, 2013; Khan *et al.*, 2015; Gibert *et al.*, 2019). In addition to improving plant growth, fungal endophytes help plants many other ways. For instance, tomato plants inoculated with the fungal endophytes *Ampelomyces* sp. and *Penicillium* had improved plant growth, stress endurance, recuperation rate as well as crop production compared to non-inoculated plants. All these results demonstrate that fungal endophytes can benefit their host plants.

I found significant variations in the fungal endophyte communities that resulted in variations in terpene concentrations and plant biomass among white spruce genotypes. Overall, 76% of inoculated families showed higher concentrations of monoterpenes and improved foliar biomass, and 56% of inoculated families were found to have higher concentrations of sesquiterpenes. I propose that the white spruce family may explain the variance that exists within fungal endophyte communities (Vincent *et al.*, 2016). In a common garden experiment where multiple genotypes of balsam poplar (*Populus balsamifera* L.) were growing, host genotype was shown to be a primary driver of the composition of the leaf-associated fungal endophytic community (Bálint *et al.*, 2013). Similarly, Rajala *et al.* (2013) found significant differences in the needle fungal community between clones of Norway spruce. The effects of plant genotypes on endophytic fungal communities appears to be common even among annual plants. For instance, Sapkota *et al.* (2015) reported that plant genetics play a significant role in the formation of the fungal community on wheat, barley, cereal, and rye. All these studies demonstrate that plant's genetic composition can influence the endophytic fungal assemblages (Whitham *et al.*, 2006; Zimmerman & Vitousek, 2012; Barker *et al.*, 2018). Currently, it is unknown what aspect of white spruce genotypes affect the fungal communities.

Increase in the concentrations of plant defense metabolite likely negatively effects on the eastern spruce budworm defoliation (Sumarah *et al.*, 2005a). Likewise, improved biomass suggests that endophytes can boost planting stock output in forest nurseries. Nevertheless, since fungal endophyte communities are complex and interactions with host plants might vary depending on the ecological situation (Schulz & Boyle, 2005), these in vitro effects need to be confirmed in a natural setting (González-Teuber, 2016). The current study is one of the few that links host-plant resistance features with fungal endophyte populations in a forest tree species.

The discovery of ecological drivers of plant-fungal endophyte interactions is a critical step in understanding fungal community patterns in forests.

Table 4.1: The individual monoterpenes (ng mg^{-1}) in 30 treated and control families/genotypes of *Picea glauca* foliage. The units are based on fresh weight.

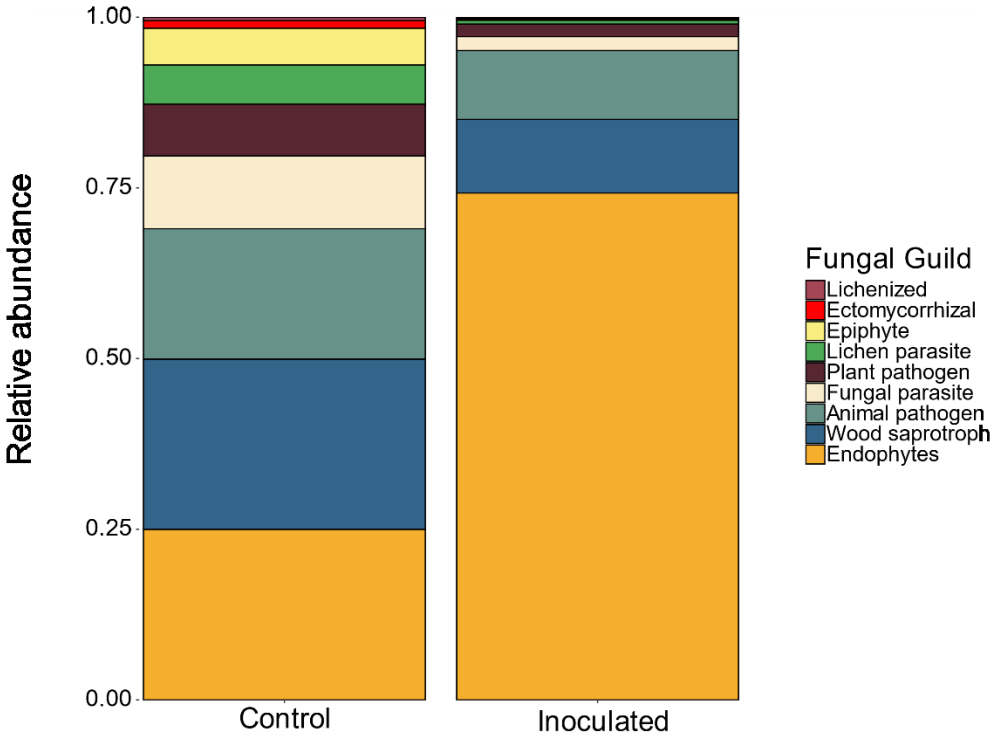
Group	Families	α-Pinene	Camphene	β-Pinene	β-Myrcene	Limonene	3-Carene	Terpinolene	Camphor	α-Terpineol	α-Terpinene	Borneol	endo-Borneol	Linalool	Sabinene hydrate	Bornyl acetate	Geranyl acetate	β-Citral	β-Phellandrene	Verbenone	Eucalyptol	Tricyclene	Sabinene	Camphene hydrate	Citronellol	Citronellol acetate	Total monoterpenes
Inoculated	128	664.7	1011	120.9	909.7	766.2	4.58	129.3	2126	20.9	0.9	84.8	3.21	12.1	16.12	2454.2	63.58	4.38	11.1	7.8	23.1	80.9	18.2	1.74	60.15	16.41	8612
Control	128	152.2	235.75	30.21	243	178.3	0.39	28.24	252.36	3.7	0	10	0	2.61	2.11	852.39	90.18	3.66	3.09	0.2	2.9	18.3	0.44	0	25.87	9.575	2145.5
Inoculated	133	604.6	932.99	128.6	655	663.9	1.83	113.7	2243.5	21.2	0.443	57.3	3.09	8.78	15.7	1335.5	147.6	7.54	11.6	5.3	12	73.5	3.59	2.64	46.99	22.31	7119.2
Control	133	608.2	937.98	115.4	843.5	588.1	2.37	112	2214.1	16.1	0.46	52.8	2.48	11	14.71	1416.9	171.7	9.33	16	4.1	11.2	73.8	3.33	1.71	49.06	24.12	7300.6
Inoculated	143	545.5	862.75	111.2	854.8	1072	1.7	110.5	2305.2	19.4	0.588	44.6	4.85	14.9	14.92	1696.1	43.23	12.3	65	5.8	15.8	64.8	3.2	2.49	73.79	11.8	7956.6
Control	143	577.9	891.25	109.9	904.7	882.1	1.6	106.4	2240.9	14.5	0.49	41.3	3.1	15.3	14.85	1791.7	84.89	5.41	14.1	5.2	10.3	67.6	2.77	1.87	109.3	19.93	7917.4
Inoculated	148	652.1	1023	125.2	1140	743.9	2.6	111.9	2424.8	16.9	0.638	46.8	3.87	10.2	15.85	2290.6	167.2	28.1	18	11	10.8	80.2	3.19	3.13	43.85	19.65	8993.6
Control	148	430	664.71	85.45	645.3	450.3	3.62	82	1287.1	10.3	0.401	28.4	2.29	10.6	10.79	2048.6	153.7	16.4	10.5	5.2	4.57	47.8	2.05	0.94	51.78	21.47	6074.3
Inoculated	156	786.8	1192.2	167.1	883.9	1131	4.54	149.6	3518.4	38.3	0.968	122	4.29	24.5	28.27	2847.8	62.78	4.75	33	15	45.4	109	6.88	7.46	73.7	16.53	11275
Control	156	611.3	1000.9	137.7	745.2	917.1	1.95	143.4	3294.7	33.8	0.844	78.6	1.28	16.6	24.8	1751	90.04	7.39	47.2	11	24.8	75.1	4.77	3.49	85.32	15.94	9123.9
Inoculated	157	560.4	879.61	116.5	671.1	870.5	1.56	107.4	1971.7	21.24	0.395	54.2	0.37	11.4	16.61	2163.3	49.94	5.08	33.9	4.5	15.6	65.4	3.37	1.95	87.84	19.72	7733.6
Control	157	349.6	568.12	68.74	596.4	554.5	0.29	68.87	1552.3	15.26	0.08	26.3	0.26	8.47	12.06	1245.7	38.91	1.65	7.45	3.4	19.9	40.7	2.43	0.56	78.03	20.71	5280.6
Inoculated	158	280.9	454.7	53.41	303.9	400.1	0.51	50.59	1124.4	9.13	0.099	16.4	2.16	5.43	8.575	1178.4	119.4	2.56	7.23	3.3	7.21	33.5	1.6	0	28.06	24.78	4116.4
Control	158	326.1	507.13	63.34	347.1	536.5	0.55	58.98	957.16	12.53	0	15.6	1.26	5.47	9.139	1552.5	12.34	3.77	69	1.6	18.3	37.9	2.75	0.38	17.13	6.407	4563
Inoculated	170	785.9	1226.2	174.3	898.4	975.2	3.23	163.8	2961.5	25.45	0.929	52.3	5.6	12.7	27.21	3134.5	239.5	22.9	38.3	3.8	20.9	98.1	4.88	5.94	64.98	36.14	10983
Control	170	460.4	755.69	98.47	601.8	764.4	1.56	105.3	1513.1	17.93	0.428	29.3	3.86	8.23	15.57	2529.7	188.8	10.6	45.7	3.6	16.9	58.9	3.43	2.7	51.33	49.18	7336.8
Inoculated	176	666.8	1023.4	137.6	516.3	986	7.36	109.6	2494.2	24.47	0.738	49	3.35	24.4	21.3	1976.3	190.7	11.5	21.5	7.4	20.5	79.8	4.21	2.32	141.3	44.96	8565
Control	176	375.6	625.94	99.82	376.3	539.2	3.9	68.19	1239.7	9.006	0.425	17	0.8	14.8	8.696	1221.3	101.5	6.05	21.2	4.9	0	46.2	1.57	0.86	111.4	18.55	4912.9
Inoculated	178	802.7	1275.3	162.5	1032	1394	2.98	166.6	3383.4	22.88	1.076	70.7	0.83	15.5	23.54	2603.6	113.3	5.71	16.7	16	7.79	97	3.71	3.01	63.83	30.33	11315
Control	178	56.15	415.24	179.9	904.7	449.3	2.77	173.1	1698	23.78	0.89	76.2	0.64	19	27.3	3012.2	176.2	10	67.3	11	5.86	108	3.74	3	11.32	25.33	7460.3
Inoculated	180	758.9	1180	171.5	848.1	1089	2.66	155.9	3087.2	44.81	0.873	56.8	2.59	22.1	28.97	2465.5	72.8	14	16.9	9.5	51.6	90.7	6.46	3.2	119.1	22.49	10322
Control	180	292.6	455.98	57.76	338.1	425.5	4.42	47.78	763.4	8.337	0.067	17.1	1.66	5.49	7.048	1382.6	60.65	9.87	8.1	0.8	8.38	33.9	1.77	0.23	39.2	13.68	3984.5
Inoculated	183	610.4	951.14	129.2	560.3	965	1.64	107.6	2300.6	20.81	0.663	53.4	3.55	13	17.04	2056.3	29.13	2.12	18.2	6.3	18.2	75.5	3.64	3.13	11.77	11.49	7970.1
Control	183	360.5	606.25	75.24	232.8	494.8	0.49	72.38	1320.9	9.981	0.222	41.7	2.44	7.57	10.25	1575.9	76.26	3.82	10.5	3.3	1.55	46.7	1.6	0.78	8.342	19.38	4983.7
Inoculated	188	379.2	609.67	69.44	595	607.9	0.78	62.61	1198.5	8.859	0.455	58.7	0.65	6.42	9.797	1762.3	101.1	39.6	16.5	3.3	7.2	53.2	1.91	9.36	48.63	15.21	5666.4
Control	188	402.8	638.32	72.73	1022	755.4	1.37	46.54	1517.4	9.358	0.397	57.9	3.4	9.32	10.42	1420.7	155.8	13.4	14.8	8.4	5.95	53.4	2.12	4.95	87.78	19.97	6334.8
Inoculated	190	719.3	1091	173.6	1056	1013	9.49	130.9	2709.6	28.11	1.13	56.7	2.8	17.1	22.92	2674.6	48.89	7.66	42.7	8.6	34.7	85.8	5.61	6.16	57.77	14.35	10019
Control	190	473.8	770.77	95.58	548.5	534.6	3.4	95.08	1443.3	15.57	0.64	27.1	2.57	9.5	13.03	2314.5	76.09	8.63	19.2	5.6	9.7	60.6	2.93	1.88	57.9	19.67	6610.1
Inoculated	195	626.7	1026.9	126.9	847.1	949	2.07	128.6	2759.7	19.56	0.843	46	3.06	10.2	19.79	1876.9	110.7	3.04	12.1	6.3	25.7	79.3	4.38	2.29	63.31	31.33	8781.8
Control	195	448	722.98	93.15	664.6	687	1.11	90.31	1733.6	12.09	0.474	36.5	0.59	8.87	13.1	1615.6	96.46	2.4	8.56	5.1	8.29	54.5	2.69	1.65	73.48	40.93	6422.1
Inoculated	201	500.8	788.86	94.86	670	650.6	3.11	95.7	1993.7	11.41	0.634	56.7	0.26	9.86	14.43	1772.7	94	9.88	9.17	4	0	59.7	1.94	3.92	70.67	23.79	6940.7
Control	201	556.2	856.37	121.6	596.9	684.1	14.3	110.9	1750.2	14.11	1.033	34.8	2.75	9.03	14.19	2352.3	51.47	13	12.6	4	6.77	64.5	3.01	2.92	63.13	19.45	7359.6
Inoculated	203	408.9	643.39	82.48	441.6	697.5	1.03	82.03	1531	10.55	0.398	40	3.37	6.7	11.92	1608.2	35.88	1.02	8.12	5.1	5.64	47.1	2.21	1.1	51.77	15.28	5742.4
Control	203	186.2	289.86	35.67	371.3	354.2	0.34	30.55	707.64	5.004	0.144	13.8	0.94	3.5	4.505	688.82	22.82	0.44	8.06	1.7	3.43	21.5	0.93	0.39	9.173	10.13	2771.1
Inoculated	1586	471.6	674.7	117.2	817.6	568.3	3.55	69.1	1100.1	14.86	0.874	35.8	1.63	9.7	10.33	1829.1	142.4	24.6	16.5	3.2	13.6	51.1	3.29	1.96	80.16	30.66	6092.1
Control	1586	217	333	39.34	456	182.7	3.02	32.28	284.71	6.997	0.262	14.6	0.11	3.03	2.909	995.04	113.4	10.5	4.47	0.7	5.27	27	1.01	1.88	31.44	23.8	2790.5

