Spatial characteristics of volatile communication and the role of soil resources in pine defenses

by

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Thesis abstract

Recent unprecedented climate change has increased the frequency and severity of tree-killing *Dendroctonus* bark beetles (Coleoptera: Curculionidae, Scolytinae). Understanding factors and mechanisms influencing host plant-bark beetle interactions at a landscape level such as plant defenses, edaphic conditions, plant nutrients, and plant-plant interactions will be important to determine the impact of bark beetle outbreaks and tracking their invasion success. I studied the range expansion of mountain pine beetle (*Dendroctonus ponderosae* Hopkins; MPB) into the novel host jack pine (*Pinus banksiana* Lamb.) in the boreal forest of western Canada. I investigated the effect of soil moisture on the existence of gradients in the chemical defenses along jack pine stems by using a MPB associated phytopathogenic fungus, *Grosmannia clavigera*. I further tested whether soil moisture gradients can affect non-structural carbohydrate (NSC) mobilization and allocation to two main classes of chemical defenses in jack pine, monoterpenes and diterpene resin acids. I found that constitutive NSC production increased with stem height, diterpene resin acid concentrations decreased, and monoterpene concentrations did not vary. With increasing soil moisture, NSC production decreased, monoterpene concentrations increased, and diterpene resin acid concentrations did not vary. At an induced level, trees on the sites with higher soil moisture developed smaller necrotic lesions and had higher monoterpene concentrations by mobilizing local NSC reserves. Diterpene resin acid concentrations did not vary with soil moisture but differed at each stem height. I also compared MPB host acceptance and brood production in jack pine cut bolts from trees on sites with different soil moisture, and nutrient concentrations. Host acceptance and brood production were greater in bolts from the site with lower soil moisture and higher phloem nitrogen concentration.

Finally, I tested whether pines interact and cooperate by using volatile defense compounds, and whether such interactions correlate with the spatial characteristics of sites, and tree attributes. I studied the constitutive and induced responses in non-attacked lodgepole pines within 30 m radii of pines attacked by MPB. I found that pines interacted with chemotypically related trees only. These results suggest that pines discriminate between volatile cues from kin and strangers, and the emitters likely aid only chemotypically related pines by emitting specific blends of volatiles that can only be deciphered by the receiving kin.

Preface

This thesis presents three data chapters (Chapters 2, 3, and 4) that are either published, or submitted for publication. All studies represent collaborative work with Dr. Nadir Erbilgin of the University of Alberta. I was responsible of all experimental designs, data collection and analyses, and writing of this thesis as the following chapters (manuscripts). Dr. Erbilgin was involved with concept formation for the original research, advice on research design, and help with manuscript composition. I followed all obligatory permits and protocols enforced by the Federal Government of Canada and the Provincial Government of Alberta for these studies.

Chapter 2 of this thesis has been submitted for publication as A. Hussain, G. Classens, S. Guevara-Rozo, J. A. Cale, R. Rajabzadeh, N. Erbilgin, "Spatial variation in soil available water holding capacity alters carbon mobilization and allocation to chemical defenses in jack pine". I was responsible for the experimental design, data collection and analysis, running the chemical analyses, and writing the manuscript. Dr. Erbilgin was involved in concept formation, assisted with experimental design, and manuscript composition. Dr. Cale helped with diterpene resin acid chemical analysis, Rajabzadeh helped with monoterpene chemical quantification, and Classens and Guevara-Rozo helped with sample preparation.

Chapter 3 of this thesis has been published as A. Hussain, G. Classens, S. Guevara-Rozo, N. Erbilgin, "Soil available water holding capacity can alter the reproductive performance of mountain pine beetle (Coleoptera: Curculionidae) in jack pine (Pinales: Pinaceae) through phloem nitrogen concentration" in Environmental Entomology. I was responsible for experimental design, data collection and analysis, running soil and tree phloem nutrient analyses, soil available water holding capacity analysis and monoterpene chemical analysis, and writing the manuscript. Dr. Erbilgin was involved in concept formation, assisted with field research

design and manuscript composition, and Classens and Guevara-Rozo helped with sample preparation, beetle rearing and collection.

Chapter 4 of this thesis has been published as A. Hussain, J. C. Rodriguez-Ramos, N. Erbilgin, “Spatial characteristics of volatile communication in lodgepole pine trees: evidence of kin recognition and intra-species support” in Science of the Total Environment, 692:126–135. I was responsible for field research design, data collection and analysis, running chemical analyses, and writing the manuscript. Dr. Erbilgin was involved in concept formation, assisted with research design and manuscript composition. Rodriguez-Ramos helped with the fieldwork, and Rajabzadeh helped with monoterpene chemical quantification.

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Chapter 1

Thesis Introduction

1.1 Range expansion by mountain pine beetle

Over the past four decades, climate change has significantly increased the suitability of pine habitats for mountain pine beetle (*Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae, Scolytinae; MPB) (Logan et al. 2003; Kirilenko and Sedjo 2007; Raffa et al. 2017) which has amplified the beetle's access to pines at higher elevations and in more north-eastern stands in western North America. The current MPB outbreak, which has expended from its epicentre in central British Columbia into western Alberta, is the largest insect epidemic on record (Kurz et al. 2008). Trees on millions of ha have already been affected (Kurz et al. 2008; Raffa et al. 2008; Bentz et al. 2010; Safranyik et al. 2010). Historically, MPB is an obligate herbivore that has co-evolved with lodgepole pine (*Pinus contorta*) and periodically its populations have burst into extensive recurrent outbreaks (Bentz et al. 2010; Safranyik et al. 2010; Cale et al. 2017, 2019). In its natural range, MPB is also known to feed on other pine species, including sugar pine (*Pinus lambertiana* Dougl.), western white pine (*Pinus monticola* Dougl. Ed. D. Don), whitebark pine (*Pinus albicaulis Engelmann*), and ponderosa pine (*Pinus ponderosa*, P. Laws. Ex C. Laws) (Wood SL 1982).

Currently, as the MPB is spreading eastwards and beyond 60° North, it is threatening jack pine (*Pinus banksiana* Lamb.) dominated boreal forests (Cullingham et al. 2011; Erbilgin et al. 2014; Erbilgin 2019) which could have serious socioeconomic and ecological repercussions (Dhar et al. 2018). This invasion will jeopardize ecosystem function across Canada's boreal zone

which represents 75% of the country's forests or 28% of the global boreal forests (Wulder et al. 2007). Jack pine is the most widespread and the northern most pine species in North America's boreal zone (Rudolph and Laidly 1990; Cullingham et al. 2011). The species symbolizes a 4,000 km long corridor between western and eastern pine species in North America (Rudolph and Laidly 1990; Taft et al. 2015). Jack pine's ecological importance is also obvious by the fact that 150 bird species, representing 50% of all bird species in Canada live in jack pine's native range (Rudolph and Laidly 1990). The MPB invasion also poses serious threats of reduced carbon uptake by forests and increased emissions by decaying dead trees (Kurz et al. 2008). The massive tree mortality caused by MPB is turning forests from carbon sinks to carbon sources and the aggregate impact of the beetle outbreak in the affected region during 2000–2020 has been forecasted to be 270 mega tons of carbon (Kurz et al. 2008; Ghimire et al. 2015).

In the endemic stage, a female MPB launches the attack by identifying aging, or stressed trees as they are easy to colonize due to compromised defenses. A successful entry into the tree is followed by biosynthesis of the aggregation and anti-aggregation pheromones *trans*-verbenol and verbenone respectively from the host monoterpene, α-pinene (Wood DL 1982; Blomquist et al. 2010). The beetles also vector several phytopathogenic fungal species including *Grosmannia clavigera, Ophiostoma montium*, and *Leptographium longiclavatum* that are involved both in overcoming host defenses and in providing nutrition to developing beetles (Six 2003). In post-attack trees, as the larvae feed on the phloem, extensive larval tunneling takes place at right (90°) angles to the maternal galleries which girdles the trees to death. By consuming phloem and colonization of the phloem by fungal symbionts, bark beetles obtain important nutritional benefits that may enable faster development and higher survival of the brood (Whitney and Spanier 1982; Six and Paine 1998; Goodsman et al. 2012; Cale et al. 2017).

Understanding the spatial variation in edaphic conditions is particularly important in the MPB's recently expanded range in jack pine forests. Throughout jack pine's extensive boreal range, widespread spatial heterogeneities in soil types, soil nutrients, and soil available water holding capacity (AWHC) exist (Cayford et al. 1983; De Jong and Sheilds 1988; Visser 1995). To ensure successful host colonization and reproduction, beetles require tree death through depletion of its primary resources (i.e., non-structural carbohydrates) that fund the production of secondary defense compounds (Wiley et al. 2016; Roth et al. 2018). However, the integral variations in edaphic conditions can potentially affect these balances, subsequently influencing beetle population dynamics differentially at the landscape level.

1.2 Defense in pines

Sessile plants took millions of years to evolve abilities to counter threats by dynamically regulating their morpho-physiological responses including changes in gene expression, biosynthetic pathways, and resource allocation patterns (Raffa et al. 2008; Labandeira and Currano 2013). The coexistence among plants and insects has further refined these interactions into often very intricate relationships (Ehrlich and Raven 1964), for example, herbivory by specialist insects (Becerra and Venable 1999), and pollination and seed dispersal by selective organisms (Jordano 1987).

Some plant defense strategies are constitutive in nature (i.e., pre-formed), such as specialized cell walls, waxy epidermal cuticles, shells, trichomes, thorns and different bark types, and other defenses are inducible and can function independently or in agreement with constitutive defenses (Green and Ryan 1972; Edwards and Wratten 1985; Dixon and Paiva 1995;

Francheshi et al. 2005; Karban 2011; War et al. 2012; Mason et al. 2019). Inducible plant defenses are based on temporary chemical arbitrations initiated by a stressed plant thereby making the plant more irregular and less suitable location for insect herbivores or pathogens (Raffa et al. 2005; Bohlmann 2012; Moreira et al. 2014; Klutsch et al. 2017b; Erbilgin 2019). Most insects, however, have evolved counter-resistance traits that may offset these plant defenses. In fact, some insects can even sequester the highly specialized plant defense compounds for their own benefit, for example, MPB biosynthesis of aggregation and anti-aggregation pheromones from the host monoterpene, α-pinene (Wood DL 1982; Blomquist et al. 2010; Erbilgin et al. 2014).

To date, inducible defense mechanisms have been confirmed in most plant species investigated. The number of inducible compounds in the 20-30% of higher plants studied so far has already exceeded >100,000 (Kessler 2015). These compounds include nitrogen containing alkaloids, nonprotein amino acids, amines, cyanogenic glycosides and glucosinolates or non-nitrogen containing terpenes, flavonoids, polyacetylenes and phenylproponoids (D'Auria and Gershenzon 2005).

Conifers, including lodgepole pine and jack pine, produce complex oleoresins that contain chemicals such as terpenes (Keeling and Bohlmann 2006; Boone et al. 2011; Raffa et al. 2017; Erbilgin 2019). Among the terpenes of the pine oleoresins, monoterpenes and diterpene resin acids are the highest in concentrations (Keeling and Bohlmann 2006; Cale et al. 2019; Chiu et al. 2019b). The existing evidence suggests both monoterpenes and diterpene resin acids play important complementary roles in the defense systems of the pines (Trapp and Croteau 2001; Franceschi et al. 2005; Keeling and Bohlmann 2006; Chiu et al. 2019b; Erbilgin 2019).

Diterpene resin acids have stronger anti-fungal but weaker anti-beetle properties than monoterpenes (Phillips and Croteau 1999; Kopper et al. 2005; Boone et al. 2013). Monoterpenes are toxic to bark beetles and are likely the primary pine defense compounds used against MPB (Franceschi et al. 2005; Raffa et al. 2017). Their effects on phytophagous insects may include difficulty in host location, delayed mating behaviour, decreased fecundity, delayed larval development, increased toxicity and increased attack by insect parasitoids (Shelton 2004; Erbilgin et al. 2017a; Erbilgin 2019).

1.3 Suitability of jack pine as a host for mountain pine beetle colonization

Host suitability has been described to demonstrate similarities in the secondary metabolites between an invading insect's historical and novel hosts (Berenbaum 1995; Becerra 1997; Murphy and Feeny 2006; Erbilgin 2019). Mountain pine beetle is strongly dependent on host suitability for successful colonization and reproduction (Safranyik et al. 2010; Cullingham et al. 2011). Like lodgepole pine, jack pine phloem also contains α-pinene that the newly arrived female beetles hydroxylate to produce their pheromones *trans*-verbenol and verbenone (Pitman et al. 1968; Keeling and Bohlmann 2006; Blomquist et al. 2010; Chiu et al. 2019a). However, α-pinene was reported to be more abundant in the novel host, which may result in the production of more *trans*-verbenol, the primary aggregation pheromone of MPB (Clark et al. 2014; Rosenberger 2017).

Similarly, another secondary compound, 3-carene was higher in abundance in the lodgepole x jack pine hybrid trees (Hall et al. 2013; Clark et al. 2014). In a greenhouse experiment, 3-carene released by jack pine seedlings was found to be three times higher in concentration than by lodgepole pine seedlings (Lusebrink et al. 2011). In the field trials, the

mixture of *trans*-verbenol and 3-carene increased beetle capture in two pine species (Erbilgin et al. 2014). In another study by Lusebrink et al. (2016), myrcene was more abundant in water stressed jack pine trees. Myrcene synergizes with beetle aggregation pheromones, and thus is important to beetle mate finding and reproduction as well as overwhelming host tree defenses (Raffa et al. 2013).

Likewise, limonene is toxic to most *Dendroctonus* species including MPB; however, its concentration in stressful conditions, for example, water deficit only increases in lodgepole pine. Despite their short maternal galleries, female brood beetles emerging from jack pine had more fat content than those emerging from lodgepole pine bolts (Lusebrink et al. 2016). Lusebrink et al. (2013) reported that beetles reared in bolts from trees that had faced drought emerged with greater fat content. In short, jack pine might facilitate MPB populations not only because of the composition of its secondary compounds, but also because of potential stresses it faces in its harsh boreal growing conditions.

1.4 Spatial variation in carbon allocation to defenses and the role of available water holding capacity

Plants are the primary sources of carbon for all terrestrial life. However, how plants invest fixed carbon is still not very clear (Gershenzon 1994; Goodsman et al. 2013; Wiley et al. 2016). In vascular plants, most of the photosynthetic and stored carbon needs to be mobilized as NSC in the phloem to support growth and metabolism. In pines, NSC mobilization, their relationship with distance from the source (canopy), and NSC deficient lower stem vulnerability was demonstrated by Goodsman et al. (2013). Soil water availability is a central resource affecting plant fitness. Water stress in plants can alter NSC production and allocation, and thus, the

concentration, composition, and distribution of both primary and secondary metabolites (Mundim and Pringle 2018). Such imbalances are especially more critical for plants under stressful conditions because like growth and reproduction, the production of defense compounds is metabolically costly that acts as a carbon sink in trees (Gershenzon 1994).

Although, numerous studies have focused on soil water and plant responses, the majority of such field studies have assessed soil water availability at the time of sampling or over a short period of time, thus their results do not reflect the historical patterns in water availability and other soil gradients that may influence physiological responses in plants, including NSC metabolism, storage and defense chemicals. In this context, soil available water holding capacity (AWHC) is a more appropriate proxy to test the effects of water availability on plant responses because AWHC is more relevant to predict the long-term soil characteristics that influence vegetation growth and development (Reynolds et al. 2000; Piedallu et al. 2011). The soil AWHC refers to the difference between field capacity (the maximum amount of water the soil can hold), and permanent wilting point (the threshold where a plant can no longer extract any water from the soil) (Kirkham 2014).

As NSC fund defensive responses in trees, variations along tree stems may result in differential responses to MPB (Cole and Amman 1983) or its associated fungi (Goodsman et al. 2013). These gradients may potentially be associated to the lower stem as the weakest stem points for initial MPB attacks (Cole and Amman 1983). Therefore, measuring NSC availability and defense compound concentration along tree stems across different AWHC regimes may further disclose important patterns in the health of tissues in different stem sections at a landscape level.

1.5 Role of available water holding capacity, and soil and phloem nutrients in mountain pine beetle success

Unlike MPB's historical hosts, jack pine seems to be better adapted to various abiotic stresses due to its water-conserving nature and its ability to grow on well-drained and nutrient-poor boreal soils (Rudolph and Laidly 1990; Moore et al. 2000). The region extending eastwards from Lake Winnipeg to Newfoundland is subject to lake and coastal influences and hence, receives more annual precipitation than the western continental region extending from the Yukon to Manitoba (Price et al. 2013). Moreover, widespread spatial heterogeneities in soil types, soil nutrients, and AWHC are also known to exist (Cayford et al. 1983; De Jong and Sheilds 1988; Visser 1995). These variations have key effects on forest productivity and the occurrence of insect outbreaks (Price et al. 2013). Currently, we lack critical information to assess how variations in the growing conditions of jack pine could influence host selection behaviour and reproductive performance of MPB in the novel host.

The nutritional value of a host tree is critical for herbivores as it can affect their growth and reproduction, several metabolic processes, cellular structure, and genetic coding. Bark beetles in particular face such challenges as the phloem they consume is generally very low in important nutrients, such as nitrogen, phosphorus and potassium (Mattson 1980; Ayres et al. 2000; Sterner and Elser 2002; Goodsman et al. 2012; Plassard 2018; Guevara-Rozo et al. 2019).

1.6 Spatial characteristics of volatile communication in pines

Plants also mediate aboveground interactions with other organisms in the same or different taxa, and environments by releasing volatile organic compounds (VOC) that are intended to reduce losses due to attacking insects and pathogens (Kessler and Baldwin 2002; Crepy and Casal 2015;

Sampaio et al. 2016; Kollist et al. 2018). However, plant-plant communication is a topic of considerable debate, typically focused on the ecological relevance of such interactions that are mostly verified in controlled environments, but very little observed in nature (Barbosa et al. 2009). Furthermore, we have a limited understanding of how plants can perceive these signals and distinguish them from conspecifics. This is further complicated by little research done on exploring the role of spatial characteristics of sites, plant attributes and other contrasting field conditions that can potentially impact these interactions by affecting the dispersal and concentrations of VOC plumes (Thistle et al. 2011; Lowman and Schowalter 2012; Zitouna-Chebbi et al. 2015).

Because airborne VOC are carried by the wind, these interactions essentially require three components: an 'emitter', a 'receiver' and a 'field' where the exchange of information (VOC) occurs (Baldwin and Schultz 1983; Sampaio et al. 2016; Kollist et al. 2018). Nevertheless, all three components have their own limitations under field conditions, especially when working with large woody plants, like conifers. For example, complex forest conditions to detect tree responses, and numerous methodological difficulties associated with the size and longevity of trees.

Interestingly, as closely related plants are more likely to host common insects and pathogens, these VOC-mediated interactions have been demonstrated to be more common among closely related plants (Baldwin and Schultz 1983; Dudley and File 2007; Barbosa et al. 2009; Heil and Karban 2009; Karban and Shiojiri 2009; Crepy and Casal 2015). However, these differential responses require a mechanism to distinguish between related and non-related plants. In this context, VOC emissions of some plants, especially pines cluster into chemotypes, defined

as ‘chemically distinct individuals of a species within a population that are morphologically indistinguishable’ (Keefover-Ring et al. 2009; Pieruschka and Schurr 2019). As chemotypes are heritable, they are a reliable way to predict relatedness in plants (Hanover 1971; Axelrod and Hamilton 1981; Karban et al. 2014a). However, to date no study has yet tested VOC-mediated communication, kin recognition or support in pines.

1.7 Thesis aims

I had two major aims for this thesis research: (1) understanding the role of edaphic conditions in influencing susceptibility of jack pine to MPB; (2) understanding spatial characteristics of volatile communication in pines. The first two projects (Chapters 2 and 3) in this thesis used mature jack pine trees and their associations with multiple growing conditions in the field to investigate how soil water availability and soil nutrients influenced NSC mobilization, allocation, and tree susceptibility to MPB. The third project (Chapter 4) used mature lodgepole pine trees as a model species to understand spatial characteristics of volatile communication in pines. Although I planned to use jack pine in this study (Chapter 4) as well, after an exhaustive search in 2015 and 2016, I could not find the right number of recently infested pure jack pine stands, as required by my experimental design. Therefore, I decided to study volatile communication in lodgepole pine trees instead of jack pine trees.

I hypothesized that NSC mobilization and allocation to defense responses in jack pine will be negatively influenced by deteriorating growing conditions, which will in turn lead to greater susceptibility of jack pine and greater reproductive success of MPB. More specifically, the following objectives were tested: (1) exploring the relationship between growing conditions and chemical defenses in jack pine; (2) understanding differential pathogen-induced responses

mediated by growing conditions; (3) exploring how soil resources influence carbon dynamics of jack pine trees; (4) identifying how these changes impact overall MPB success; (5) examining how pines communicate by using the induced volatile organic compounds; (6) how volatile communications in pines are influenced by spatial characteristics of sites and tree attributes. The information gathered through these projects will help us better understand the further range expansion of MPB at landscape level, and the spatial characteristics of tree-tree communication at stand level.

In Chapter 2, I studied the interactions between spatial variations in concentrations of NSC availability and defense compounds in the different sections (height) of jack pine tree stems. I further explored these interactions by relating them to soil water availability at constitutive and induced levels of chemical defenses. Fungal inoculations by *Grosmannia clavigera* induced tree defense. I also evaluated vulnerability to the MPB-associated phytopathogenic fungus (*G. clavigera*) at the lower stem (1.3 m) and studied if soil water availability plays in pine vulnerability. Next, I related differences in terpene concentrations to the differences in local and distant NSC reserves. Results from this research demonstrate that soil water availability can indeed, alter defenses and vulnerability by influencing tree NSC dynamics. I also show that jack pine relies on local NSC resources to fund the induced responses against *G. clavigera.* The chapter titled "Spatial variation in soil available water holding capacity alters carbon mobilization and allocation to chemical defenses in jack pine" and is currently under review; please also see Preface.

In Chapter 3, in order to validate my findings in the Chapter 2, I studied MPB host acceptance and brood production success in cut bolts of jack pine trees. I specifically focused on

the relationship between soil and phloem resources, and MPB success (i.e., host acceptance, maternal gallery lengths, and brood production). I collected 30 bolts (15 bolts/site) from 30 trees (15 trees/site) on two sites with significantly different soil water availability. I found that host acceptance by and brood production of MPB were greater in bolts from the site with lower water availability. These bolts also had higher concentrations of phloem nitrogen that interacted with soil water availability. These results confirm that MPB success is higher in potentially stressed host trees, as extensively reported in the literature. This chapter is titled "Soil available water holding capacity can alter the reproductive performance of mountain pine beetle (Coleoptera: Curculionidae) in jack pine (Pinales: Pinaceae) through phloem nitrogen concentration" and has been published in Environmental Entomology (please also see Preface).

In Chapter 4, I investigated the stability of chemotypic expression in pines, and characterized any volatile organic compound (mostly monoterpenes) mediated pine interactions at stand level. This study was specifically aimed at exploring the aboveground MPB-mediated information sharing in pines using volatile cues to cooperate with chemotypically related kin. These interactions have been confirmed in many other plant species, but not in long living trees, including pines. Three objectives motivated me to pursue the following questions (1) How stable are lodgepole pine chemotypes at the constitutive level? (2) Are herbivory informing interactions general in lodgepole pine, or only effective in individuals of related chemotypes? (3) Can site aspects and distance between the attacked and non-attacked trees influence such interactions? In a field study involving 201 mature lodgepole pine trees, I investigated variations in the concentrations of defense compounds in neighbouring conspecifics with distance from a central signaling tree (i.e., MPB attacked, ~12 months ago), and site aspects. I found that the attacked pines interacted with neighbouring non-attacked pines. I further found that these interactions

were spatially restricted by the distance separating the two pine trees, and site aspects. Interestingly, I also found that these interactions only occurred between chemotypically related trees, i.e., I found evidence of kin recognition and support in lodgepole pine trees. This chapter is titled “Spatial characteristics of volatile communication in lodgepole pine trees: evidence of kin recognition and intra-species support” and has been published in Science of the Total Environment (please also see Preface)

Chapter 2

Spatial variation in soil available water holding capacity alters carbon mobilization and allocation to chemical defenses in jack pine (*Pinus banksiana*)

2.1 Abstract

Carbohydrate metabolism in trees has received considerable attention due to its role in global forest decline. However, carbohydrate mobilization, storage, and allocation show large variation along tree stems. Currently, we have a limited understanding of the causes or consequences of carbohydrate variation on plant chemical defenses. I measured non-structural carbohydrates (NSC), monoterpenes, and diterpene resin acids along jack pine (*Pinus banksiana*) stem before inoculating with a phytopathogenic fungus in sites with different soil available water holding capacities (AWHC). I then assessed induced responses in trees by measuring lesion areas (necrotic tissues), NSC mobilization, and terpene concentrations along different stem heights. Before inoculation, NSC concentrations increased with stem height, diterpene resin acid concentrations decreased, and monoterpene concentrations did not vary. However, with increasing AWHC, NSC concentrations decreased, monoterpene concentrations increased, and diterpene resin acid concentrations did not vary. After inoculation, trees on the high AWHC sites developed smaller lesions, mobilized local NSC, and induced higher monoterpene concentrations in phloem and lesions. Diterpene resin acid concentrations did not vary with AWHC but differed at each stem height. Inoculation density showed no difference. I conclude that gradients in soil

AWHC affect stem vulnerability in jack pine by influencing carbon mobilization, allocation, and utilization.

Keywords: defense compounds, mountain pine beetle, range expansion, sink-source, soil water.

2.2 Introduction

Forests play critical roles in the Earth's carbon, energy and water cycles, and provide important ecosystem services and essential commodity goods such as fiber and wood (Seidl et al. 2017). However, climate change through influencing natural and anthropogenic disturbance regimes is threatening the sustainability of forests (Trumbore et al. 2015; Seidl et al. 2017; Moreno-Fernández et al. 2019). For instance, drought and climate change induced tree mortality events have been documented across multiple forest types (Jactel et al. 2012; Gaylord et al. 2013; Hart et al. 2014; Allen et al. 2015; McDowell et al. 2018; Kono et al. 2019). Most field studies with drought focus on the availability of soil water at the time of sampling or over a short period of time (1-3 years) which does not reflect the historical patterns in water availability and other soil gradients that may be influencing physiological responses in plants, including non-structural carbohydrate (NSC) metabolism and defense chemicals. In this study, I used soil available water holding capacity (AWHC) as a proxy to test the effects of water availability on plant responses because AWHC is more relevant to predict the long-term soil characteristics, including water availability, influencing vegetation growth and development (Reynolds et al. 2000; Piedallu et al. 2011).

Carbon allocation to storage pools, growth, metabolism, and biosynthesis is an internal process that allows plants to meet the flexible demands for resources. Such allocations are

especially critical for trees under stressful conditions because like growth and reproduction, the production of defense compounds is metabolically costly that acts as a carbon sink in trees (Gershenzon 1994). Carbon can be allocated differentially within a tree, depending on its life history strategies, herbivory pressures, and other evolutionary processes (Herms and Mattson 1992; Goodsman et al. 2013; Klutsch et al. 2017a). In general, carbon allocations are thought to be driven by source-sink balance (Herms and Mattson 1992). Therefore, production of defense compounds may account for balances between growth and defense along availability gradients of common resources (Koricheva et al. 1998; Koricheva 2002).

Constitutive defenses represent a 'fixed cost' for a tree, therefore, the production of induced defenses at carbon sinks may require mobilization of NSC along tree stems to meet their needs (Koricheva et al. 1998; Goodsman et al. 2013; Klutsch et al. 2017a; Raffa et al. 2017). It is well established that source-sink disruption such as defoliation or pathogen infection can alter NSC mobility in plants (Vanderklein and Reich 1999; Li et al. 2002; Galiano et al. 2011). However, abiotic factors such as soil water availability are also known to influence carbon transport through xylem and phloem by limiting their ability to transport carbon, water, and nutrients, and hence, could potentially alter source-sink relationships (Morgan 1984; Woodruff and Meinzer 2011; Lemoine et al. 2013; Hartmann 2015).

In conifers, NSC reserves, primarily comprised of starch, are converted to soluble sugars and transported to carbon sinks throughout the tree (Woodruff and Meinzer 2011; Goodsman et al. 2013; Raffa et al. 2017; Huang et al. 2019). Transport of NSC over longer distances within a tree is an important factor recognized to be affecting many functions including defenses (Knoblauch and Peters 2010; Lahr and Krokene 2013; Wiley et al. 2016; Huang et al. 2019).

Conifers are presumed to channel NSC from source tissues to sinks in order to support the production of carbon-rich chemical defenses and provide additional energy for the recovery of wounded tissues (Roitsch et al. 2003; Goodsman et al. 2013; Cale et al. 2019; Huang et al. 2019). However, it is unclear how NSC resources from local and distant sources are allocated to sinks, and if such a translocation is influenced by differences in soil AWHC.

The recent unprecedented range expansion by mountain pine beetle (MPB), *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae) into the jack pine (*Pinus banksiana*) forests of western North America (Erbilgin et al. 2014; Erbilgin 2019) motivated me to study the vulnerability of mature jack pine trees along their stems to MPB, as was reported for a historical host of MPB (Goodsman et al. 2013). Throughout its range, jack pine trees grow in environments that show spatial heterogeneity in soil types, soil nutrients, and AWHC (Cayford et al. 1983; De Jong and Sheilds 1988; Visser 1995). However, how these variations affect carbon mobilization and allocation to chemical defenses is unknown. Beetles require tree death through depletion of its resources (i.e., NSC) to ensure successful host colonization and reproduction (Wiley et al. 2016; Roth et al. 2018). After identifying and landing on a suitable host, beetles bore through the outer bark of the host and inoculate the phloem and xylem with their symbiotic fungi, including *Grosmannia clavigera* (Six 2003). Beetles must first encounter the constitutive defenses in the phloem (Franceschi et al. 2005) but as the beetle colonization continues, the trees also respond to the attack by producing additional defense chemicals. This is generally followed by the formation of resin-filled necrotic lesions that are a tree's response to the fungal infection and involves local autolysis of parenchyma cells, and an increased secretion of defense compounds, rendering the phloem no longer suitable for larval or fungal development (Franceschi et al. 2005; Keeling and Bohlmann 2006). Lesion size in trees is considered a good

predictor of resistance to a pathogen, and smaller lesions are suggested to specify more capable defenses (Bonello et al. 2006; Krokene et al. 2008; Goodsman et al. 2013).