Inoculated	1917	339.6	570.01	84.3	556.5	441.7	3.06	91.2	1278.6	12.95	0.578	43.7	2.16	7.89	11.83	2098.6	104	5.59	19.3	6.5	7.29	47.7	2.06	4.15	33.92	22.4	5795.4
Control	1917	222.5	388.09	48.75	333.1	280.9	0.84	49.36	700.38	8.117	0.262	21.1	1.48	4.86	6.678	1381.8	76.4	4.13	5.93	3.6	8.54	28.6	1.52	0.53	20.45	27.2	3625.2
Inoculated	1918	565.2	856.38	117.1	1128	592.2	1.38	85.7	1815.4	13.35	1.002	46.6	2.13	8.35	12.37	1828.4	363.3	13.5	17.6	4.4	7.26	75.4	2.65	8.52	93.89	67.01	7727.3
Control	1918	235.5	343.38	55.56	389.7	324.2	0.36	33.29	395.53	5.171	0.536	14.6	0.52	5.18	4.564	1117	128.3	3.53	24.1	2.2	3.14	30.7	1.1	4.49	41.61	35.34	3199.5
Inoculated	1924	313.6	486.91	65.03	566.5	493.7	0.67	57.98	1072.6	8.021	0.327	21.1	1.36	8.75	9.19	1298.3	42.56	3.78	11.8	1.2	0	38.1	1.27	1.13	50.06	22.32	4576.1
Control	1924	74.47	108.92	14.23	198	116.8	0.19	12.68	64.796	0.804	0.075	1.7	0.14	1.4	0.561	501.62	87.18	0.23	17.2	0	0.84	8.32	0.37	0	24.39	32.77	1267.7
Inoculated	1951	333	505.2	77.67	493.7	445.2	6.37	54.35	1097.8	19.16	0.394	23.6	1.23	7.59	10.28	1296.2	41.79	2.73	13.2	2.9	36.4	38.5	3.01	1.83	41.54	13.67	4567.4
Control	1951	304.9	462.97	66.29	493.7	391.1	4.45	47.56	783.23	19.19	0.37	42	0.72	8.55	10.06	1447.5	63.3	3.64	13.7	1.4	34.8	42.2	3.33	5.44	12.85	13.24	4276.5
Inoculated	1952	445.9	703.43	87.41	834.1	806.6	1.62	74.26	1768	21.27	0.742	62	5.38	11.6	14.88	1937.8	48.28	5.82	62.7	5.6	40.8	52.2	3.54	1.13	79.46	24.65	7099.4
Control	1952	266.1	429.73	48.5	423.6	448.8	0.13	41.69	839.23	12.73	0.163	14.9	1.93	7.74	7.053	1206.4	59.41	2.45	5.55	2.3	22.8	32.8	1.96	0.39	90.46	35.54	4002.3
Inoculated	1976	414.6	643.26	96.21	555.1	560.9	1.6	77.38	1195.2	9.769	0.614	36.1	1.62	10.2	10.15	1951	69.45	13.4	8.29	4.4	6.49	49.1	2.3	1.86	74.37	21.27	5814.7
Control	1976	475.7	776.35	99.38	678.9	702.8	1.27	91.8	1346.3	10.56	0.566	44.8	2.66	10.9	11.15	2085.1	42.64	18.2	11	4	1.55	66.3	2.31	3.37	89.04	17.75	6594.4
Inoculated	1978	369	587.57	105.6	468.7	779.3	1.66	56.9	1507.9	10.68	0.255	34.7	3.11	10.4	10.8	1628.5	34.3	3.78	12.4	3.6	16.7	45.1	2.28	0.48	64.81	16.69	5775.3
Control	1978	154.1	440.21	94.57	205.1	153.1	1.34	96.41	601.59	13.07	0.839	60.2	2.72	10.3	13.73	2095.7	44.05	11.9	13.8	5.4	23.9	61.2	3.11	1.63	70.58	13.45	4191.9
Inoculated	1980	381.1	583.3	84.94	481.2	589.1	1.37	70.05	1331.1	11.94	0.502	48.4	1.93	6.79	13.04	2140.3	28.56	1.41	9.02	4.7	14.1	47	2.21	1.79	48.72	13.8	5916.4
Control	1980	270.1	421.62	49.91	388.5	335	0.63	42.52	510.57	4.707	0.154	19.4	1.62	4.95	6.47	1827.7	99.06	1.09	5.35	2.1	2.29	34.8	0.99	0.25	35.78	21.71	4087.5
Inoculated	1986	661.7	976.07	144.5	850.3	1199	1.67	109.6	2609.1	23.54	1.12	69.9	2.89	13.4	19.8	1988.1	96.88	28.4	68.5	3.5	41.9	76	4.19	3.16	81.8	26.82	9102.3
Control	1986	379.3	573.87	72.22	569.5	418.3	0.51	59.08	976.01	10.57	0.66	26.2	1.69	5.34	8.005	1602.9	206.1	21.1	8.73	1.6	19.3	41.9	2.47	0.43	12.41	12.07	5030.4
Inoculated	2105	677.4	1021.6	139.4	1352	1280	6.6	123.5	2679.2	19.58	0.855	72.9	6.3	17.5	19.43	1757.4	143.7	19	28.3	7.2	20.5	87.1	5.01	8.4	125	21.36	9639.1
Control	2105	321.1	531	76.28	708.9	631	1.37	58.78	1201.2	9.869	0.32	41.9	5.32	9.56	11.02	1091.5	267.8	14	14.4	4.7	10.5	42.8	2.62	3.96	96.99	17.48	5174.3
Inoculated	2106	570.1	904.84	160.2	778.2	825.6	1.76	136.9	2310.9	28.22	0.771	83.7	6.91	14.1	19.1	2179.3	79.63	20.4	16.1	18	20	77.7	4.45	6.71	71.58	23.26	8358.5
Control	2106	355.4	579.72	99.09	357.4	500.9	1.17	89.5	1203.2	14.81	0.359	34.5	4.06	7.61	13.22	1711	141.5	6.65	9.22	5.5	12.7	46.1	3.19	3.42	73.27	18.72	5292.2
Inoculated	2113	535.7	781.86	113.9	851.7	748.2	1.61	104.3	1770.1	18.07	0.669	52.8	3.86	11.9	16.27	2207.5	92.23	7.68	11.7	10	31.9	65.5	5.28	2.86	46.9	10.66	7503.4
Control	2113	332.6	503.07	72.86	855.8	468.8	1.35	63.75	1006.3	7.283	0.371	27.7	2.29	7.77	9.284	1603	79.98	7.05	8.29	6.4	2.09	39.8	2.03	1.62	52.43	24.65	5186.6

Table 4.2: The individual sesquiterpenes (ng mg^{-1}) in 30 inoculated and control families/genotypes of *Picea glauca* foliage. The units are based on fresh weight.