Soil water availability can limit conifer defenses by influencing resin flow and its composition (Niinemets 2015; Arango-velez et al. 2016; Lusebrink et al. 2016; Mundim and Pringle 2018; Sevanto 2018). The cumulative effects of abiotic and biotic stresses on trees will be reflected in their abilities to maintain constitutive defenses and the ability to mount effective inducible defenses (Franceschi et al. 2005; Gaylord et al. 2013; Netherer et al. 2015). As oleoresins represent both physical and chemical defense systems against MPB, understanding their chemistry is considered important in bark beetle–host evolution (Trapp and Croteau 2001; Franceschi et al. 2005; Keeling and Bohlmann 2006; Raffa et al. 2017; Erbilgin 2019). In this context, monoterpene and diterpene resin acid concentrations are suitable candidates to measure chemical defenses as tree oleoresins are mostly composed of these two subclasses of terpenes (Trapp and Croteau 2001; Keeling and Bohlmann 2006). The composition and concentrations of these compounds further outline a complex set of constraints that need to be addressed in order to efficiently assess host tree defense capacity and how this capacity could be affected by edaphic conditions and insect attack dynamics (Bohlmann 2012).

The objective of this study was to determine whether gradients in constitutive and induced defenses, and vulnerability exist along jack pine stem, and if these relate to AWHC of soils. The soil AWHC refers to the difference between field capacity (the maximum amount of water the soil can hold), and permanent wilting point (the threshold where a plant can no longer extract any water from the soil) (Kirkham 2014). Water available to trees is generally held by energy forces that range from 100 to 1500 kPa, and theoretically, plants cannot recover once the

permanent wilting point is exceeded (Fowells and Means 1990). I further aimed to relate differences in terpene concentrations to the differences in local NSC reserves and distance from carbon sources. I quantified defense by measuring monoterpene and diterpene resin acid concentrations in the phloem, and monoterpenes in the lesions. I quantified NSC reserves by measuring the concentrations of soluble sugars and starch in the phloem tissues. As evidence of direct vulnerability in jack pine trees, I compared lesion areas formed as a result of inoculations with live *G. clavigera* cultures.

2.3 Material and methods

2.3.1 Experimental design and sampling

Soil moisture content in the entire range of jack pine is spatially very diverse, ranging between 3 to 17 % by weight in some of the well-stocked stands (Rudolph and Laidly 1990). Generally, the species can grow in nutritionally poor sandy dry soils to moderately nutritious well drained loamy sands (Rudolph and Laidly 1990). As the conditions for jack pine growth are well documented, I selected six sites in Lac la Biche, Alberta (Canada) on relatively dry soils to better represent the species. The selected sites were of natural origin (i.e., open pollinated) with similar climatic conditions, mean elevation of 618 m, and a soil type of loamy sand Degraded Dystric Brunisol (Kocaoglu and Brunelle 1975). There were no signs of any management activities such as pruning or harvesting on the sites; however, these forests have been subjected to natural biotic and abiotic disturbances such as wildfire, and infection by dwarf mistletoe (*Arceuthobium americanum*). Dwarf mistletoe infection is the most common biotic disturbance along with a less frequent occasional (over 100 years) abiotic disturbance wildfire. I measured soil moisture content of the sites by using an electromagnetic sensor (ML3 Theta Probe, Delta-T Devices,

UK). Briefly, after removing the litter layer, I inserted the probe into the soil up to a depth of 25 cm at four points on each site. I repeated the measurements four times for each of the four sampling points per site. I calculated the mean soil moisture content of each site before selecting potentially different sites for available water holding capacity (AWHC). I did not perform any formal statistical tests on the probe readings as I was more specifically interested in the AWHC of our sites (i.e., long-term soil water availability). Considering AWHC instead of soil moisture content also helped us rule out biases due to any recent inconsistent and patchy rainfall in the region.

Two sites with the lowest soil moisture (mean 2.51 ±0.33% SE) were located at (N 55.059, W 112.024; N 55.067, W 111.996), two with medium soil moisture (3.81 ±0.51%) at (N 55.057, W 112.028; N 55.082, W 111.986), and another two with highest soil moisture (5.51, ±0.42%) were located at (N 55.079, W 111.990; N55.075, W111.993). On each site, I selected 15 mature jack pine trees with no apparent aboveground signs or symptoms of insect or pathogen activity. The mean diameter at breast height (1.3 m) of trees was 23.22 cm (±0.19). I sampled all trees for phloem tissues (3 cm x 3 cm) at 1.3 m and 5 m stem heights in North and South cardinal directions using a 3 cm wide flame-sterilized chisel using 96% ethanol (Fisher-Scientific, Pittsburgh, PA, USA). I performed the preliminary stand evaluation, the first round of tree and soil sampling, and tree inoculation on July 7 and 8, 2015.

I used a single isolate of *G. clavigera* (EL033) to inoculate the trees at 1.3 m (about 4 cm right of the tissue collection point). I sub-divided the 15 trees into three groups of five trees. I did not inoculate trees in the first group as they served as control. Trees in second and third groups received 4 and 16 inoculations of live *G. clavigera* mycelial culture (252,000 cells ml−1)

respectively. The inoculation protocol followed a similar procedure as described in (Rice et al., 2007). I made all inoculations in a 0.03 m2 rectangle on the north aspect of the trees (Lusebrink et al., 2016). After six weeks, I collected phloem samples (about 4 cm right of the lesions). At the same time, I exposed the lesions caused by fungal inoculations at 1.3 m, measured fungal growth, and sampled them for chemical analyses. I pooled together phloem samples from each tree by height and sampling time, however, I handled lesion samples separately. I separately wrapped all samples in aluminum foils and flash froze them in liquid N before storing them at –40°C in the laboratory.

I collected soil samples from each site for nutrient analysis and AWHC assessment. Briefly, I established one 15 m x 30 m rectangular grid on each site and selected four random but evenly distributed sampling points within each grid. After removing the litter layer, I used a soil auger to collect soil samples to a depth of 25 cm at each of four points. I transferred soil samples into Ziploc bags and kept on dry ice before storing at –40°C in the laboratory. Each sample weighed approximately 250 g.

2.3.2 Assessment of soil available water holding capacity

To assess AWHC, I used pressure plate chambers (Extractor 1500F2, Soil Moisture Equipment Corp., Santa Barbara, CA, USA) as described in Klute (1986). Briefly, I subsampled all four soil samples from each site four times (n=16 for each site) and sieved them with a 2 mm round-hole sieve. I then took 25 g of soil samples in the retaining rings and placed them in the pressure chambers and saturated them by gently pouring in water to slightly cover the retaining rings. After 24h, I removed excess water and pressurized the chambers (100 kPa for field capacity and 1500 kPa for permanent wilting point). After 48h, I removed the samples from the chambers and

weighed immediately. Finally, I oven dried the samples at 105°C for 24h and weighed again for differences. I calculated AWHC of soil samples as a difference between field capacity and permanent wilting point. I reported the results volumetrically in cm^3 of water per cm^3 of soil.

2.3.3 Nutrient analysis

I analyzed total soil calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, sulfur, and zinc at the University of Alberta (http://nral.ualberta.ca). Briefly, I exposed phloem samples by removing their outer bark, oven dried subsamples at 40°C for 24h, and ground them by using a Wiley Mini Mill fitted with a 40 mesh (0.4 mm) screen (Wiley, Thomas Scientific, Swedesboro, NJ, USA). I microwave assisted digested the samples in nitric acid, followed by the analysis using an inductively coupled plasma-optical emission spectrometer (ICP-OES) on an iCAP 6300 DUO (N. America) apparatus (Thermo Fisher Corp., Cambridge, U.K). I determined total phloem and soil nitrogen contents by the dumas combustion method by using the Costech Model EA 4010 Elemental Analyzer (Costech International Strumatzione, Florence, ITL). I ran every tenth sample twice to validate our results and reported total phloem nutrients as % of dry weight of tissue samples, and total soil nutrients as g kg^{-1}.

I also determined plant available soil nutrients at the same facility. Briefly, I extracted available potassium and phosphate by using 5 g of oven dried soil with 50 ml of Modified Kelowna. I extracted chloride and sulfate by using 10 g of field moist soil with 20 ml of Milli-Q water. I extracted ammonium and nitrate by using 5 g oven dried soil samples with 2M KCl and filtering through Fisherbrand™ Q2 filter paper (Fisher Scientific). I analyzed the plant available nutrients by using Dionex Ion Chromatography DX 600 (Dionex, Sunnyvale, USA), and reported as mg kg^{-1}.

2.3.4. Non-structure carbohydrate analysis

I oven dried subsamples of the flash frozen phloem tissues at 100°C for 1h to stop enzymatic conversion of starch to sugars, and then dried continuously for 72h at 70°C. I then ground the dried samples by using a Wiley Mini Mill fitted with a 40 mesh (0.4 mm) screen. I followed the protocol described by Chow and Landhäusser (2004). Briefly, I extracted water-soluble sugars from 50 mg of the prepared subsamples in 80% hot ethanol and reacted them with phenol-sulfuric acid before colourimetric measurement by using a spectrophotometer (Pharmacia LKB Ultrospec III, Sparta, NJ, USA) at a wavelength of 490 nm. I enzymatically digested starches in the remaining residual pellet and combined the resultant glucose hydrolyzate with peroxidase-glucose oxidase/o-dianisidine (colour reagent) before measuring glucose hydrolyzate (starch) concentrations at a wavelength of 525 nm. I reported NSC results as μg mg^{-1} of DW tissue.

2.3.5 Monoterpene analysis

I ground the remaining phloem samples in liquid N by using a cryogrinder (SPEX Sample Prep Freezer Mill 6770, NJ, USA). Sample preparation and analysis followed a similar protocol as described by Erbilgin et al. (2014). Briefly, I extracted subsamples of 100 ± 2 mg of the ground tissues twice in 0.5 ml pentane and 0.004% tridecane as an internal standard in 1.5ml micro-centrifuge tubes. After adding the solvent, I vortexed the extracts for 30s at 3,000 rpm, sonicated for 10min, centrifuged at 13,000 rpm for 15min at 4°C, and then placed in a –40C° freezer for 2h. I transferred the extracts into gas chromatography (GC) vials and injected 1 μl at a split ratio of 10:1 in a coupled GC-mass spectrometer (GC-MS; 7890A-5975C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP Innowax column (ID 0.25 mm; length 30 m; Agilent Tech.). I used helium as the carrier gas at a flow rate of 1.1 ml min^{-1}. The temperature program started at

55 °C (held for 1min) then ramped by 40°C min^{-1} to 65°C (held for 1min), then 40°C min^{-1} to 75°C (held for 30s), then 7 °C min^{-1} to 130°C, and then 20°C min^{-1} to 250°C (held for 30s). I used the following standards to quantify extracts, mainly monoterpenes: α-pinene, β-pinene, 3-carene, myrcene, limonene, *p*-cymene, camphor, 4-allyanisole, borneol (Fluka, Sigma-Aldrich, Buchs, Switzerland; chemical purity >95%), γ-terpinene, α-terpinene, pulegone, terpineol (Sigma-Aldrich, St. Louis, MO, USA; >95%), ocimene, terpinolene, bornyl acetate (>95%), camphene (SAFC Supply Solutions, St. Louis, MO, USA; >80%), and β-phellandrene (Erbilgin lab; >90%). I identified compounds by comparing their retention times and mass spectra with those of the commercial standards and quantified through calibration curves using the standards generated from analyses of a serial of three dilutions of known quantities of standards and calculated as μg mg^{-1} of fresh tissue.

2.3.6 Diterpene resin acid analysis

I quantified diterpene resin acids using a multiwavelength detection approach described by Kersten et al. (2006). Briefly, I ground phloem samples in a cryogrinder and extracted diterpene resin acids from 100 ± 2 mg in 1.5ml micro-centrifuge tubes by using 1 ml of HPLC-grade methanol as a solvent. The vortexed samples for 30s, sonicated for 10 min and then left in the dark at room temperature for 24h. The following day, I centrifuged the samples at 18,000 rpm and the collected the supernatants in 2 ml GC vials. I analyzed the extracted samples for diterpene resin acids by using an ultra-performance liquid chromatograph (1290 Infinity, Agilent Tech.) fitted with a Zorbax Eclipse Plus C18 RRHD column (2.1 x 150 mm, 1.8μm; Agilent Tech.) and a diode array detector. I used 10 μL injection volume for the analysis. As a mobile phase, I used a binary isocratic system consisting of 85% HPLC-grade methanol and 15% of an

aqueous solution of acetic acid (1.7% v/v) flowing at a rate of 0.4 ml min^{-1} and heated to 40ºC. I quantified the compounds by using analyte absorbance at wavelengths of 240, 268, and 282 nm. I calculated standard curves used to quantify diterpene resin acids from dilutions prepared from analytical standards of abietic acid (purity >94%), dehydroabietic acid (>99%), levopimaric acid (>96%), neoabietic acid (>99%), and palustric acid (>91%) purchased from CanSynth (Toronto, ON, CAN). I reported results as µg mg^{-1} of FW.

2.3.7 Statistical analysis

I used R v3.4.3 (R Core Team 2017) for all statistical analyses. For monoterpenes, I used a subset of five compounds, including α-pinene, β-pinene, limonene, myrcene, and 3-carene that represented >95% of the total concentration (Erbilgin 2019). I found all other compounds either too low to quantify, or only in a few samples. I checked data for the assumptions of homoscedasticity and normality by using Levene's and Shapiro–Wilk tests, respectively. Where necessary, I either (log+1) or BoxCox transformed data prior to analysis. Our statistical models included mixed models and PERMANOVAs (9,999 permutations) for multivariate analyses followed by univariate analysis using either ANOVAs, t-tests or their non-parametric alternatives. I conducted Tukey's HSD tests or Dunn's multiple comparisons test with Benjamini-Hochberg method to examine pair-wise differences for significant main effects or significant interactions. I performed PERMANOVAs with the 'adonis' function and checked the differential dispersion between test groups with a permutation-based test for homogeneity of dispersion 'PERMDISPER' by using the 'betadisper' function in the 'vegan' package (Oksanen et al. 2017), followed by ANOVAs and visual inspections of the mean distances to centroids. To visualize significantly different PERMANOVA results, I conducted Canonical Analysis of

Principal Coordinates (CAP) with the 'capscale' function in the 'vegan' package and tested the correlation between canonical principal coordinates, variables, and test groups through a permutational test. I performed separate tests for constitutive and induced NSC, monoterpenes and diterpene resin acids at 1.3 m and 5 m stem heights, or for any multivariate comparisons involving these two stem heights.

I compared phloem nitrogen concentration at 1.3 m among trees by fitting a linear mixed model in the 'lme4' package with the 'lmer' function (Bates et al. 2015). I used AWHC as a fixed effect and sites as a random effect. I fitted a separate linear mixed model to compare lesion areas in trees among all sites, and used AWHC, total constitutive monoterpene, total constitutive diterpene resin acid, total constitutive NSC and phloem nitrogen concentrations at 1.3 m as fixed effects and sites as a random effect. I used the function ANOVA in the 'car' package to determine the significance of the fixed effects (Fox and Weisberg 2018). I further used *post-hoc Tukey's* HSD test to identify differences in lesion areas in trees. I performed this test by applying the general linear hypothesis function 'glht' function of the 'multcomp' package to the model (Hothorn et al. 2008). For the proportional variations in induced NSC, monoterpenes and diterpene resin acids, I pooled the four and sixteen inoculation densities together as they did not vary and compared with the control trees at 1.3 m and 5 m stem heights. To address the differential scaling of induced defense compounds and allocation of NSC due to variations in the edaphic conditions, I focused on the proportional induction. I calculated proportional induction in the concentrations of NSC as [(Constitutive – Induced) / Induced)] and proportional induction in the concentrations of defense compounds as [(Induced – Constitutive) / Constitutive)]. I found copper in some soil samples, but only in trace amounts, therefore I excluded it in the analysis. I constructed all figures using non-transformed data.

2.4 Results

2.4.1 Soil AWHC and soil and phloem nutrient analyses

Available water holding capacity (AWHC) varied among categories of sites (ANOVA, $F_{2,21}$ =127.4, P<0.001). *Post-hoc* comparisons using Tukey's HSD test indicated that the High, Medium, and Low sites had highest, intermediate and lowest AWHC, respectively (Suppl. Table 2.1). None of the sites within a category varied. Sites also varied in available nutrient concentrations including sulfate, phosphate, total available nitrogen, and total available nutrients (Suppl. Table 2.2). *Post-hoc* comparisons using Tukey's HSD tests indicated that the Low AWHC sites had highest concentrations of phosphate, total available nitrogen, and total available nutrients, and lowest concentration of sulfate. Ammonium, chloride, soluble and extractable nitrates, and total nitrates did not vary among sites.

Among the total soil nutrients, concentrations of sulfur, calcium, zinc, iron, sodium, and total soil nutrients varied, however, no differences existed in nitrogen, phosphorus, potassium, magnesium and manganese concentrations (Suppl. Table 2.3). *Post-hoc* comparisons using the Tukey's HSD tests indicated that the High AWHC sites had significantly higher concentrations of zinc, sulfur, iron and total soil nutrients. The Medium AWHC sites had higher concentration of sodium. The concentration of calcium was significantly lower at the Medium AWHC sites however, no differences existed between Low and High AWHC sites.

The phloem tissues collected from trees varied in concentration of nitrogen (% of dry weight) (LMER: $\chi^2_{(2)}$=14.31; P<0.001). *Post-hoc* Tukey's HSD test indicated that trees on the Low AWHC sites had significantly higher concentration of phloem nitrogen than those on the High AWHC sites. Trees on the Medium AWHC sites had marginally higher concentration of

phloem nitrogen than those at the High AWHC sites. I did not find any differences among trees at the Low and Medium AWHC sites (Suppl. Fig. 2.1, Suppl. Table 2.4).

2.4.2 Constitutive non-structural carbohydrates

One-way PERMANOVA indicated significant correlations in the concentrations of constitutive NSC and AWHC both at 1.3 m and 5 m (Suppl. Table 2.5). CAP analysis visually displayed the clustering of sites at both stem heights and showed that constitutive NSC mostly associated with High AWHC sites at 1.3 m (Fig. 2.1A, B). However, at 5 m they mostly associated with Low AWHC sites. Univariate analysis confirmed that concentrations of constitutive soluble sugars and total NSC varied both at 1.3 m and 5 m (Suppl. Table 2.6). Concentrations of constitutive starch did not vary at any stem height. *Post-hoc* pair-wise comparisons using Tukey's HSD tests revealed that both 1.3 m and 5 m stem heights at the Medium AWHC had lowest concentration of constitutive soluble sugars compared to Low and High AWHC sites (Fig. 2.1C, D; Suppl. Table 2.6). I did not find any difference between Low and High AWHC sites. In addition, concentration of total NSC was highest in trees at the Low AWHC sites both at 1.3 m and 5 m stem heights compared to Medium AWHC sites. Trees on High AWHC sites showed no difference at either stem height. Although, the 5 m stem height had higher concentrations of constitutive soluble sugars and total NSC than 1.3 m stem height on all sites (Fig. 2.1C, D; Suppl. Table 2.7), the differences between the two heights varied among sites (Fig. 2.1E, Suppl. Table 2.8). The concentration of starch did not vary. *Post hoc* Dunn's pair-wise multiple comparisons tests revealed that trees on the Low AWHC sites had highest difference in concentrations of both soluble sugars and total NSC at 5 m stem height than those on the sites

with Medium AWHC. However, there were no differences between Low and High or Medium and High AWHC sites.

2.4.3 Constitutive monoterpenes

One-way PERMANOVAs indicated significant differences in concentrations of constitutive monoterpenes among sites at 1.3 m but not at 5 m stem heights (Suppl. Table 2.9). CAP analysis visually displayed the clustering of sites. The analysis showed that constitutive monoterpenes mostly associated with High AWHC sites at 1.3 m stem height (Fig. 2.2). Univariate analysis showed that concentrations of constitutive α-pinene, 3-carene, and total monoterpenes differed among sites at 1.3 m stem height (Suppl. Table 2.10). *Post-hoc* comparisons using Tukey's HSD tests confirmed that concentration of total monoterpenes was lowest in trees sampled at Medium AWHC sites and highest at Low and High AWHC sites (Fig. 2.3). I found no difference between Low and High AWHC sites. The High AWHC sites had highest concentration of α-pinene and 3-carene, and the Medium AWHC sites had lowest concentrations of these two monoterpenes. I found no difference in concentrations of α-pinene or 3-carene between Low and High AWHC sites. Overall, I did not find differences in concentrations of constitutive monoterpenes between the two stem heights ($F_{1,178}$=0.86, P=0.46).

2.4.4 Constitutive diterpene resin acids

One-way PERMANOVAs indicated that AWHC did not influence concentrations of constitutive diterpene resin acids at 1.3 m or 5 m stem heights (Suppl. Table 2.11). However, I found significant differences between the two stem heights ($F_{1,178}$=10.35, P=0.001). Univariate analysis confirmed that concentrations of constitutive palustric acid, abietic acid, neoabietic acid,

levopimaric acid, and total diterpenes were higher at 1.3 m compared to 5 m stem height (Fig. 2.4; Suppl. Table 2.12). The concentration of dehydroabietic acid did not vary.

2.4.5 Induced non-structural carbohydrates

At 1.3 m stem height, proportional differences in concentrations of induced NSC varied with treatment and AWHC (Suppl. Table 2.13). At 5 m stem height, I only detected a moderate effect of AWHC but not the treatment effect. I did not detect any interactions between these two main effects at any stem height. Univariate analysis indicated significant differences in the proportional variation of sugar concentrations between treated and control trees at 1.3 m on the High AWHC sites but not at Medium AWHC or Low AWHC sites. I detected no differences in sugar concentrations at 5 m for any of the sites. The proportional variation in concentration of starch did not vary between treated and control trees at any site category or stem height. Total NSC too only varied at 1.3 m between treated and control trees at the High AWHC sites, but not on any other site category (Fig. 2.5; Suppl. Table 2.14). I did not detect any differences in total NSC concentrations at 5 m for any site category.

The proportional differences in concentrations of NSC varied between 1.3 m and 5 m stem heights, and with the main effect AWHC (Suppl. Table 2.15). I did not detect any treatment effect on differences or interaction among the main effects. Univariate analysis indicated significant differences in proportional concentrations of total NSC and sugars for both control and treated trees between 1.3 m and 5 m stem heights on all sites (*t*-test, $P<0.05$). The concentration of starch did not vary between stem heights (*t*-test, $P>0.05$) or control and treated groups on any site category (*t*-test, $P>0.05$).

2.4.6 Induced monoterpenes

Two-way PERMANOVA indicated that proportional differences in concentrations of induced and constitutive monoterpenes significantly correlated with AWHC but not with fungal treatment at 1.3 m (Suppl. Table 2.16). I did not find a significant interaction between the two main effects. CAP analysis indicated a clear separation between groups of trees on the different site categories. Results showed that trees on the High AWHC sites highly associated with proportional variations in concentrations of induced monoterpenes (Fig. 2.6). Univariate analysis at 1.3 m showed that proportional differences in concentrations of induced monoterpenes varied with AWHC including total monoterpenes, α-pinene, β-pinene and myrcene (Suppl. Table 2.17). I found no difference for limonene, and myrcene and 3-carene showed marginal differences. None of the monoterpenes varied among control groups (P>0.05). *Post-hoc* comparisons using Tukey's HSD tests revealed that proportional variations in concentrations of total monoterpenes and α-pinene were highest in trees sampled at the High AWHC sites than those at Low and Medium AWHC sites (Fig. 2.7; Suppl. Table 2.17). The latter two site categories showed no difference. For β-pinene, the High AWHC sites had highest proportional variation compared to Medium AWHC, however, the Low AWHC sites showed no difference.

At 5 m, I neither observed any significant correlation of proportional variation in concentrations of induced monoterpenes with AWHC nor with fungal treatment or an interaction between these two main effects (Suppl. Table 2.16). The proportional differences in concentrations of induced and constitutive monoterpenes however, differed between 1.3 m and 5 m stem heights and correlated with AWHC (Suppl. Table 2.18). Interaction between the two main effects, heights and AWHC was also significant. For treated trees, univariate analysis of

proportional differences in concentrations of induced monoterpenes between 1.3 m and 5 m stem heights revealed significant differences for β-pinene at Low AWHC sites, and limonene and myrcene at Medium AWHC sites. At the High AWHC sites, I detected significant differences for all monoterpenes including total, α-pinene, β-pinene, limonene, myrcene and 3-carene (Suppl. Table 2.19). Among control groups, 3-carene at Low AWHC, limonene at Medium AWHC, and total monoterpenes, α-pinene and 3-carene at High AWHC sites differed between the two stem heights (Suppl. Table 2.20).

2.4.7 Induced diterpene resin acids

The proportional differences in concentrations of induced and constitutive diterpene resin acids did not vary with fungal treatment, AWHC or with their interaction either at 1.3 m or 5 m stem heights (Suppl. Table 2.21). However, they differed between 1.3 m and 5 m stem heights (Suppl. Table 2.22). Univariate analysis showed significantly higher proportional differences in concentrations of dehydroabietic acid at 1.3 m than at 5 m stem height, and palustric acid at 5 m than at 1.3 m stem height (Suppl. Table 2.23). None of the other diterpene resin acids showed significant differences.

2.4.8 Interaction between tree chemistry and lesion areas

Available water holding capacity significantly correlated with lesion areas (LMER: $\chi^2_{(2)}$=7.38; *P*=0.02). *Post-hoc* Tukey's HSD test indicated that trees on the Low AWHC sites had significantly larger lesions than trees on the High AWHC sites (Fig. 2.8, Suppl. Table 2.24). Total constitutive monoterpenes (LMER: $\chi^2_{(1)}$=0.39; *P*=0.52), total constitutive diterpene resin acids (LMER: $\chi^2_{(1)}$=2.05; *P*=0.15), total constitutive NSC (LMER: $\chi^2_{(1)}$=0.17; *P*=0.67), phloem

nitrogen (LMER: $\chi^2_{(1)}$=1.90; *P*=0.15), fungal inoculation density (LMER: $\chi^2_{(1)}$=0.30; *P*=0.57), and DBH (LMER: $\chi^2_{(1)}$=0.82; *P*=0.36) did not correlate with fungal lesion areas.

In contrast, concentrations of induced monoterpenes in lesion samples significantly correlated with soil AWHC (Suppl. Table 2.25). Inoculation density (4 vs 16) did not influence monoterpene concentrations. CAP analysis visually displayed clustering of the groups and showed that induced monoterpenes in lesions mostly associated with High AWHC sites (Fig. 2.9A). One-way ANOVAs indicated that concentrations of total monoterpenes, α-pinene, limonene and myrcene differed among lesions sampled from trees on different sites (Suppl. Table 2.26). *Post-hoc* comparisons using Tukey's HSD tests revealed lesions sampled from trees on the High AWHC site had highest concentrations of total monoterpenes, α-pinene, limonene and myrcene compared to Low AWHC and Medium AWHC sites (Fig. 2.9B; Suppl. Table 2.26). I found no differences for these monoterpenes among trees on the Low AWHC and Medium AWHC site. Moreover, I did not find differences in concentrations of β-pinene and 3-carene among sites.

2.5 Discussion

Through this study, I demonstrated that AWHC can influence carbon-defense dynamics along jack pine stem and hence, the effects of a fungal pathogen infection on pine metabolites. I provided several lines of evidence to support the importance of AWHC for carbon allocation to defense compounds and validated our results by comparing direct vulnerability in jack pine by linking fungal lesions to both AWHC and defense compounds.

2.5.1 Role of AWHC in NSC-defense interactions before fungal infection

I found variation in NSC concentrations along jack pine stem, with greater concentrations occurring at the upper portions, supporting an earlier study (Goodsman et al. 2013). Monoterpene concentrations did not vary along jack pine stem, whereas diterpene resin acids did. Trees in the Low AWHC sites had the highest NSC levels at 5 m, relative to trees in the other AWHC categories. Monoterpenes also showed variation among sites only at 1.3 m height, having positive correlation with AWHC of soils. In contrast, AWHC of soils had no effect on diterpene resin acid concentrations.

Our results generally support the expectation of variations in NSC and defense compounds along the gradients of AWHC and stem height. As maximal tree radial growth in Alberta occurs in summer, NSC gradients likely originate from carbon budgeting involving competing growth sinks along tree stems (Minchin and Lacointe 2005; Woodruff and Meinzer 2011). Abiotic factors like soil AWHC can influence NSC mobility in the phloem (e.g., embolism by callose deposition) that could potentially alter source-sink relationships. This may potentially weaken the phloem's long-distance transportation ability, causing bottleneck effects at the source (Morgan 1984; Lemoine et al. 2013; Hartmann 2015). Furthermore, the impact of water availability on terpene production is well documented (Grote et al. 2009; Lusebrink et al. 2011 2016; Arango-velez et al. 2016). As proposed by the source-sink framework, high NSC availability and strong growth sinks near the source likely amplify soluble sugar mobilization which may positively influence monoterpene synthesis, thus masking any potential constraints imposed by soil AWHC (Goodsman et al. 2013). This is consistent with findings by Honkanen et al. (1999) who reported positive correlations between growth rate and monoterpene concentrations in *Pinus sylvestris*. However, soil AWHC did not influence diterpene resin acid concentrations even though they varied along jack pine stem. These results suggest that the

interaction between NSC and defense is more complex than I initially realized, and stresses the importance of considering the influence of site conditions on source-sink dynamics in pines.

2.5.2 Role of AWHC in NSC-defense interactions after fungal infection

Fungal inoculations altered the interactions observed at the constitutive level and produced new patterns of interactions between NSC and defense chemicals along jack pine stem. NSC concentrations increased at 1.3 m height of trees on the High AWHC sites, whereas no differences were found at 5 m height. The High AWHC sites also had highest proportional monoterpene concentrations at 1.3 m, whereas proportional diterpene resin acid concentrations did not correlate with soil AWHC after fungal infection.