Group	Families	Germacrene D-4-ol	Germacrene D	β-Elemene	γ-Cadinene	Oplopanone	α-Cadinene	Total sesquiterpene
Inoculated	128	187.9	22.3	6.3	4.11	32.9	17.1	270.8
Control	128	49.9	15.4	0.2	0	4.78	2.2	72.6
Inoculated	133	180.7	49.5	5.1	1.8	19.7	10.6	267.5
Control	133	194.3	31.5	5.5	1.85	17.8	9.6	260.6
Inoculated	143	189.8	19.3	6.4	1.75	20.8	17.8	256.1
Control	143	151.5	11.8	5.3	1.71	16.1	7.77	194.2
Inoculated	148	162.4	13.3	3.6	2.1	18	8.9	208.4
Control	148	114.5	16.9	3.3	1.2	15	6.71	157.7
Inoculated	156	274.4	19.2	8.1	4.0	29.5	16.9	352.3
Control	156	257.6	25.4	6.9	3.8	19.3	15.6	328.7
Inoculated	157	145.3	12.3	4.7	2.8	23.3	15.6	204.2
Control	157	63.5	7.5	1.0	0.2	13.3	3.61	89.2
Inoculated	158	159.9	5.5	4.3	2.6	10.1	10.2	192.6
Control	158	161.9	11.5	4.3	2.3	12.1	9.5	201.7
Inoculated	170	161.0	17.5	4.5	2.5	25.2	12	222.8
Control	170	154.1	9.7	3.9	1.8	15.5	8.6	193.8
Inoculated	176	236.3	23.4	7.3	5.0	35	19.9	327.0
Control	176	163.4	25.9	4.5	3.4	14.4	14.4	226.1
Inoculated	178	316.5	30.8	9.7	6.0	31	24.6	418.6
Control	178	146.1	35.5	8.9	4.7	30.7	20.4	246.3
Inoculated	180	189.2	10.0	5.3	3.2	21.1	12.8	241.8
Control	180	69.6	7.2	1.7	0.9	8.3	5.5	93.5
Inoculated	183	177.4	21.3	5.8	4.5	26.6	17.5	252.9
Control	183	122.6	12.0	3.5	2.6	16	10.7	167.5
Inoculated	188	100.8	10.6	3.0	2.6	17.9	9.4	144.5
Control	188	97.39	19.3	3.2	2.91	22.9	12.8	158.5
Inoculated	190	150.3	21.5	4.8	4.5	25.3	15.8	222.2
Control	190	131.2	17.6	3.9	3.53	12.4	11.4	180.1
Inoculated	195	145.8	23.0	4.7	4.51	24	17	219.1
Control	195	132.4	17.4	3.7	3.1	18.1	13	187.8
Inoculated	201	185.1	27.3	5.2	4.6	19.9	17.1	259.2
Control	201	141.9	30.9	4.9	6.7	21.4	22	228

Inoculated	203	134.7	14.9	4.2	4.1	18.5	16.6	193.1
Control	203	81.5	6.9	2.7	4.4	9.7	14.6	120
Inoculated	1586	204.1	24.4	7.3	10.2	14.4	30.5	290.9
Control	1586	95.0	21.7	3.902	6.08	2.75	19.7	149.1
Inoculated	1917	108.3	24.7	3.5	4.4	11.6	14.2	166.71
Control	1917	98.8	16.0	2.6	3.9	8.3	13.7	143.5
Inoculated	1918	176.8	22.5	6.5	7.0	15.6	21.9	250.5
Control	1918	93.7	12.7	2.5	3.7	7.7	12.3	132.8
Inoculated	1924	72.3	16.8	1.7	1.8	6.9	7.1	106.6
Control	1924	50.2	7.7	0.6	1.5	0.6	5.6	66.4
Inoculated	1951	76.4	20.1	2.4	2.6	11.1	9.5	122.2
Control	1951	67.5	7.3	1.5	1.6	8.1	7.0	93.2
Inoculated	1952	168.8	24.2	6.1	7.9	24.1	31.4	262.8
Control	1952	105.1	28.5	3.5	4.6	8.3	15.5	165.6
Inoculated	1976	178.3	20.4	5.5	6.2	15.2	20.6	246.3
Control	1976	141.0	13.1	4.3	3.9	17.2	16	195.6
Inoculated	1978	115.3	20.3	3.7	3.5	18.3	14.7	175.9
Control	1978	131.8	16.1	4.4	5.3	14.2	18.7	190.5
Inoculated	1980	73.2	7.6	1.7	2.5	12.2	10.9	108.3
Control	1980	112.0	30.2	3.4	3.3	10.1	12.7	171.7
Inoculated	1986	156.4	26.7	5.4	5.1	21.2	19.8	234.8
Control	1986	98.0	8.5	3.1	3.39	7.96	12.2	133.3
Inoculated	2105	203.8	29.1	6.7	2.05	25.1	14.6	281.4
Control	2105	147.0	21.6	4.6	0.8	13.7	7.2	195.0
Inoculated	2106	183.2	16.8	5.8	1.1	21.8	6.9	235.8
Control	2106	111.7	13.6	3.3	0.1	12.1	3.0	144.0
Inoculated	2113	176.9	27.2	5.8	1.9	14.2	9.0	235.2
Control	2113	152.1	32	4.8	1.0	7.3	5.2	202.6


1.00
0.75
0.50
0.25
0.00
Relative abundance
Control
Inoculated
Fungal Guild
Lichenized
Ectomycorrhizal
Epiphyte
Lichen parasite
Plant pathogen
Fungal parasite
Animal pathogen
Wood saprotroph
Endophytes

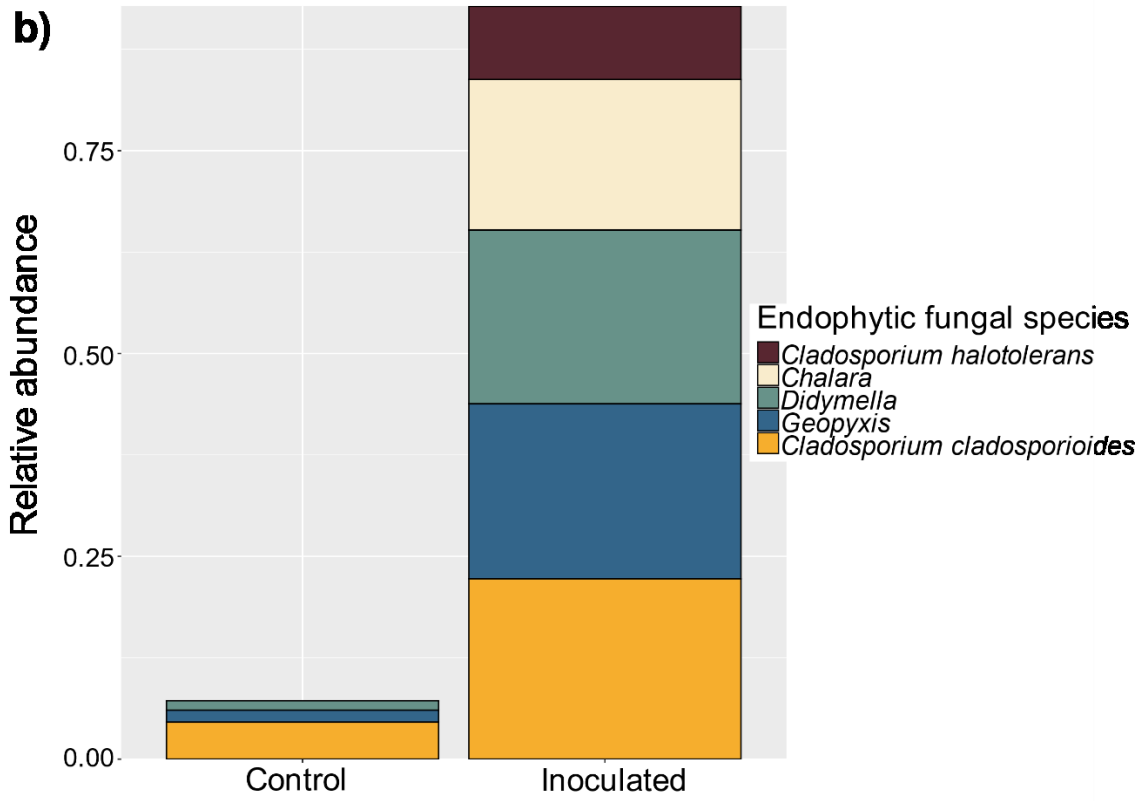

b)
0.75
0.50
0.25
0.00
Relative abundance
Control
Inoculated
Endophytic fungal species
Cladosporium halotolerans
Chalara
Didymella
Geopyxis
Cladosporium cladosporioides

Figure 4.1. Relative abundance of (a) fungal guilds and (b) foliar endophytic fungal species identified in *Picea glauca* following control and inoculated families based on FUNGuild database (Nguyen *et al.,* 2016).