Even though I successfully induced chemical defenses in jack pine (i.e., evident from the increased proportional concentrations of terpenes), there was no difference between different densities of fungal inoculations. This result may potentially highlight the novelty of the host–pathogen relationship as they lack any prior co-evolutionary encounters, thus any patterns observed on co-evolved hosts (e.g., lodgepole pine) may not be precisely applicable in jack pine (Tsui et al. 2012; Cale et al. 2019; Erbilgin 2019). These findings further suggest that jack pine lacks the ability to distinguish between low and high intensity pressures from at least certain novel biotic agents and allocates resources and defenses equally, supporting earlier studies (Cale et al. 2017, 2019; Klutsch and Erbilgin 2018).

I found evidence to support that AWHC of soils can influence carbon allocation in jack pine which may modulate induced defense response to a fungal infection (Klutsch et al. 2017b). Particularly, trees on the High AWHC sites responded to the infection by allocating NSC to sinks at 1.3 m. However, no differences emerged at 5 m, suggesting that jack pine trees mobilized

local carbon resources for defense at sinks depending on the AWHC of soils (Woodruff and Meinzer 2011; Lemoine et al. 2013). These results are consistent with findings in other pine species (Guérard et al. 2007; Goodsman et al. 2013). Because of carbon allocation, the High AWHC sites also had highest proportional monoterpene concentrations at 1.3 m. Although proportional total diterpene resin acid concentrations did not vary with the AWHC of soils, dehydroabietic acid was proportionally higher at 1.3 m, however, I suspect that abietadienoic acids in the wound underwent oxidation reactions as they encountered the air, resulting in the production of dehydroabietic acid (Gref and Ericsson 1985).

2.5.3 Cascading effects of AWHC on fungal infection through altered NSC-defense interactions

Trees on the Low AWHC sites had larger lesion areas than those on the High AWHC sites. Typically, larger lesions indicate higher susceptibility of trees to fungal infection (Bonello et al. 2006; Krokene et al. 2008; Goodsman et al. 2013; Klutsch et al. 2017b). These results could be explained by the changes in NSC concentrations and defense chemicals, and their interactions, in response to the infection as I found proportionally higher concentrations of soluble sugars and total NSC at 1.3 m in inoculated trees compared to control trees, and higher NSC was associated with higher induced monoterpene concentrations in lesions in trees on the High AWHC sites. No such differences were observed on any other sites. Overall, these results reflect ability of trees to endure bark beetle and pathogen attacks by allocating NSC reserves available to support chemical defenses (Långström et al.1992; Goodsman et al. 2013; Klutsch et al. 2017a; Roth et al. 2018; Cale et al. 2019). However, soil conditions limiting resource acquisition may alter or delay such allocation, resulting in slower defense response and higher tree susceptibility. Finding negative correlation between lesion size and soil AWHC supports this suggestion. Likewise, I

observed a bottleneck effect (i.e., reduced ability to mobilize resources to the point of infection) in the NSC concentrations in trees at 5 m at the Low AWHC sites, which also demonstrate that AWHC of soils can influence the ability of trees to respond to the infection. However, since I did not quantify other defense compounds such as phenolic glycosides or diterpene resin acids in the lesions, I are very cautious to propose that longer lesions in trees on the Low AWHC sites resulted from the reduced ability of trees to mobilize resources, and hence lower amounts of defense monoterpenes were produced at the sinks.

In conclusion, like all conifers, jack pine produces carbon-based mixtures of terpenes dedicated to serve as physical and chemical defenses against insects and pathogens (Keeling and Bohlmann 2006; Boone et al. 2011; Raffa et al. 2017; Erbilgin 2019). Thus, efficient NSC mobilization to sinks is critical for synthesizing defense compounds against bark beetles (Gaylord et al. 2013; Goodsman et al. 2013; Wiley et al. 2016; Raffa et al. 2017; Roth et al. 2018) and seems to be an adaptive response in pines (Goodsman et al. 2013; Klutsch et al. 2017a, b). However, water availability can influence the flow of these defenses in trees by hindering phloem and xylem transport and metabolic functions (Morgan 1984; Woodruff and Meinzer 2011; Lemoine et al. 2013; Hartmann 2015; Arango-velez et al. 2016; Lusebrink et al. 2016). I showed that the concentrations of monoterpenes were evenly distributed along the stems of jack pine trees, but with the creation of carbon sinks by fungal infection, their ability to mobilize local resources to replenish the declining resources at the sinks varied depending on the AWHC of soils. Trees on the High AWHC sites appear to be more effective in deploying their resources and consequently, their chemical defenses were more pronounced. In contrast, trees on the Low AWHC sites do not seem to respond to the infection similarly, likely due to limited NSC reserves, resulting in greater fungal infection. Based on these results I hypothesize that a

tree's ability to mobilize NSC to meet the needs of the local sinks is negatively correlated with soil moisture. This hypothesis provides a mechanistic explanation why drought stressed trees are more likely to be killed by bark beetles (Gaylord et al. 2013; Lahr and Krokene 2013; Wiley et al. 2016; Klutsch et al. 2017b; McDowell et al. 2018).

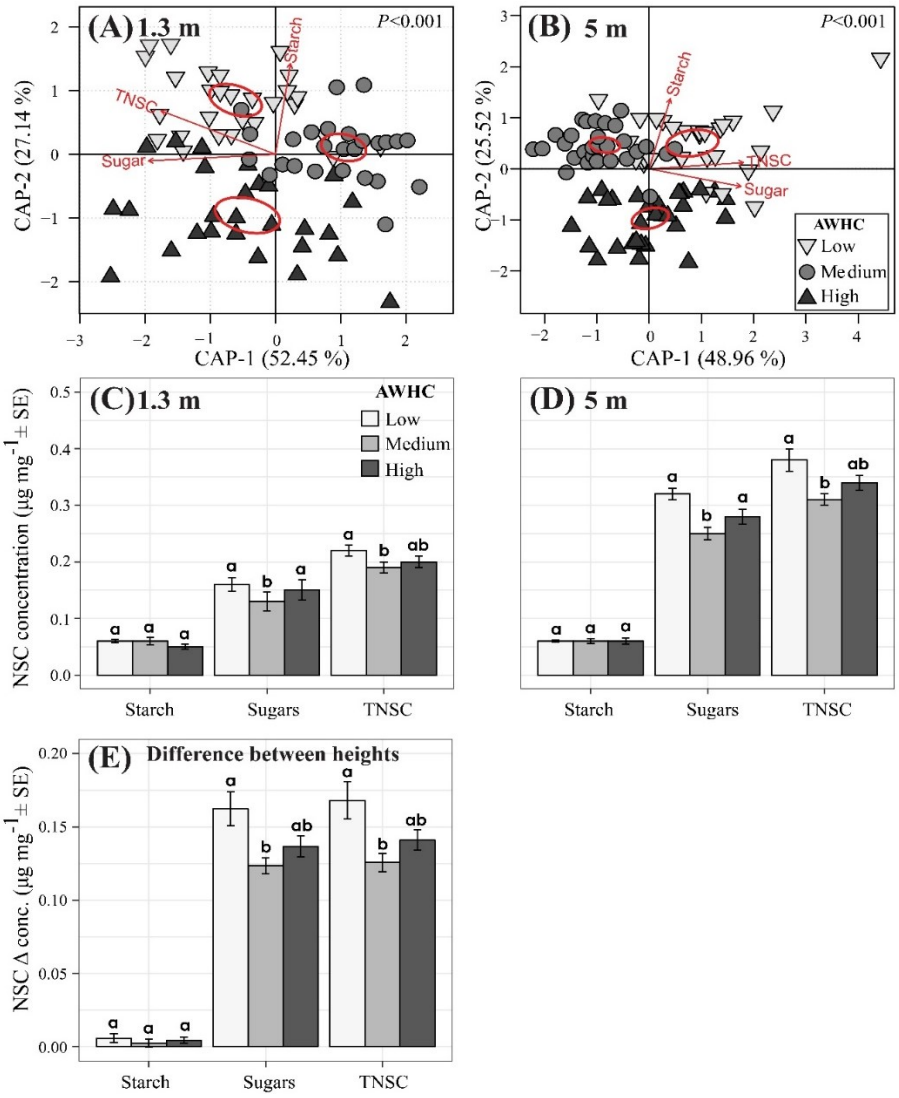


Fig. 2.1 Constitutive non-structural carbohydrate (NSC) profile of the experimental *Pinus banksiana* trees on selected sites categorized as Low, Medium or High AWHC (available water holding capacities). Canonical analysis of principal coordinates of constitutive NSC concentrations at (A) 1.3 m and (B) 5 m shows the separation of sites based on the differences in their AWHC (Suppl. Table 2.5). *P*-values indicate the significant effect of AWHC on sample separation. Ellipses represent the 95% confidence intervals around group centroids using standard errors. Arrows indicate the contribution of individual variables to the CAP axes. Concentrations of constitutive NSC compared at (C) 1.3 m, and (D) 5 m heights. (E) Differences in the concentration of constitutive starch, sugars and Total NSC (TNSC) at 5 m height after subtracting the concentrations at 1.3 m height. Different letters indicate significant differences at α=0.05 in one-way ANOVAs.

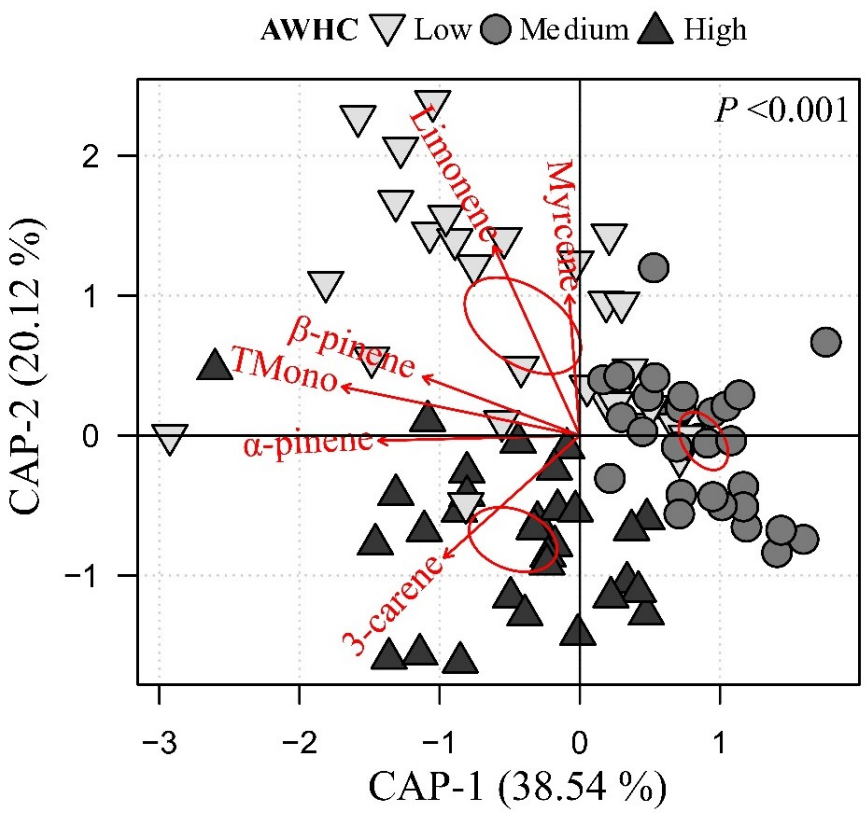


Fig. 2.2 Canonical analysis of principal coordinates of the concentrations of the constitutive monoterpenes in *Pinus banksiana* phloem tissues sampled at 1.3 m height based on the differences in the available water holding capacities (AWHC) of soils: Low, Medium and High sites (Suppl. Table 2.9). *P*-value indicates the significant effect of AWHC on sample separation. Ellipses represent the 95% confidence intervals around group centroids using standard errors. Arrows indicate the contribution of individual variables to the CAP axes. TMono = Total monoterpenes.

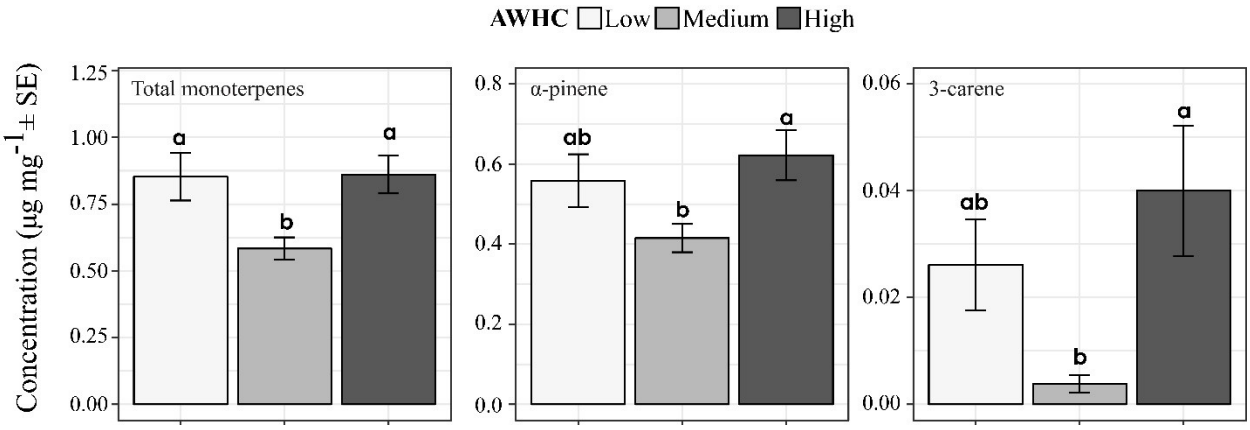


Fig. 2.3 Concentrations of constitutive monoterpenes at 1.3 m height in *Pinus banksiana* trees on selected sites categorized as Low, Medium or High AWHC (available water holding capacities). Different letters indicate significant differences among means at α=0.05 in one-way ANOVA.

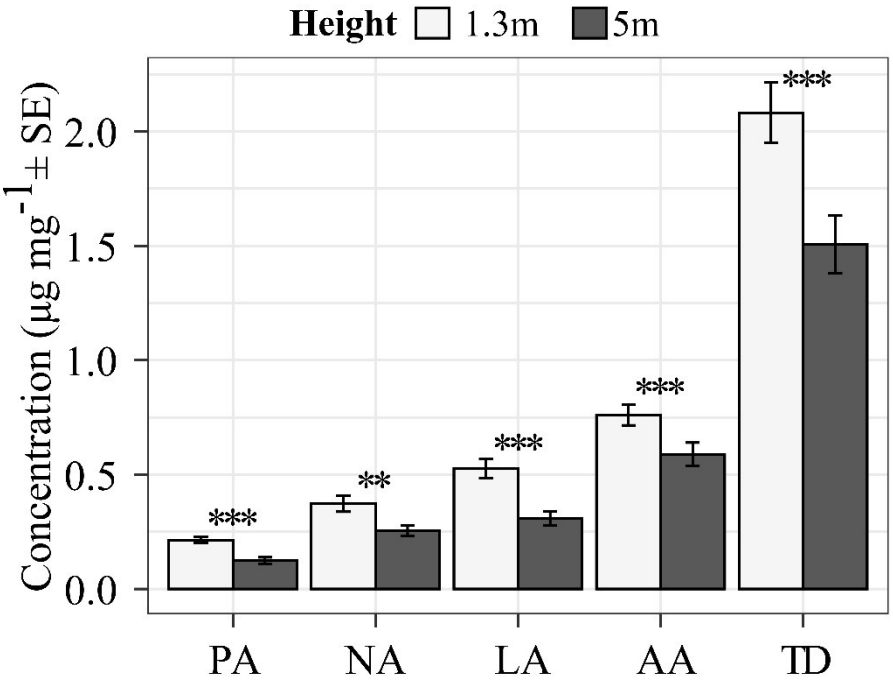


Fig. 2.4 Concentration of constitutive diterpene resin acids compared between 1.3 m and 5 m heights along the stems of *Pinus banksiana* trees sampled on sites in Lac la Biche, Alberta. Sites were categorized as Low, Medium and High AWHC (available water holding capacities). '**' indicates P = 0.01–0.001; '***' $P < 0.001$. PA=Palustric acid, NA=Neoabietic acid, LA=Levopimaric acid, AA=Abietic acid and TD=Total diterpenes.