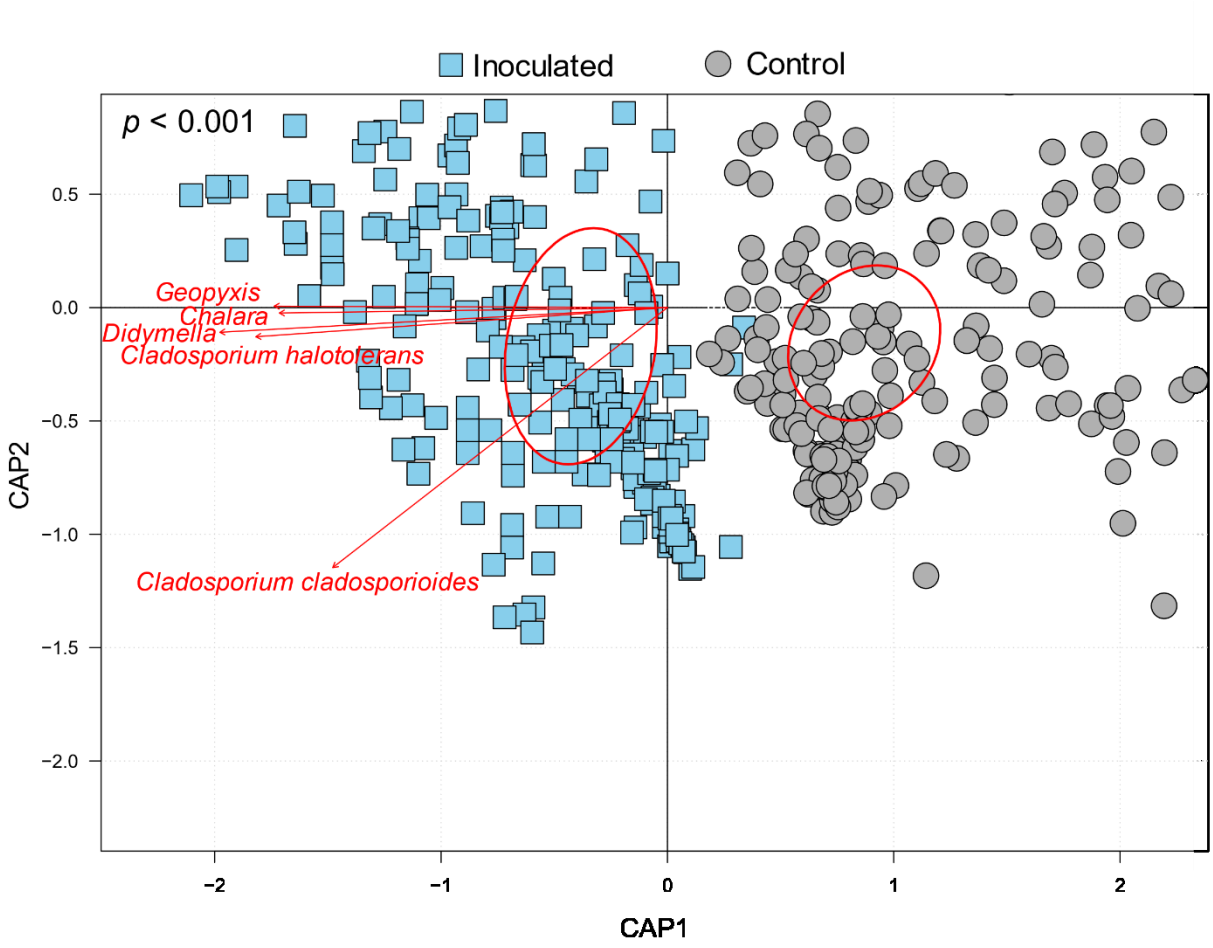


Figure 4.2. Canonical Analysis of Principal Coordinates (CAP) plot the distribution of individual endophytic fungal abundance in *Picea glauca* inoculated and control families. Red vectors represent individual fungal endophytes. PermANOVA was conducted between control and inoculated endophytic fungal abundance. (p <0.001).

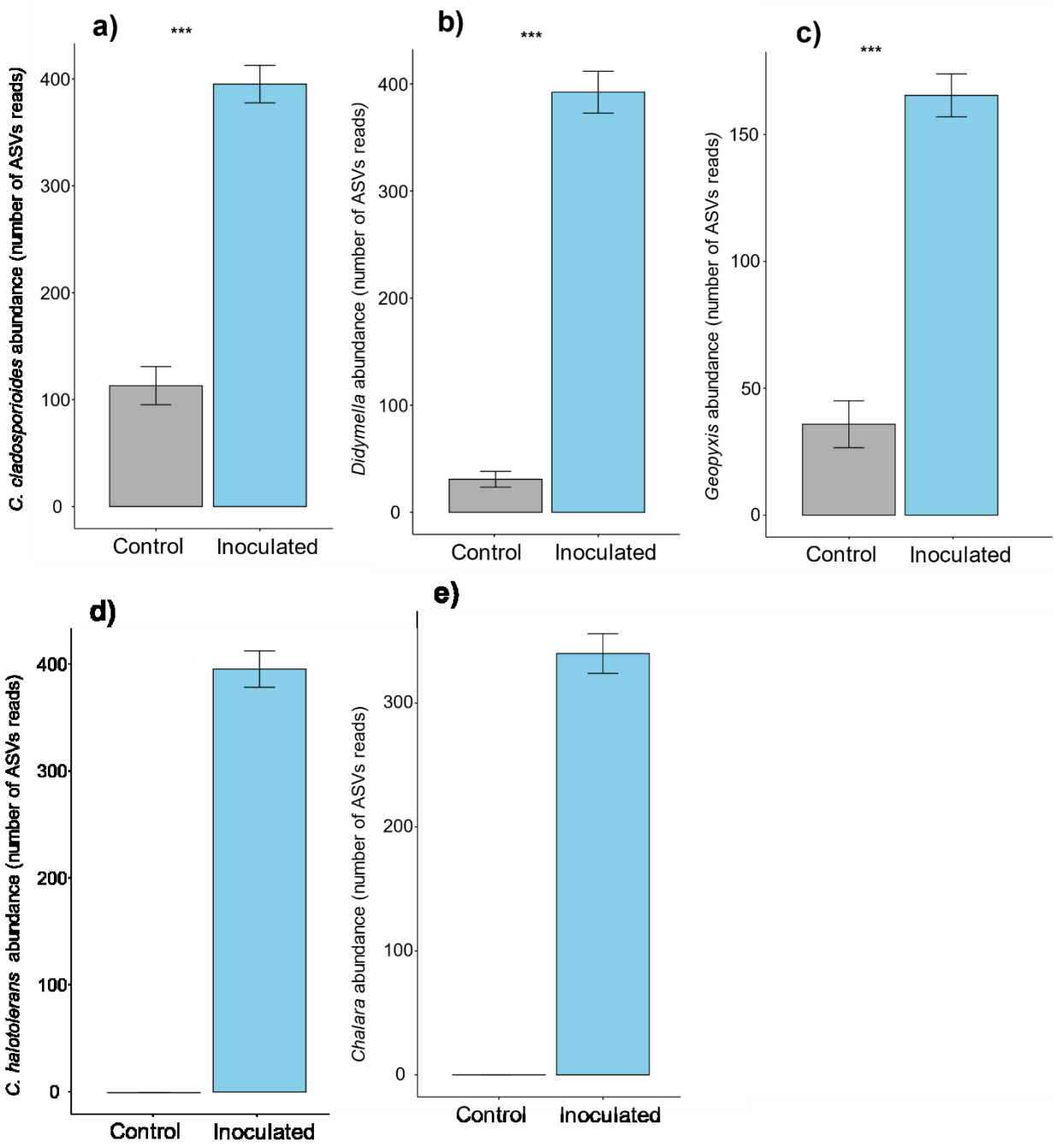


Figure 4.3. Mean (±SE) endophytic fungal read abundance of (a) *Cladosporium cladosporioides* (b) *Didymella* (c) *Geopyxis* (d) *Cladosporium halotolerans*, and (e) *Chalara.* The two-sample t-test was done for comparing control and inoculated groups. The asterisks show significant differences at $p < 0.01$.