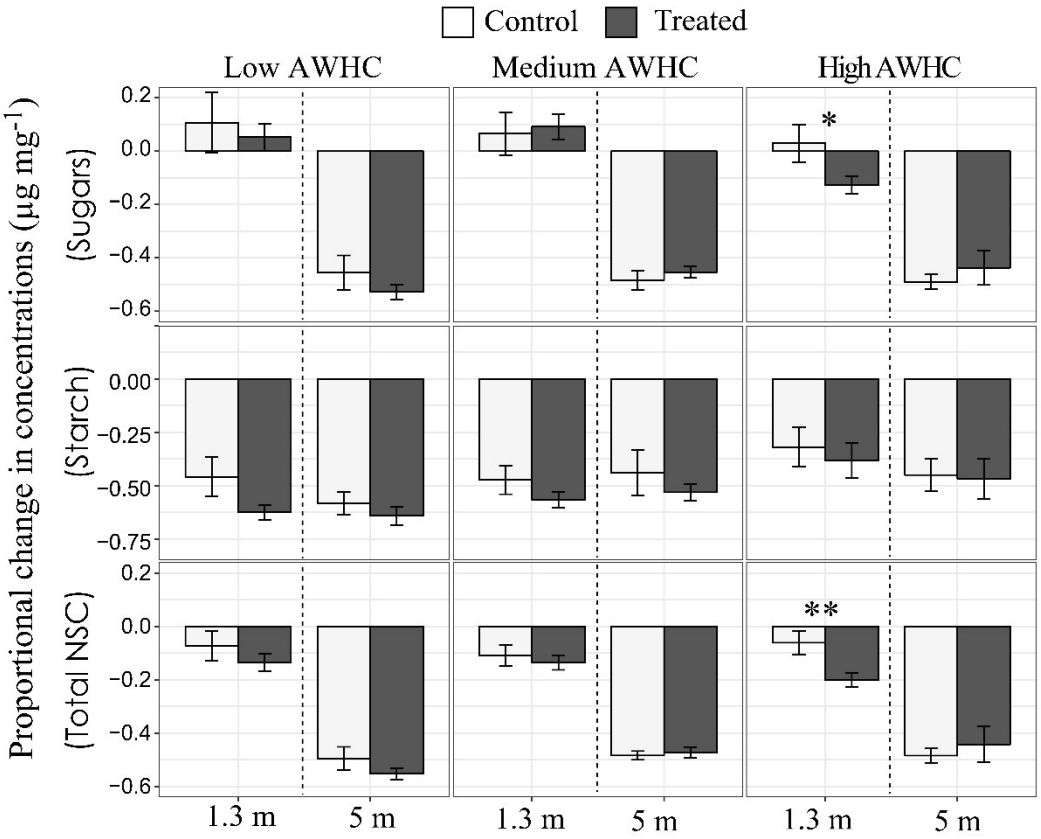


Fig. 2.5 Comparison of proportional differences in the concentrations of non-structural carbohydrates (NSC) at 1.3 m and 5 m heights between control and treated *Pinus banksiana* trees sampled at sites categorized as Low, Medium and High AWHC (available water holding capacities). Treated trees were inoculated with live mycelia (252,000 cells ml^{-1}) of *Grosmannia clavigera* at 1.3 m height and control trees were not inoculated. '*' indicates P = 0.05–0.03; '**' $P < 0.03$. Supporting information is reported in (Suppl. Tables 2.13 and 2.14).

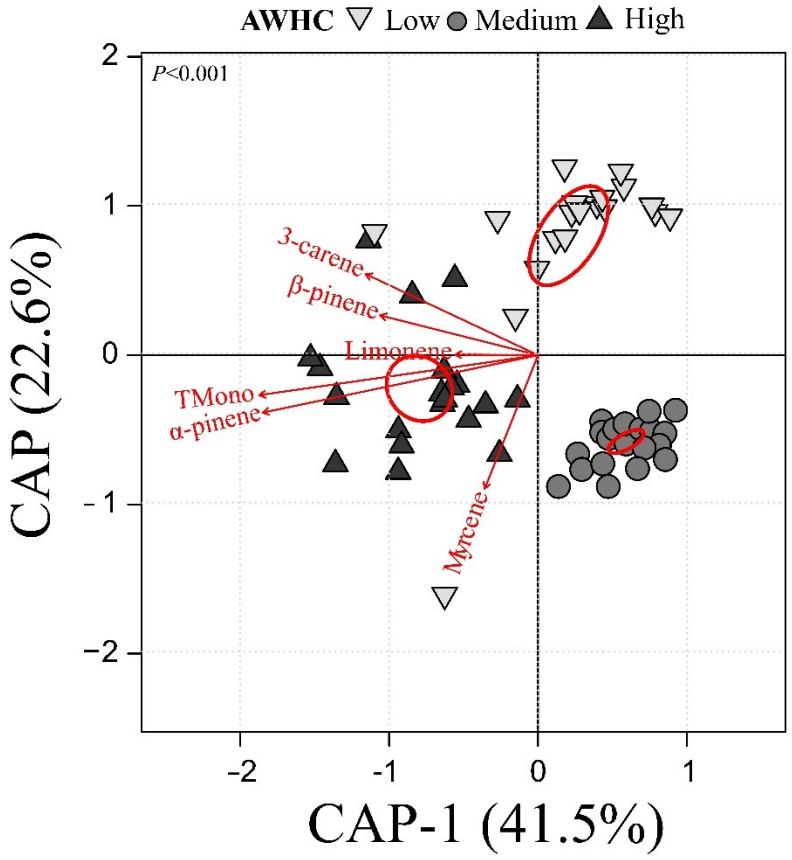


Fig. 2.6 Canonical analysis of principal coordinates of the proportional differences in the concentrations of constitutive and induced monoterpene concentrations in *Pinus banksiana* phloem tissues. The induction was caused by inoculation with live mycelia (252,000 cells ml^{-1}) of *Grosmannia clavigera* in the treated trees or by mechanical wounding in the control trees at 1.3 m height. The separation is based on the differences in the available water holding capacities (AWHC) of sites: Low, Medium and High (Suppl. Table 2.16). *P*-value indicates the significant effect of AWHC on sample separation. Ellipses represent the 95% confidence intervals around group centroids using standard errors. Arrows indicate the contribution of individual variables to the CAP axes TMono = Total monoterpenes.

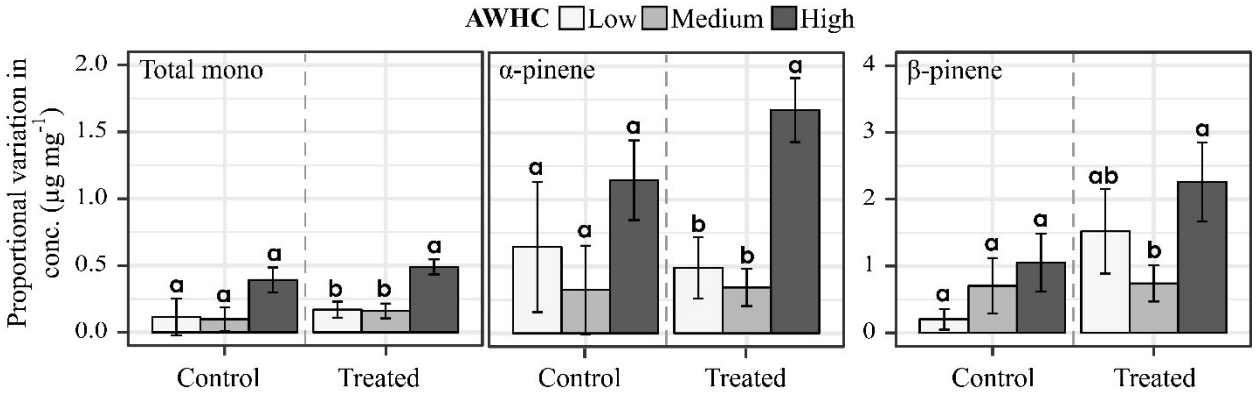


Fig. 2.7 Proportional differences in the concentrations of induced and constitutive monoterpenes in the phloem tissues of *Pinus banksiana* compared between control and treated trees. Trees were sampled 1.3 m aboveground, and treated trees were inoculated with live *Grosmannia clavigera* mycelia and control trees were left uninoculated (252,000 cells ml^{-1}). Sites were categorized as Low, Medium and High AWHC (available water holding capacities). Different letters indicate significant differences between means at α=0.05 in one-way ANOVA. Total mono= Total monoterpenes.

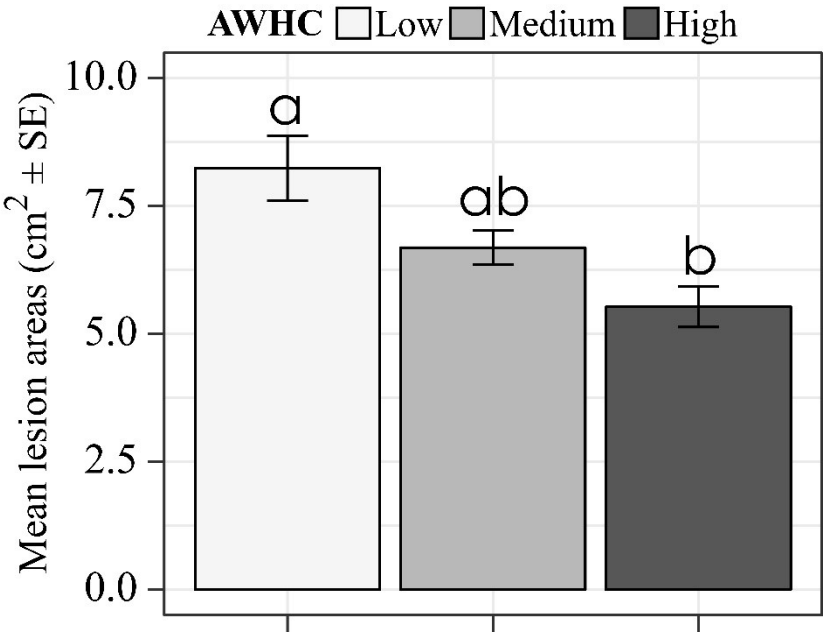


Fig. 2.8 Mean lesion areas compared among *Pinus banksiana* trees on sites that were characterized as Low, Medium and High AWHC (available water holding capacities). Trees were inoculated with live mycelia (252,000 cells ml^{-1}) of *Grosmannia clavigera* 1.3 m aboveground and the lesion areas were measured six weeks later (Suppl. Table 2.24). Different letters indicate significant differences between means at α=0.05 in a one-way ANOVA.

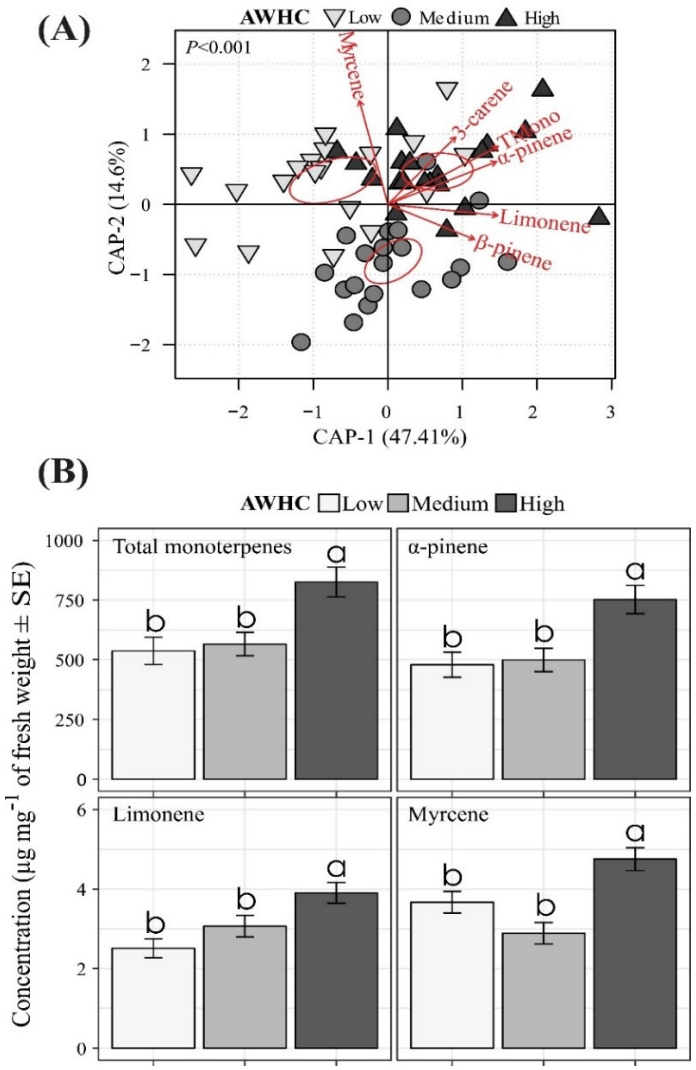


Fig. 2.9 Concentrations of induced monoterpenes vary in the necrotic lesions caused by *Grosmannia clavigera* inoculation (252,000 cells ml^{-1}) in *Pinus banksiana* with soil characteristics. (A) Canonical analysis of principal coordinates (CAP) of induced monoterpene concentrations at 1.3 m height. Sites were categorized as Low, Medium and High AWHC (available water holding capacities). *P*-value indicates the significant AWHC effect on sample separation. Ellipses represent the 95% confidence intervals around group centroids using standard errors. Arrows indicate the contribution of individual variables to the CAP axes. TMono = Total induced monoterpenes. (B) Concentrations of induced monoterpenes in the lesion samples of *P. banksiana*. Separate one-way ANOVAs were performed to compare sites for differences followed by pairwise *post-hoc* Tukey's HSD tests (Suppl. Table 2.26). Different letters indicate significant differences between means at α=0.05 in one-way ANOVAs.

Suppl. Table 2.1 One-way ANOVA table showing the differences in available water holding capacity (AWHC) of soil samples at sites that were categorized as Low, Medium and High sites.