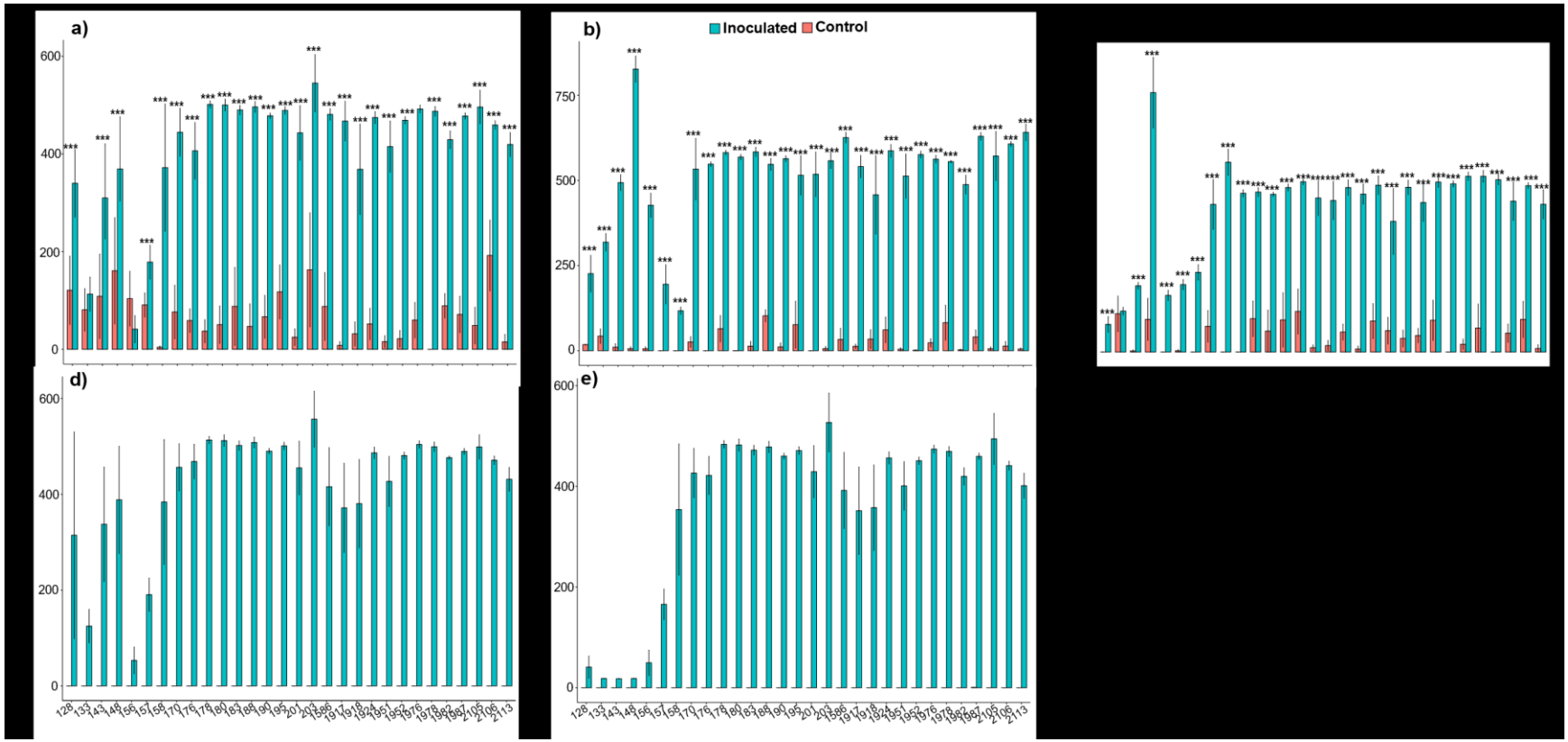


Figure 4.4. Mean (±SE) endophytic fungal read abundance of (a) *Cladosporium cladosporioides* (b) *Didymella* (c) *Geopyxis* (d) *Cladosporium halotolerans*, and (e) *Chalara* among 30 *Picea glauca* families. The two-sample t-test was done for comparing each control and inoculated families separately. The asterisks show significant differences at $p < 0.01$.

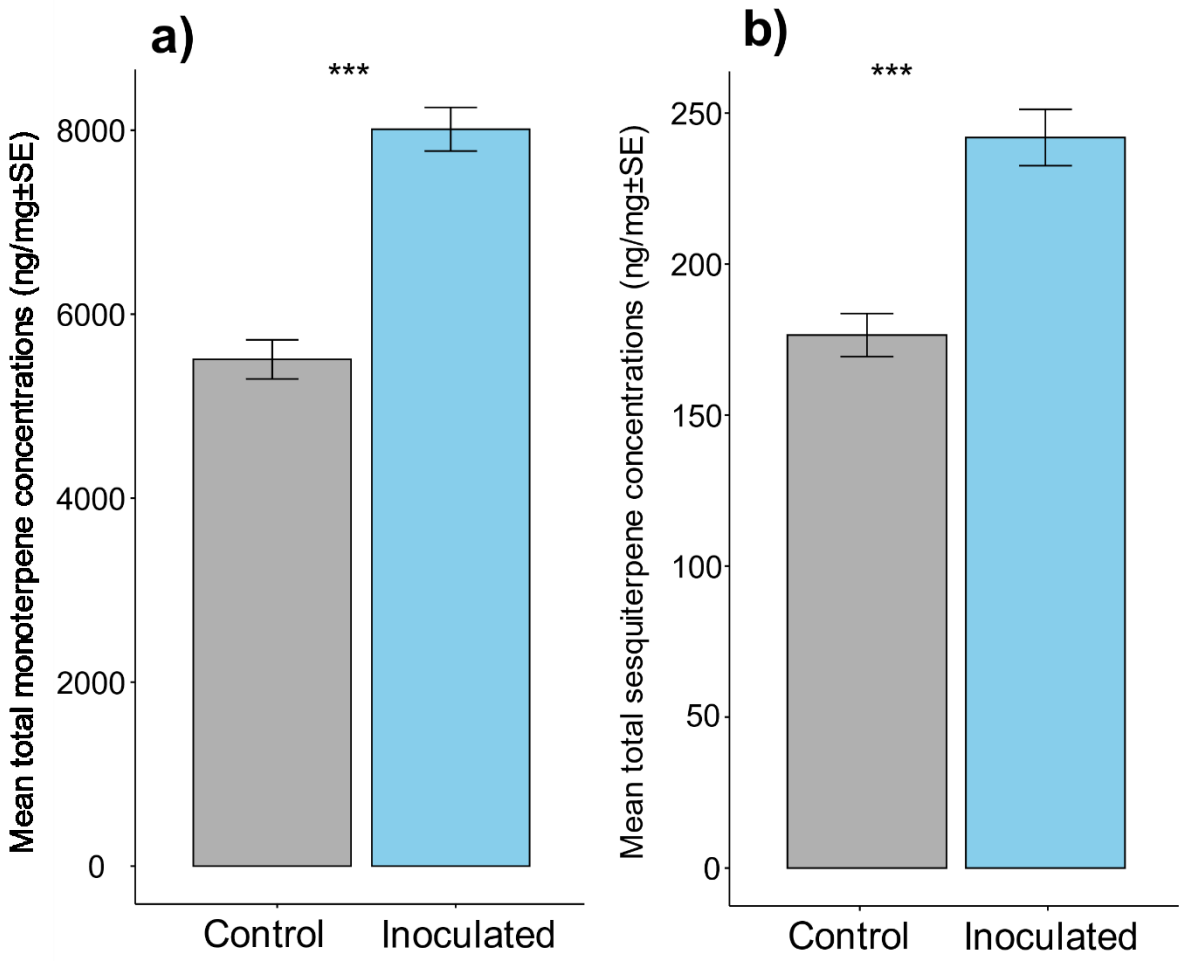


Figure 4.5. Mean (±SE) concentrations of **(a)** total monoterpenes and **(b)** total sesquiterpenes *Picea glauca* seedlings. The two-sample t-test was conducted to compare control and inoculated groups. The asterisks show significant differences at $p < 0.01$.

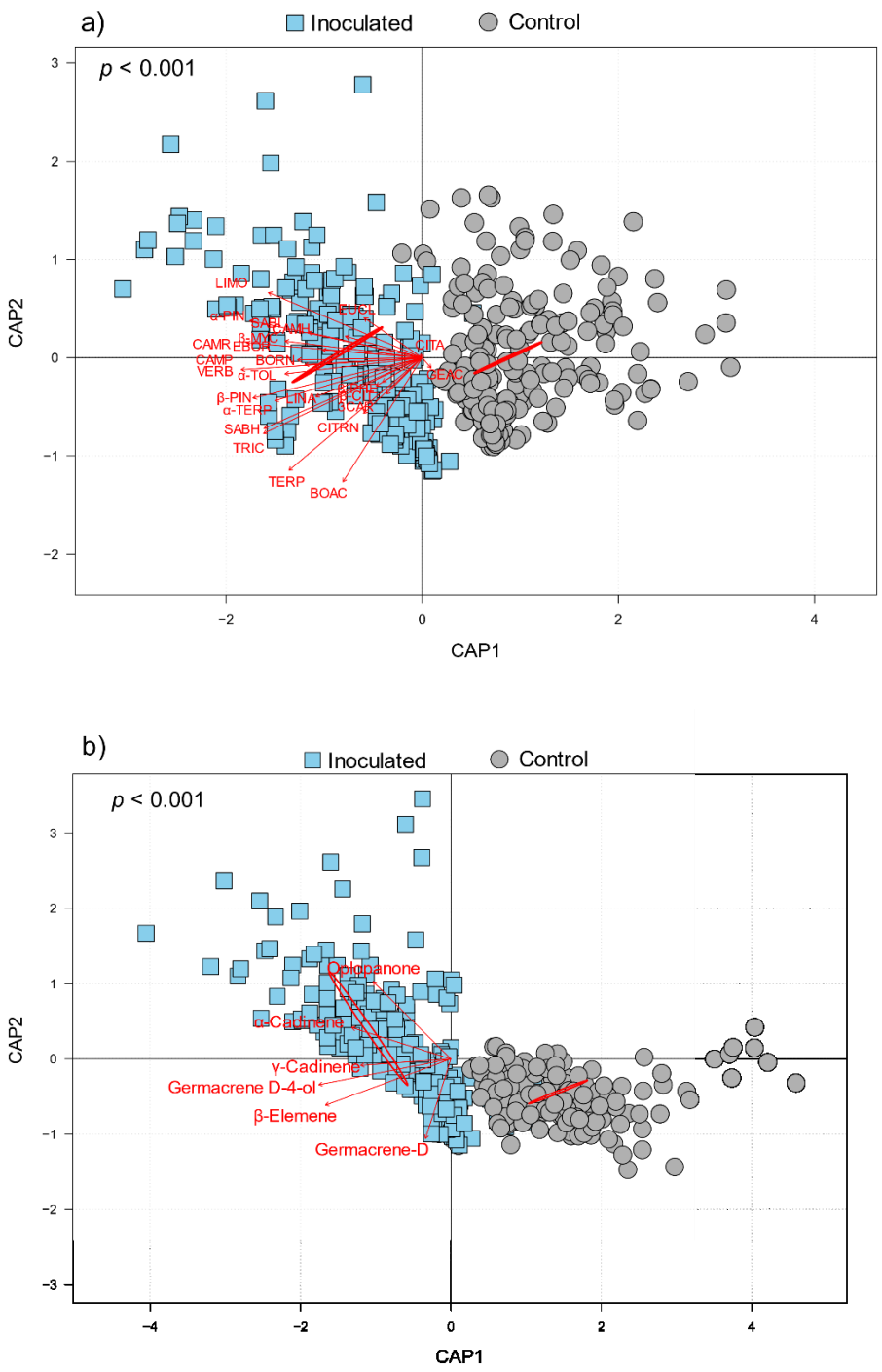


Figure 4.6. Canonical Analysis of Principal Coordinates (CAP) plot the distribution of (a) individual monoterpenes and (b) individual sesquiterpenes in *Picea glauca* inoculated and control seedlings of *Picea glauca*. PermANOVA was conducted between control and inoculated endophytic fungal abundance. [(a) monoterpenes: p <0.001; (b) sesquiterpenes: p <0.001].