Pair-wise comparison	Mean difference	SE	df	*P*	95% Confidence Interval	
					Lower bound	Upper bound
Low AWHC-Medium AWHC	0.05	0.007	21	<0.001	0.03	0.07
Low AWHC-High AWHC	0.12	0.007	21	<0.001	0.10	0.13
Medium AWHC-High AWHC	0.06	0.007	21	<0.001	0.04	0.08

Suppl. Table 2.2 Mean concentrations (mg kg^{-1}) of available soil nutrients at sites in Lac la Biche, where *Pinus banksiana* trees were sampled. Sites were categorized as Low, Medium or High AWHC (available water holding capacity).

	Mean(±SE) concentration (mg kg^{-1})			ANOVA		
Nutrients	Low AWHC	Medium AWHC	High AWHC	*F*	df	*P*
Sulfate	0.28(±0.02) b	0.62(±0.10) a	0.45(±0.08) a	10.96	2	<0.001
Phosphate	1.42(±0.31) a	0.55(±0.05) b	0.55(±0.06) b	5.38	2	0.012
Total available nitrogen	3.41(±0.19) a	2.58(±0.23) b	3.28(±0.20) ab	4.73	2	0.021
Total available nutrients	5.42(±0.38) a	4.00(±0.24) b	4.59(±0.18) ab	6.35	2	0.006
Ammonium	1.89(±0.20)	1.26(±0.20)	1.68(±0.14)	3.03	2	0.061
Chloride	0.31(±0.05)	0.24(±0.04)	0.30(±0.08)	0.87	2	0.43
Soluble nitrate	0.24(±0.06)	0.13(±0.04)	0.20(±0.04)	1.52	2	0.242
Extractable nitrate	1.27(±0.17)	1.20(±0.10)	1.36(±0.07)	0.42	2	0.652
Total nitrates	1.52(±0.18)	1.33(±0.10)	1.56(±0.09)	0.89	2	0.421

Suppl. Table 2.3 Mean concentrations (g kg^{-1}) of total soil nutrients at sites in Lac la Biche, where *Pinus banksiana* trees were sampled. Sites were categorized as Low, Medium or High AWHC (available water holding capacity).

	Mean(±SE) g kg^{-1}			ANOVA		
Nutrient	Low AWHC	Medium AWHC	High AWHC	*F*	df	*P*
Sulfur	0.04(±0.00) ab	0.03(±0.00) b	0.05(±0.00) a	5.36	2	0.013
Calcium	0.28(±0.02) a	0.18(±0.02) b	0.35(±0.05) a	9.48	2	0.001
Zinc	0.02(±0.00) b	0.02(±0.00) b	0.03(±0.00) a	8.42	2	0.002
Iron	3.58(±0.19) b	4.22(±0.86) ab	5.33(±0.53) a	4.24	2	0.028
Sodium	0.02(±0.00) ab	0.02(±0.00) a	0.01(±0.00) b	6.93	2	0.004
Total nutrients	5.09(±0.20) b	5.58(±1.03) b	7.22(±0.63) a	4.96	2	0.017
Nitrogen	0.32(±0.02)	0.27(±0.02)	0.31(±0.02)	2.37	2	0.117
Phosphorus	0.32(±0.02)	0.27(±0.03)	0.35(±0.03)	2.53	2	0.103
Potassium	0.11(±0.01)	0.12(±0.01)	0.13(±0.02)	0.11	2	0.895
Magnesium	0.25(±0.02)	0.27(±0.04)	0.28(±0.02)	0.42	2	0.660
Manganese	0.11(±0.02)	0.17(±0.09)	0.19(±0.04)	3.80	2	0.052

Suppl. Table 2.4 *Post-hoc* Tukey's HSD test showing pair-wise comparison of phloem nitrogen concentration among trees on the experimental sites. Sites were categorized as Low, Medium or High AWHC (available water holding capacity).

Groups compared	Estimate	SE	*Z*	df	*P*
Low AWHC–Medium AWHC	0.01	0.010	1.64	91	0.101
Low AWHC–High AWHC	0.04	0.010	3.77	91	<0.001
Medium AWHC–High AWHC	0.02	0.010	2.16	91	0.061

Suppl. Table 2.5 Results of the one-way PERMANOVAs of constitutive non-structural carbohydrates compared in *Pinus banksiana* phloem tissues sampled at 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the number of residuals of each *F* test.

Sampling height	Factor	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
1.3 m	$AWHC_{89}$	6.91	0.13	2	<0.001	0.118
5 m	$AWHC_{89}$	8.45	0.16	2	<0.001	<0.001

Suppl. Table 2.6 One-way ANOVA table of constitutive non-structural carbohydrates (NSC) compared in *Pinus banksiana* phloem tissues sampled at 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). ANOVA results show comparisons among sites, accompanied by means and standard errors. *P*-values indicate significant differences at α=0.05 as indicated by *post-hoc* Tukey's HSD tests or Dunn's multiple comparisons tests with Benjamini-Hochberg method.

Sampling height	NSC	Mean(±SE) concentration (μgmg^{-1} of dry weight)			ANOVA		
		Low AWHC	Medium AWHC	High AWHC	*F* †	df	*P*
1.3 m	Soluble sugars	0.16 (±0.00) a	0.13 (±0.00) b	0.15 (±0.01) a	10.29	2	<0.001
	Starch	0.06 (±0.00)	0.06 (±0.00)	0.05 (±0.00)	3.80 †	2	0.149
	Total NSC	0.22 (±0.01) a	0.19 (±0.00) b	0.20 (±0.01) a	7.06	2	0.001
5 m	Soluble sugars	0.32 (±0.01) a	0.25 (±0.01) b	0.28 (±0.01) a	10.21	2	<0.001
	Starch	0.06 (±0.00)	0.06 (±0.00)	0.06 (±0.00)	2.76 †	2	0.251
	Total NSC	0.38 (±0.01) a	0.31 (±0.01) b	0.34 (±0.01) ab	10.43	2	<0.001

†Kruskal-Wallis rank sum test was conducted when the assumptions of the analysis were not met. The numbers indicate Kruskal-Wallis χ^2 values.

Suppl. Table 2.7 Results of Wilcoxon rank sum tests (with continuity correction) comparing the concentrations of constitutive non-structural carbohydrates (NSC) in *Pinus banksiana* phloem tissues between at 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). *P*-values indicate significant differences at α=0.05.

		Mean (±SE) concentrations (μg mg^{-1})			
Variable	Sites compared	1.3 m	5 m	*W*	*P*
Soluble sugars	Low AWHC	0.16 (±0.00) b	0.32 (±0.01) a	890.5	<0.001
	Medium AWHC	0.13 (±0.00) b	0.25 (±0.01) a	898.5	<0.001
	High AWHC	0.15 (±0.01) b	0.28 (±0.01) a	893.5	<0.001
Starch	Low AWHC	0.06 (±0.00)	0.06 (±0.00)	519.1	0.301
	Medium AWHC	0.06 (±0.00)	0.06 (±0.00)	498.5	0.465
	High AWHC	0.05 (±0.00)	0.06 (±0.00)	505.5	0.411
Total NSC	Low AWHC	0.22 (±0.01) b	0.38 (±0.01) a	896.5	<0.001
	Medium AWHC	0.19 (±0.00) b	0.31 (±0.01) a	897.5	<0.001
	High AWHC	0.20 (±0.01) b	0.34 (±0.01) a	888.5	<0.001

Suppl. Table 2.8 Kruskal–Wallis analysis of variance followed by Dunn's pair-wise multiple comparison tests with Benjamini-Hochberg method of the differences in the concentrations of constitutive non-structural carbohydrates (NSC) in the phloem tissues of *Pinus banksiana* sampled at 1.3 m and 5 m heights on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). *P*-values indicate significant differences at $\alpha=0.05$.

Variable	Comparison	Kruskal-Wallis test		Dunn's test	
		χ^2	*P*	*Z*	*P*
Sol. sugars	Low AWHC vs Medium AWHC	15.83	0.021	2.75	0.012
	Low AWHC vs High AWHC			1.19	0.231
	Medium AWHC vs High AWHC			1.55	0.183
Total NSC	Low AWHC vs Medium AWHC	16.52	0.023	2.83	0.011
	Low AWHC vs High AWHC			0.85	0.394
	Medium AWHC vs High AWHC			1.97	0.071
Starch	Low AWHC vs Medium AWHC	2.25	0.521	0.50	0.903
	High AWHC vs Low AWHC			0.04	0.963
	Medium AWHC vs High AWHC			0.49	0.922

Suppl. Table 2.9 Results of the one-way PERMANOVAs of constitutive monoterpenes at 1.3 m and 5 m heights of *Pinus banksiana* trees on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the numbers of residuals of each *F* test.

Sampling height	Variable	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
1.3 m	$AWHC_{89}$	3.21	0.06	2	0.006	<0.001
5 m	$AWHC_{89}$	1.15	0.02	2	0.335	0.035

Suppl. Table 2.10 Constitutive monoterpene concentrations of trees compared among sites at 1.3 m height aboveground. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). *P*-values indicate significant differences at α=0.05.

Compounds	Mean(±SE) Concentration (μgmg^{-1} of fresh weight)			ANOVA		
	Low AWHC	Medium AWHC	High AWHC	*F*	df	*P*
α-pinene	0.56 (±0.07) ab	0.42 (±0.04) b	0.62 (±0.06) a	3.77	2	0.026
3-carene	0.03 (±0.01) ab	0.00 (±0.00) b	0.04 (±0.01) a	4.43	2	0.014
Limonene	0.12 (±0.02)	0.06 (±0.01)	0.07 (±0.02)	0.70	2	0.497
Myrcene	0.04 (±0.01)	0.05 (±0.01)	0.03 (±0.01)	1.17	2	0.314
β-pinene	0.06 (±0.01)	0.04 (±0.01)	0.06 (±0.01)	1.45	2	0.239
Total mono	0.85 (±0.09) a	0.58 (±0.04) b	0.86 (±0.07) a	4.92	2	0.009

Suppl. Table 2.11 Results of the one-way PERMANOVAs of constitutive diterpene resin acids at 1.3 m and 5 m heights of *Pinus banksiana* trees on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the numbers of residuals of each *F* test.

Sampling height	Variable	*F*	R^2	df	*P*	"*PERMDISPER"* (*P*)
1.3 m	$AWHC_{89}$	0.08	0.002	2	0.957	0.158
5 m	$AWHC_{89}$	0.28	0.006	2	0.782	0.409

Suppl. Table 2.12 Results of two sample *t*-tests comparing the concentrations of constitutive diterpene resin acids in *Pinus banksiana* phloem tissues between at 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Results show comparisons between heights, accompanied by means and standard errors. *P*-values indicate significant differences at α=0.05.

Compounds	Mean (±SE) concentrations (µg mg^{-1})		*t*-test		
	1.3 m	5 m	*t*	df	*P*
Palustric acid	0.21 (±0.01) a	0.12 (±0.01) b	6.31	178	<0.001
Abietic acid	0.76 (±0.05) a	0.59 (±0.05) b	3.42	178	<0.001
Neoabietic acid	0.37 (±0.04) a	0.26 (±0.02) b	3.11	178	0.002
Levopimaric acid	0.53 (±0.04) a	0.31 (±0.03) b	5.19	178	<0.001
Dehydroabietic acid	0.24 (±0.01)	0.23 (±0.02)	0.85	178	0.395
Total diterpenes	2.08 (±0.13) a	1.51 (±0.13) b	3.87	178	<0.001

Suppl. Table 2.13 Results of the two-way PERMANOVAs comparing the differences in the concentrations of induced and constitutive non-structural carbohydrates at 1.3 m and 5 m heights aboveground in *Pinus banksiana* trees on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). *P*-values indicate significant differences at α=0.05. Subscript numbers indicate the numbers of residuals of each *F* test.

Height	Variables	F	R^2	df	*P*	"*PERMDISPER*" (*P*)
1.3 m	$Treatment_{84}$	3.13	0.03	1	0.001	0.08
	$AWHC_{84}$	4.55	0.09	2	0.046	0.60
	Treatment x $AWHC_{84}$	0.44	0.01	2	0.581	
5 m	$Treatment_{184}$	0.46	0.05	1	0.651	0.98
	$AWHC_{84}$	2.04	0.04	2	0.069	0.51
	Treatment x $AWHC_{84}$	0.41	0.01	2	0.796	

Suppl. Table 2.14 Comparison of differences in the concentrations of induced and constitutive non-structural carbohydrates (NSC) between control and treated in the phloem tissues of *Pinus banksiana* trees sampled for phloem tissues at 1.3 m and 5 m heights aboveground. Treated trees were inoculated with live mycelia of *Grosmannia clavigera* at 1.3 m height and control trees were left uninoculated. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). TNSC= Total non-structural carbohydrates. *P*-values indicate significant differences at α=0.05.

		Mean (±SE) proportional difference in concentration (μg mg^{-1})									
		1.3 m		*t*-test			5 m		*t*-test		
Site	NSC	Control	Treated	*t*	df	*P*	Control	Treated	*t*	df	*P*
Low AWHC	Sugars	0.10 (±0.11)	0.05(±0.05)	0.50	28	0.617	-0.45 (±0.06)	-0.52 (±0.02)	1.04	28	0.316
	Starch	-0.45 (±0.09)	-0.62 (±0.03)	2.04	28	0.085	-0.58 (±0.05)	-0.64 (±0.04)	0.81	28	0.421
	TNSC	-0.07 (±0.05)	-0.13 (±0.03)	1.03	28	0.310	-0.49 (±0.04)	-0.55 (±0.02)	1.37	28	0.181
Med AWHC	Sugars	0.06 (±0.07)	0.09 (±0.04)	0.30	28	0.765	-0.48 (±0.03)	-0.45 (±0.02)	0.78	28	0.439
	Starch	-0.47 (±0.06)	-0.56 (±0.03)	1.31	28	0.201	-0.43 (±0.10)	-0.53 (±0.03)	0.98	28	0.333
	TNSC	-0.10 (±0.04)	-0.13 (±0.02)	0.54	28	0.591	-0.48 (±0.01)	-0.47 (±0.01)	0.39	28	0.698
High AWHC	Sugars	0.03 (±0.07) b	-0.12 (±0.03) a	2.34	28	0.026	-0.48 (±0.02)	-0.43 (±0.06)	0.55	28	0.583
	Starch	-0.31 (±0.09)	-0.38 (±0.08)	0.41	28	0.633	-0.44 (±0.07)	-0.46 (±0.09)	0.12	28	0.903
	TNSC	-0.06 (±0.04) b	-0.20 (±0.02) a	2.90	28	0.007	-0.48 (±0.02)	-0.44 (±0.06)	0.43	28	0.667

Suppl. Table 2.15 Results of the three-way PERMANOVA comparing proportional variations in the concentrations of induced and constitutive non-structural carbohydrates concentrations between 1.3 m and 5 m heights above ground in *Pinus banksiana* trees on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Treated trees were inoculated with live mycelia of *Grosmannia clavigera* at 1.3 m height and control trees were left uninoculated. *P*-values indicate significant differences at α=0.05. Subscript numbers indicate the number of residuals of each *F* test.