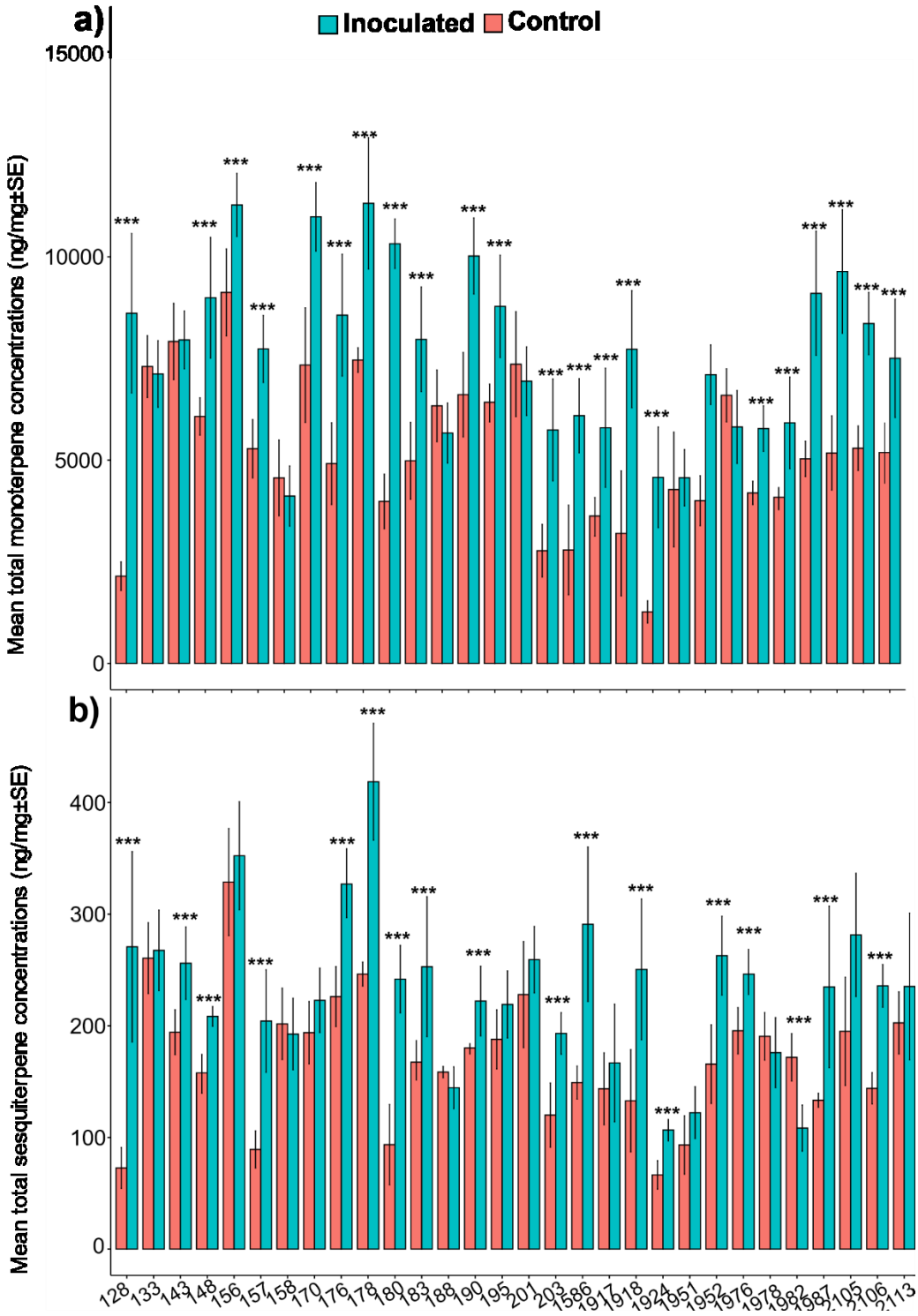


Figure 4.7. Mean (±SE) concentrations of **(a)** total monoterpenes and **(b)** total sesquiterpenes among 30 *Picea glauca* families. The two-sample t-test was conducted to compare each of control and inoculated families. The asterisks show significant differences at $p < 0.01$.

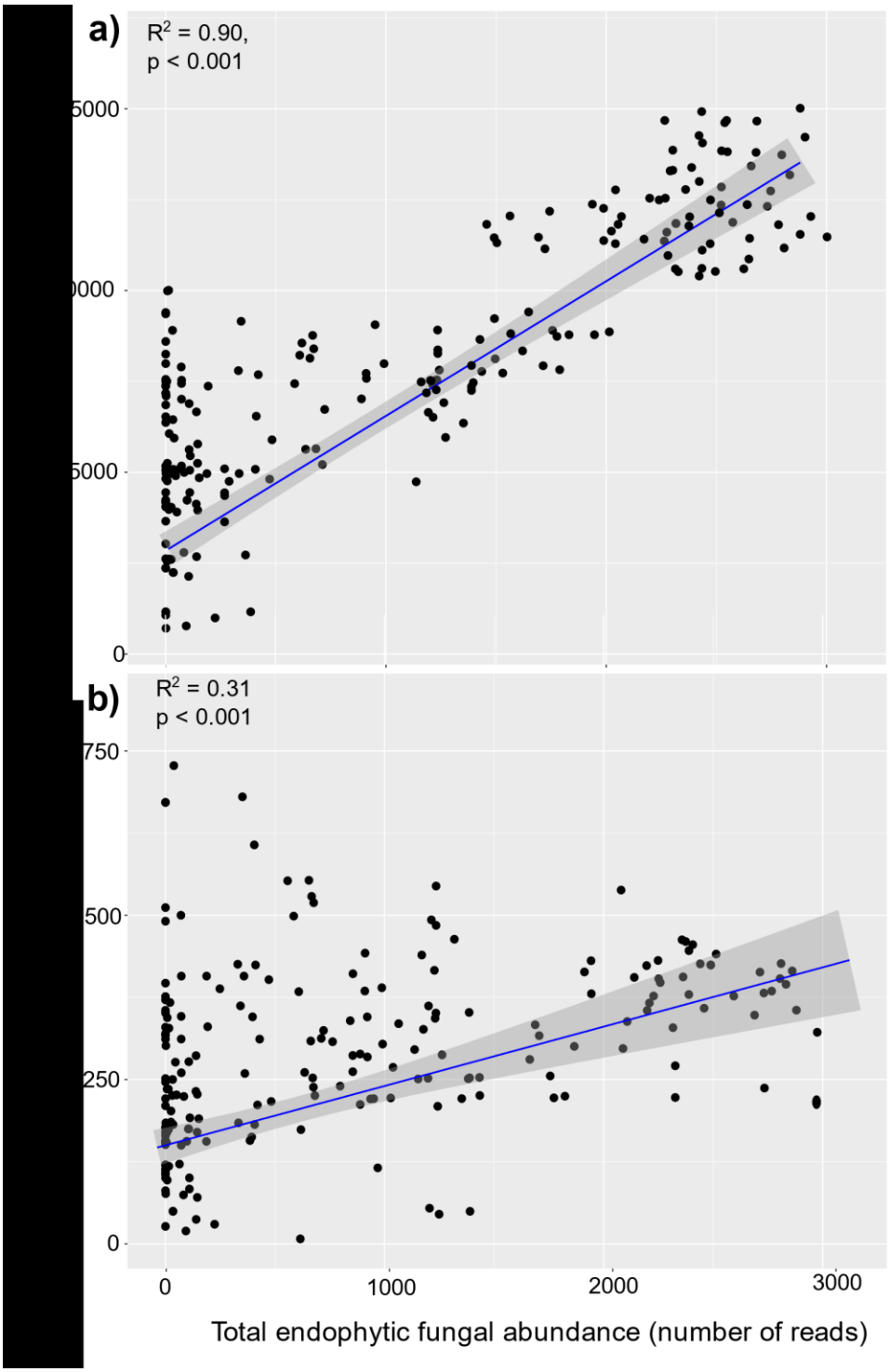


Figure 4.8. The relationship between **(a)** total fungal endophytes and total monoterpene concentrations (ng mg^{-1}), **(b)** total endophytic fungal abundance and total sesquiterpene concentrations (ng mg^{-1}) of *Picea glauca* foliage. Line and shade denote regression line and 95% confidence interval, respectively.

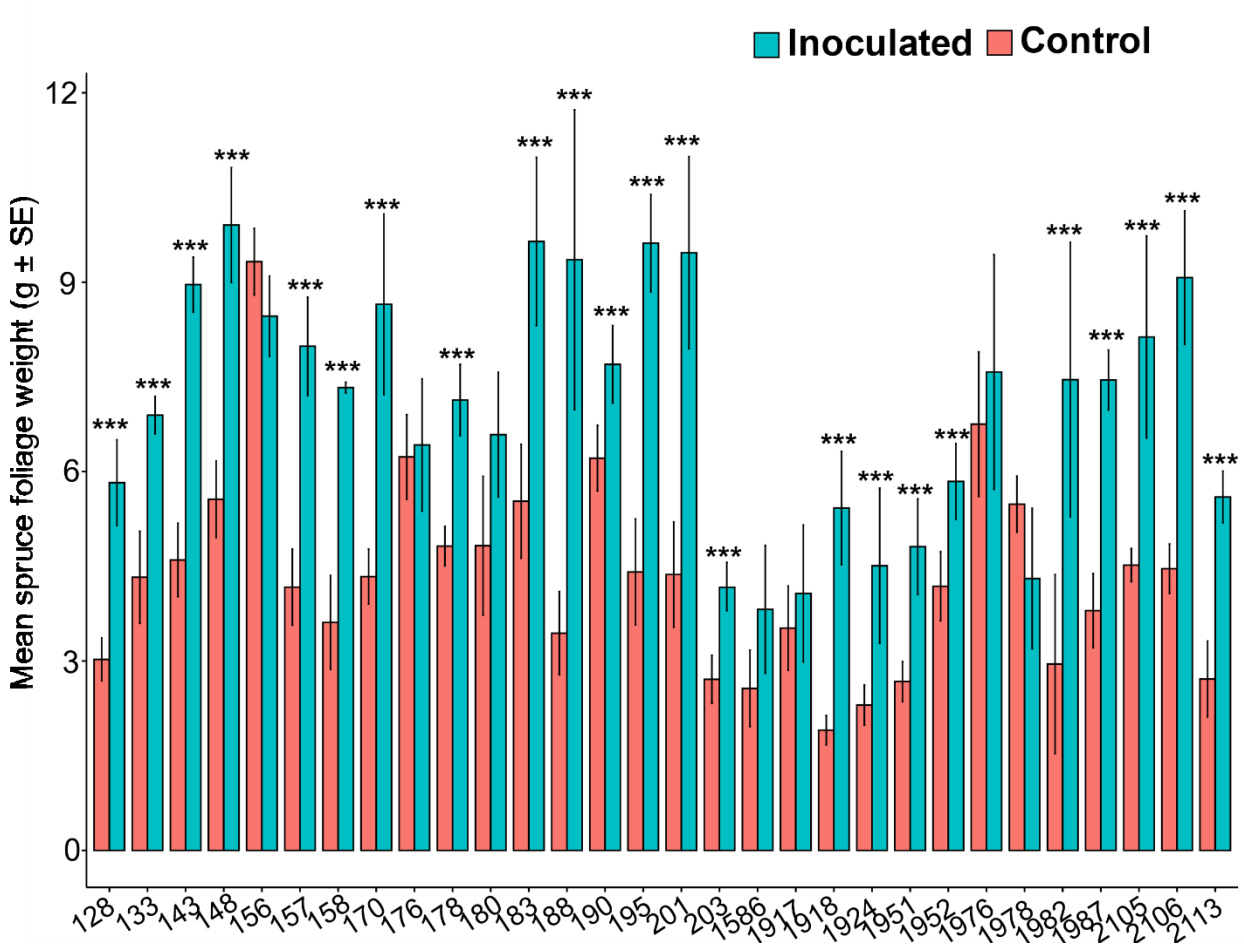


Figure 4.9. Mean (±SE) foliage biomass (g) among 30 *Picea glauca* inoculated and control (non-inoculated) families. The two-sample t-test was conducted to compare each of control and inoculated families. The asterisks show significant differences at $p < 0.01$.

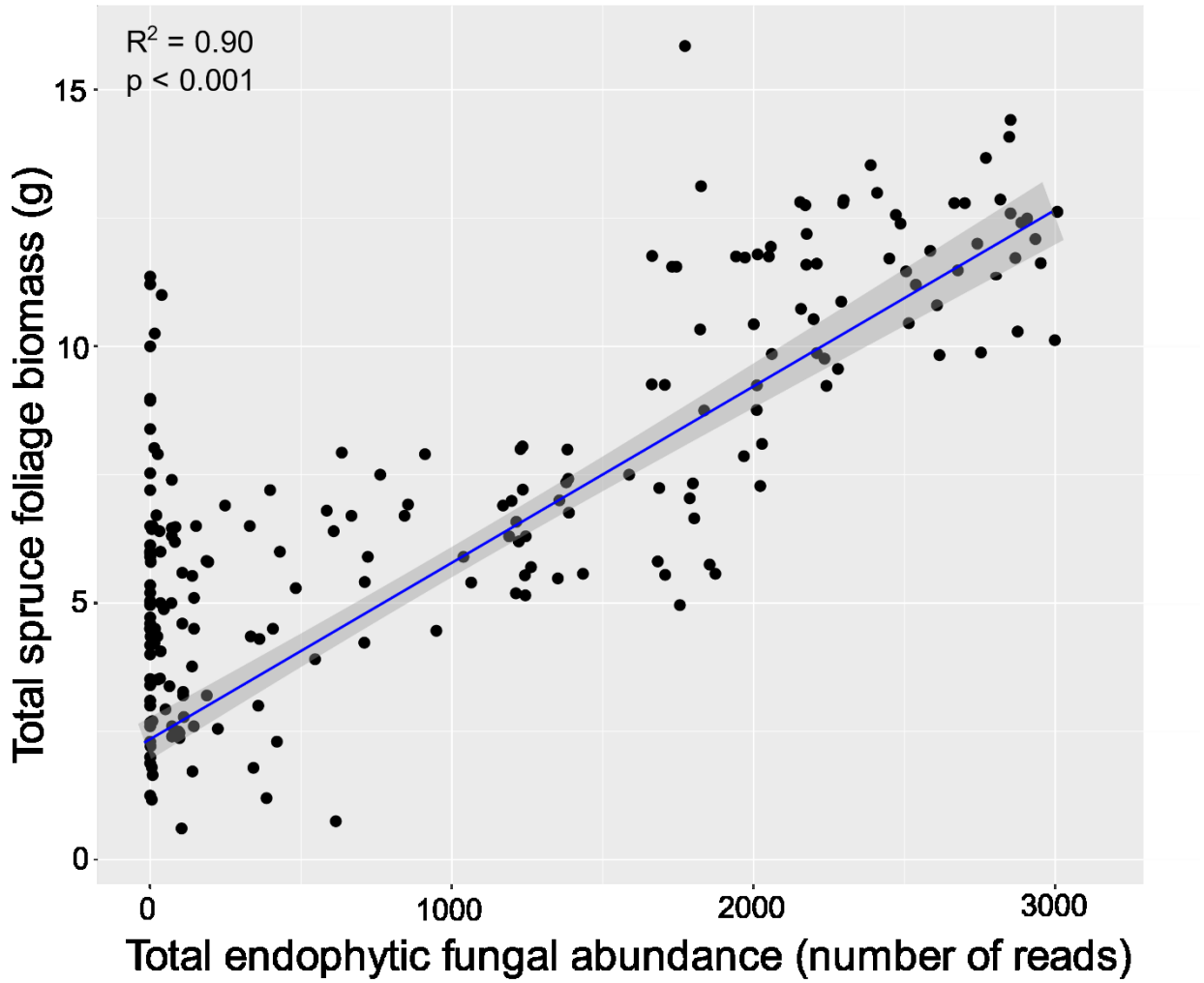


Figure 4.10. The relationship between total endophytic fungal abundance and total *Picea glauca* foliage biomass (g). Line and shade denote regression line and 95% confidence interval, respectively.

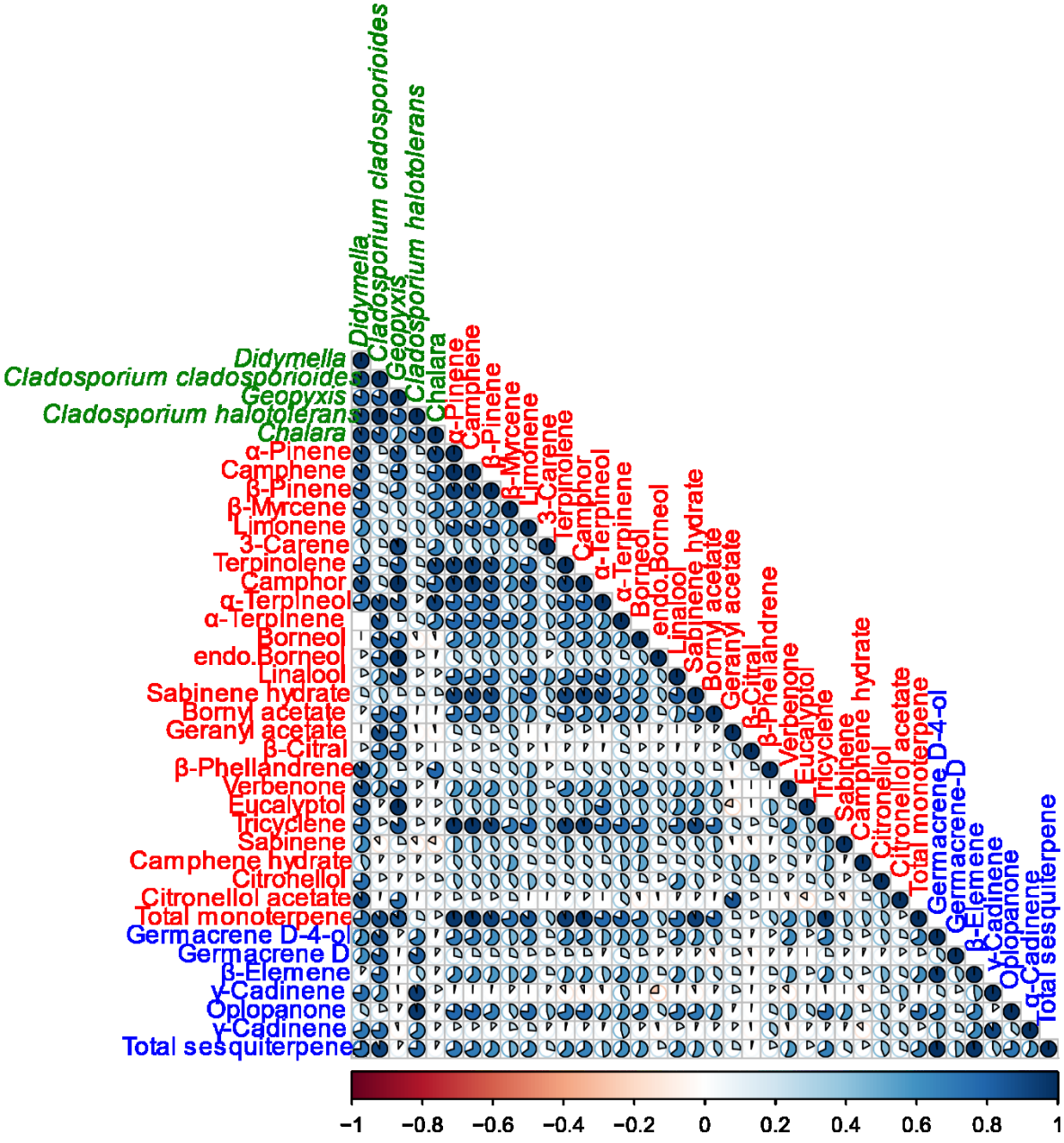


Figure 4.11. Pearson correlation pie charts (r) for individual fungal endophytes (green text), monoterpenes (red text), and sesquiterpenes (blue text) of *Picea glauca* seedlings. The darker the blue or red pie charts, the closer the r value is to either 1 or -1.

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Chapter V

Thesis Discussion

5.1 Major findings

My thesis demonstrates that the survival of the eastern spruce budworm is affected by the proportions and concentrations of monoterpenes and phenolics in white spruce foliage. White spruce families with higher proportions and concentrations of monoterpenes caused 100% larval mortality, while those with higher proportions of phenolics and lower monoterpene concentrations were less fatal to the budworm. I also show that endophytic fungi identified in white spruce foliage were found to be location- and genotype-specific, and to potentially promote the tree's production of defense chemicals such as monoterpenes and sesquiterpenes. By immediately displaying anti-herbivore defense mechanisms like poisonous mycelium or fungal VOCs, which can reduce larval survival, these fungi play a major part in improving the resistance of white spruce to herbivores like the eastern spruce budworm. In addition, the results of my research revealed that white spruce families that were inoculated with five endophytic fungi had a substantially greater abundance of endophytic fungi (90%) in comparison to the non-inoculated control group (10%). On the other hand, this abundance was genotype-specific, which indicates that different white spruce genotypes (families) can have different effects on the fungus endophytic abundance on their foliage. According to these findings, spruce genotypes that had a higher endophytic fungal abundance also had higher monoterpene and sesquiterpene concentrations, in addition to increased foliage biomass.

5.2 White spruce defense phenotypes affect the eastern spruce budworm differently

Coniferous trees' defense mechanisms against insect defoliators are significantly reliant on secondary metabolites (chemical defenses) because these metabolites are crucial components of defense mechanisms. In chapter II, I found monoterpenes and phenolics proportions varied among ten white spruce phenotypes. In general, I found strong associations between budworms and defense traits. Particularly, in the amended diet of white spruce foliage with higher concentrations and proportions of monoterpenes, the budworms died or were unable to complete their development. Recent research investigated how the spruce budworm responses to six different monoterpenes present in the foliage of balsam fir. The study reported that each monoterpene has a unique mechanism of action and set of impacts (Kumbasli & Bauce, 2013). For instance, α -pinene and -3-carene were responsible for 22% and 12% of the larval fatalities, whereas all six monoterpenes that were evaluated, including α-pinene, bornyl acetate, camphene, -3-carene, terpinolene, and tricyclene, slowed down the larval growth (Kumbasli & Bauce, 2013). This study suggested that the composition of monoterpenes and phenolics in white spruce foliage may alter the response of the eastern spruce budworm. The presence of a higher proportion of monoterpenes in spruce foliage increases the toxicity to the budworm. Overall, these findings highlight the importance of considering the differences in monoterpene and phenolic profiles when understanding the eastern spruce budworm infestations in areas with white spruce.

5.3 Foliar fungal endophytes alter white spruce terpene profiles and provide direct resistance to the eastern spruce budworm

Endophytic fungi can be beneficial to the plants they infect because they produce a variety of metabolites, such as terpenoids, which can discourage herbivores from feeding on the target plant. In chapter III, I discovered that the makeup of monoterpenes, sesquiterpenes, and fungal endophytes differed among 30 different spruce genotypes sampled on two sites. In a related study, Stefani and Bérubé (2006) identified 23 different culturable foliar fungal endophytes from seven natural white spruce stands in southern Quebec and found that endophytic fungal abundance varied among different location samples.

In this study, I was able to isolate and characterize ten different endophytic fungus morphotypes. These fungi contained 11 secondary metabolites and 13 volatile organic compounds. The composition of these metabolites varied depending on the fungal morphotype. It is known that endophytic fungi can produce secondary compounds with toxic effects on insects (Johnson & Whitney, 1994; Miller *et al.*, 2008; Kusari *et al.*, 2014). I have shown that the effectiveness of the endophytic fungi against the eastern spruce budworm depended on the amount of endophytic fungus used in the bioassays. The feeding experiment makes it abundantly clear that larger concentrations of fungus mycelia can result in a mortality rate of up to 100% of the larvae, whereas lesser amounts can result in a significant reduction in larval fitness. Similarly, Johnson and Whitney (1994) demonstrated that hyphae and hyphal extracts from several isolates obtained from *Picea mariana* increased the mortality of second-instar eastern spruce budworm (*Choristoneura fumiferana*) reared on an artificial diet.

My study also found that fungal endophytes may also regulate white spruce foliage defense chemicals. For instance, sesquiterpene-rich white spruce genotypes had more endophytic abundance. This result was expected because the fungal endophytes have been reported to produce sesquiterpenes. For instance, *Trichoderma harzianum* produced trichodermin (Lie-zhong *et al*., 2007), Xylaria sp. produced phomenone (Silva *et al.*, 2010), *Phomopsis cassiae* produced 3,12-Dihydroxycadalene, and *Epichloë typhina* produced chokols (Yoshihara *et al.*, 1985).

5.4 Foliar fungal endophytes improve the white spruce seedling terpenoid defenses and biomass

Both endophytic microbes and plants they colonize benefit from their mutualistic connection. I found that endophytic fungi can alter the foliar terpenes of white spruce seedlings. Since fungus endophytes are known to generate secondary compounds like terpenes, this was to be expected (Sumarah *et al.*, 2005; chapter III). However, the response of various families of white spruce to endophytic fungi and the synthesis of terpenes were distinct from one another. As an example, the research identified that greater concentrations of total monoterpenes were present in 23 of the inoculated spruce families when compared to the control families. In addition, 17 of the inoculated spruce families revealed greater concentrations of total sesquiterpenes when compared to the control families.

An earlier study discovered that after the fungus endophyte 5WS22E1 was introduced into nine half-sib families of white spruce seedlings, they produced a significant amount of the secondary substance rugulosin (Sumarah *et al.,* 2005). Another study was conducted in a greenhouse and examined the effect of two different kinds of fungus, *Rhizophagus intraradices*

and *Beauveria bassiana*, on the concentration of terpenoids in tomato plants as well as their defense against herbivorous insects. According to the findings, fungal inoculation led to increased amounts of certain monoterpenes and sesquiterpenes, as well as a more robust defense reaction against beet armyworm, *Spodoptera exigua* Hübne (Shrivastava *et al.*, 2015). Jallow *et al*. (2008) investigated the impact of fungal endophyte (*Acremonium strictum*) on tomato (*Lycopersicon esculentum Mill*.) terpene profiles. They found plants inoculated with endophytic fungus significantly increased trans-β-caryophyllene (sesquiterpene) as compared to endophytic-free plants.

Increased biomass production in the host plants is just one of the many benefits that endophytes provide. Other advantages include enhanced nutrient uptake, increased host plant growth, increased resilience to abiotic stress, and suppression of plant disease infection (Muthukumarasamy *et al.*, 2002). Endophytic fungi can improve plant growth by producing auxins (Nisa *et al.*, 2015). Nisa *et al*. (2015) study reveals that in response to the endophytic fungus inoculations, there was a substantial difference in the foliage fresh biomass of white spruce seedlings between the inoculated and control families. The plant growth-regulating hormone gibberellins is also produced by endophytic fungi which can facilitate shoot growth. For instance, a strain of *Penicillium citrinum* isolated from the flora of sand dunes and given the name IR-3-3 generated significantly greater amounts of gibberellins that are physiologically active. Additionally, the development of waito-c rice and *Atriplex gemelinii* seedlings was boosted by this isolated substance (Khan *et al.*, 2008).

The eastern spruce budworm defoliation is anticipated to be negatively impacted by raising the concentrations of plant defence metabolites. Higher quantities of these metabolites

have the potential to be toxic or repellent to insects like the widely recognized spruce tree defoliator known as the eastern spruce budworm.

On the other hand, there is evidence that endophytes may be able to enhance the quantity of planting material generated in forest nurseries, which is provided by the fact that there has been an increase in biomass. The increased biomass is evidence that endophytes have the potential to play a significant role in enhancing the effectiveness and productivity of forest nursery operations.

5.5 Management implications

As the eastern spruce budworm continues to spread through white spruce forests, my study examined some problems that are important to the long-term health of Canada's forest resources. White spruce shows various levels of resistance to the eastern spruce budworm, which is commonly attributed to the composition of its foliar defense compounds and fungal endophytes (Kumbasli & Bauce, 2013; Quiring *et al*., 2019). However, there is little information available on the role of fungal endophytes and secondary compounds, and most importantly, their interaction in white spruce resistance to the eastern spruce budworm. Thus, a comprehensive undertaking was needed to determine the white spruce fungal endophytes and secondary compounds diversity and to observe how such diversity can influence spruce resistance to the eastern spruce budworm.

This research contributes to the spruce budworm management by (1) identifying white spruce-resistant trees from the eastern spruce budworm so that they may be used in an effort to limit or prevent economic and ecological damage, (2) enhancing our knowledge of the interaction between white spruce secondary metabolites and their impacts on eastern spruce

budworm biology, and thus making significant contributions to a wide range of scientific disciplines, (3) understanding the role of foliar fungal endophytes altering white spruce defense metabolites, (4) identifying some endophytic communities that provide resistance to the eastern spruce budworm (i.e., causing mortality and reduced feeding), and application of these endophytes over large areas of white spruce forests during budworm outbreaks may improve tree survival. In fact, such pest management activities are already taking place in eastern Canada (Irvin Woodlands Division, New Brunswick). The information gained through my doctoral work can be valuable for understanding the roles of secondary metabolites and fungal endophytes in the evolution of white spruce resistance.

5.6 Future research recommendations

My research centered on identifying and quantifying foliar monoterpenes, sesquiterpenes, endophytic fungi from mature white spruce, and inoculation of endophytic fungi on white spruce seedlings; however, many other directions can be considered for future research. These include an interaction of ectomycorrhiza with foliar terpenes and fungal endophytes (Castaño *et al.*, 2020). In addition, studying stem endophytic fungi and how they interact with foliar fungal endophytes, as well as determining the terpenoid compositions of each, would contribute to the body of knowledge in this area.

In my research, I isolated the fungal VOCs and metabolites from endophytic fungi grown on terpenes-free PDA media. However, endophytic fungi growing on PDA amended with terpenes profiles of host tree may have provided different fungal VOC profiles. Therefore, quantifying endophytic fungal metabolite profiles based on PDA-amended media with host terpene concentrations is recommended.

In the current study, the presence of the endophytic fungus *Cladsporium* sp. led to an increase in the mortality rate of the eastern spruce budworm larvae by 100% when their diet was altered. It was found that there was a reduced incidence of *Cladsporium* sp. in seriously impacted regions of white spruce with the eastern spruce budworm in the eastern part of Canada (Stefani & Bérubé, 2006a). The next step in this research would be to observe how this endophytic fungus interacts with the eastern spruce budworm by inoculating it on established trees of white spruce in eastern Canada.

5.7 References

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