Factors	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
$Treatment_{168}$	1.51	0.01	1	0.219	0.27
$Height_{168}$	157.79	0.46	1	<0.001	0.53
$AWHC_{168}$	4.72	0.02	2	0.003	0.72
Treatment x $Height_{168}$	0.66	0.01	1	0.479	
Treatment x $AWHC_{168}$	0.36	0.01	3	0.786	
AWHC x $Height_{168}$	1.77	0.01	2	0.158	
Treatment x Height x $AWHC_{168}$	0.47	0.01	1	0.697	

Suppl. Table 2.16 Results of the two-way PERMANOVAs comparing proportional differences in the concentrations of induced and constitutive monoterpenes in *Pinus banksiana* phloem tissues sampled 1.3 m and 5 m aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the number of residuals of each *F* test. *P*-values indicate significant differences at α=0.05.

Sampling height	Variables	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
1.3 m	$AWHC_{84}$	10.40	0.18	2	<0.001	0.05
	$Treatment_{84}$	2.38	0.02	1	0.084	0.06
	AWHC x $Treatment_{84}$	1.59	0.02	2	0.171	
5 m	$AWHC_{84}$	0.86	0.01	2	0.538	0.90
	$Treatment_{84}$	1.04	0.01	1	0.398	0.04
	AWHC x $Treatment_{84}$	0.706	0.01	2	0.628	

Suppl. Table 2.17 Comparison of proportional differences in the concentrations of induced and constitutive monoterpenes in the phloem tissues of *Pinus banksiana* compared between control and treated trees. Trees were sampled 1.3 m and 5 m aboveground, and treated trees were inoculated with live *Grosmannia clavigera* mycelia and control trees were left uninoculated. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). Total mono=Total monoterpenes. *P*-values indicate significant differences at α=0.05.

Height	Compounds	Mean(±SE) concentration (μg mg^{-1} of fresh weight)			ANOVA		
		Low AWHC	Medium AWHC	High AWHC	*F*	df	*P*
1.3 m	α-pinene	0.49 (±0.22) b	0.34 (±0.14) b	1.67 (±0.24) a	11.34	2	<0.001
	β-pinene	1.52 (±0.60) ab	0.73 (±0.27) b	2.52 (±0.59) a	5.88	2	0.004
	Limonene	23.90 (±5.77)	20.15 (±5.08)	39.02 (±16.24)	0.86	2	0.428
	Myrcene	0.96 (±0.44)	0.53 (±0.11)	1.92 (±0.43)	3.06	2	0.061
	3-carene	18.75 (±10.72)	0.39 (±0.22)	21.63 (±8.73)	2.57	2	0.088
	Total mono	0.17 (±0.06) b	0.16 (±0.05) b	0.49 (±0.05) a	12.28	2	<0.001
5 m	α-pinene	0.35 (±0.11)	0.25 (±0.07)	0.59 (±0.14)	0.70	2	0.499
	β-pinene	0.33 (±0.10) a	2.42 (±0.27) a	1.25 (±0.49) a	0.86	2	0.004
	Limonene	0.32 (±0.09)	0.31 (±0.11)	1.10 (±0.81)	0.94	2	0.394
	Myrcene	0.96 (±0.34)	0.53 (±0.21)	1.92 (±0.53)	0.41	2	0.961
	3-carene	0.03 (±0.01)	0.04 (±0.01)	0.41 (±0.21)	2.57	2	0.088
	Total mono	0.39 (±0.04)	0.33 (±0.07)	0.41 (±0.07)	0.58	2	0.943

Suppl. Table 2.18 Results of the three-way PERMANOVA comparing proportional differences in the concentrations of induced and constitutive monoterpenes in *Pinus banksiana* phloem tissues sampled 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the number of residuals of each *F* test. *P*-values indicate significant differences at α=0.05.

Variable	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
$Treatment_{168}$	2.80	0.01	1	0.055	*0.02*
$Height_{168}$	7.46	0.03	1	<0.001	0.04
$AWHC_{168}$	8.70	0.08	2	<0.001	0.07
Treatment x $Height_{168}$	0.97	0.01	1	0.406	
Treatment x $AWHC_{168}$	1.47	0.01	2	0.209	
AWHC x $Height_{168}$	5.98	0.05	2	<0.001	
Treatment x Height x $AWHC_{168}$	1.13	0.01	2	0.340	

Suppl. Table 2.19 Comparison of proportional differences in the concentrations of induced and constitutive monoterpenes in the phloem tissues of the treated *Pinus banksiana* trees compared between 1.3 m and 5 m heights aboveground. Trees were inoculated with live *Grosmannia clavigera* mycelia 1.3 m aboveground. Sites were categorized as Low, Medium and High AWHC sites (available water holding capacity). Total mono= Total monoterpenes. *P*-values indicate significant differences at α=0.05.

Site	Compound	Mean (±SE) prop. difference in conc. (μg mg^{-1})		$t_{(df)}$†	*P*
		1.3 m	5 m		
Low AWHC	Total mono	0.39 (±0.14)	0.39 (±0.17)	$0.45_{(38)}$	0.467
	α-pinene	0.41 (±0.23)	0.35 (±0.14)	$-0.18_{(38)}$	0.213
	β-pinene	2.18 (±0.86) a	0.33 (±0.16) b	295†	0.002
	Limonene	0.47 (±0.31)	0.32 (±0.16)	169†	0.146
	Myrcene	0.61 (±0.21)	0.48 (±0.19)	209†	0.251
	3-carene	28.67 ±16.16)	17.30 (±10.18)	171†	0.342
Medium AWHC	Total mono	0.37 (±0.11)	0.34 (±0.17)	$0.84_{(38)}$	0.851
	α-pinene	0.34 (±0.13)	0.25 (±0.17)	$1.01_{(38)}$	0.151
	β-pinene	0.74 (±0.36)	2.43 (±1.85)	200†	0.291
	Limonene	1.08 (±0.85) a	0.32 (±0.15) b	115†	<0.001
	Myrcene	0.62 (±0.12) a	0.40 (±0.22) b	281†	0.005
	3-carene	0.76 (±0.38)	17.79 (±13.25)	112†	0.153
High AWHC	Total mono	1.76 (±0.29) a	0.42 (±0.15) b	$4.15_{(38)}$	<0.001
	α-pinene	1.93 (±0.32) a	0.60 (±0.29) b	$3.36_{(38)}$	0.003
	β-pinene	3.26 (±0.82) a	1.26 (±0.62) b	300†	0.001
	Limonene	1.70 (±0.51) a	1.10 (±0.76) b	287†	0.021
	Myrcene	2.37 (±0.59) a	0.48 (±0.29) b	300†	<0.001
	3-carene	26.66 (±12.89) a	0.84 (±0.61) b	299†	<0.001

†Wilcoxon rank sum test (when the assumptions of the analysis were not met). The numbers represent Wilcoxon test statistic.

Suppl. Table 2.20 Comparison of proportional differences in the concentrations of induced and constitutive monoterpenes in the phloem tissues of *Pinus banksiana* trees (control trees) compared between 1.3 m and 5 m heights aboveground. Trees were mechanically wounded by tissue sampling 1.3 m aboveground. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). Total mono= Total monoterpenes. *P*-values indicate significant differences at α=0.05.

Site	Compound	Mean (±SE) prop. difference in conc. (μg mg^{-1})		$t_{(df)}$†	P
		1.3 m	5 m		
Low AWHC	Total mono	0.49 (±0.33)	0.13 (±0.16)	$0.84_{(18)}$	0.406
	α-pinene	0.65 (±0.49)	0.03 (±0.11)	$0.18_{(18)}$	0.425
	β-pinene	0.20 (±0.15)	0.08 (±0.20)	$0.49_{(18)}$	0.626
	Limonene	-0.28 (±0.24)	0.51 (±0.48)	22†	0.222
	Myrcene	1.66 (±1.27)	0.49 (±0.28)	46†	0.625
	3-carene	1.79 ±2.19) b	13.91 (±4.18) a	21†	0.031
Medium AWHC	Total mono	0.24 (±0.15)	0.34 (±0.25)	$0.03_{(18)}$	0.971
	α-pinene	0.32 (±0.33)	0.37 (±0.26)	$0.69_{(18)}$	0.493
	β-pinene	0.70 (±0.42)	0.93 (±0.79)	$0.23_{(18)}$	0.981
	Limonene	0.20 (±0.00) a	0.02 (±0.11) b	80†	0.021
	Myrcene	0.37 (±0.21)	0.09 (±0.15)	61†	0.427
	3-carene	-0.07 (±0.21)	0.38 (±0.39)	24.5†	0.491
High AWHC	Total mono	0.24 (±0.15) b	0.34 (±0.25) a	$2.61_{(18)}$	0.018
	α-pinene	0.32 (±0.33) b	0.37 (±0.26) a	$2.79_{(18)}$	0.011
	β-pinene	0.70 (±0.42)	0.93 (±0.79)	$1.22_{(18)}$	0.237
	Limonene	0.20 (±0.00)	0.02 (±0.11)	37†	0.344
	Myrcene	0.37 (±0.21)	0.09 (±0.15)	60†	0.472
	3-carene	-0.07 (±0.25) b	0.38 (±0.39) a	79†	0.031

†Wilcoxon rank sum test (when the assumptions of the analysis were not met). The numbers represent Wilcoxon test statistic.

Suppl. Table 2.21 Results of the three-way PERMANOVA comparing proportional differences in the concentrations of induced and constitutive diterpene resin acids in *Pinus banksiana* phloem tissues sampled 1.3 m and 5 m aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the number of residuals of each *F* test.

Variable	Height	*F*	R^2	df	*P*	"*PERMDISPER*" (*P*)
$Treatment_{84}$	1.3 m	0.65	0.00	1	0.469	0.68
$AWHC_{84}$		1.99	0.04	2	0.118	0.41
Treatment x $AWHC_{84}$		1.48	0.03	2	0.211	
$Treatment_{84}$	5 m	1.96	0.02	1	0.155	0.57
$AWHC_{84}$		0.81	0.01	2	0.464	0.07
Treatment x $AWHC_{84}$		1.01	0.09	2	0.369	

Suppl. Table 2.22 Results of the three-way PERMANOVA comparing proportional differences in the concentrations of induced and constitutive diterpene resin acids in *Pinus banksiana* phloem tissues sampled 1.3 m and 5 m heights aboveground on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Subscript numbers indicate the number of residuals of each *F* test. *P*-values indicate significant differences at α=0.05.

Variable	*F*	R^2	df	*P*	*"PERMDISPER"* (*P*)
$Treatment_{168}$	0.52	0.01	1	0.548	0.43
$Height_{168}$	5.08	0.02	1	0.014	0.31
$AWHC_{168}$	1.88	0.02	2	0.127	0.21
Treatment x $Height_{168}$	1.82	0.01	1	0.168	
Treatment x $AWHC_{168}$	1.93	0.02	2	0.126	
AWHC x $Height_{168}$	1.19	0.01	2	0.312	
Height x Treatment x $AWHC_{168}$	0.58	0.01	2	0.621	

Suppl. Table 2.23 Comparison of proportional differences in the concentrations of induced and constitutive diterpene resin acids in the phloem tissues of *Pinus banksiana* trees compared between 1.3 m and 5 m heights aboveground. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). *P*-values indicate significant differences at α=0.05.

Compound	Mean (±SE) prop. difference in conc. (µg mg^{-1})		$t_{(df)}$†	*P*
	1.3 m	5 m		
Palustric acid	0.08 (±0.07)	0.43 (±0.09)	5381†	<0.001
Dehydroabietic acid	0.97 (±0.11)	0.13 (±0.09)	1767†	<0.001
Neoabietic acid	0.45 (±0.12)	0.21 (±0.07)	1.60 (178)	0.111
Abietic acid	0.24 (±0.07)	0.15 (±0.05)	0.62 (178)	0.535
Levopimaric acid	0.29 (±0.09)	0.33 (±0.08)	0.37 (178	0.707
Total diterpenes	0.37 (±0.09)	0.19 (±0.06)	1.58 (178)	0.115

†Wilcoxon rank sum test (when the assumptions of the analysis were not met). The numbers represent Wilcoxon test statistic.

Suppl. Table 2.24 *Post-hoc* Tukey's HSD test showing pair-wise comparison of mean lesion areas compared among *Pinus banksiana* trees on sites that were characterized as Low, Medium and High AWHC (available water holding capacity).

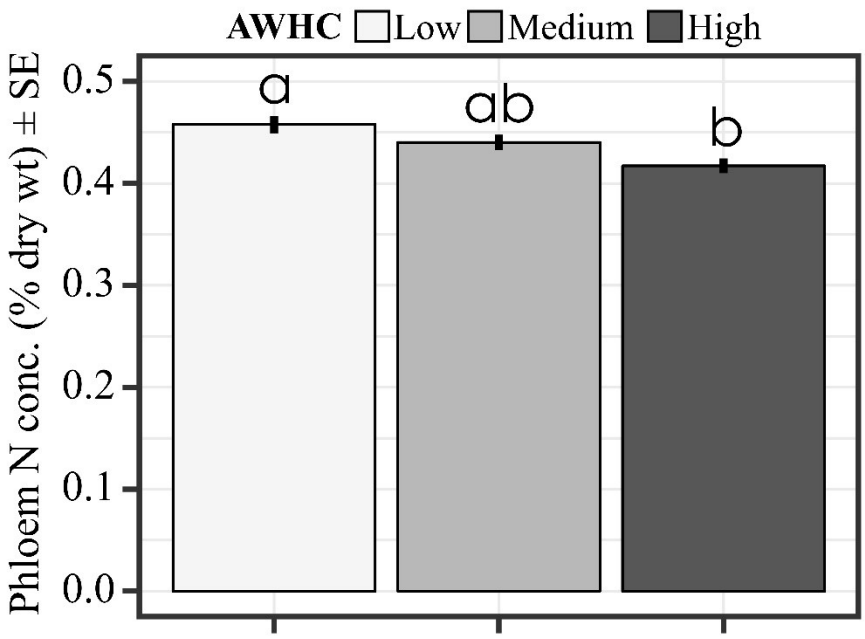
Groups compared	Estimate	SE	*Z*	df	*P*
Low AWHC–Med AWHC	1.62	1.19	1.35	6.4	0.351
Low AWHC–High AWHC	3.19	1.17	2.71	5.9	0.019
Med AWHC–High AWHC	1.57	1.16	1.35	5.6	0.351

Suppl. Table 2.25 Results of the two-way PERMANOVA comparing the concentrations of induced monoterpenes among the lesions caused by different inoculation densities (4 vs 16) of live mycelia of *Grosmannia clavigera* in *Pinus banksiana* trees on sites that were categorized as Low, Medium and High AWHC (available water holding capacity). Inoc. density = inoculation density (i.e., 4 or 16). Subscript numbers indicate the number of residuals of each *F* test.

Variable	*F*	R^2	df	*P*	*"PERMDISPER"* (*P*)
$AWHC_{56}$	4.64	0.14	2	<0.001	0.23
Inoc. $density_{56}$	1.39	0.02	1	0.191	0.51

Suppl. Table 2.26 One-way analysis of variance table of induced monoterpenes in the lesions caused by *Grosmannia clavigera* inoculations in *Pinus banksiana* trees at 1.3 m height above ground on sites categorized as Low, Medium and High AWHC (available water holding capacity). Total mono = Total monoterpenes. *P*-values indicate significant differences at α=0.05.

Compounds	Mean(±SE) concentration (μgmg^{-1} of fresh weight)			ANOVA		
	Low AWHC	Medium AWHC	High AWHC	*F*	df	*P*
α-pinene	479.23 (±52.86) b	499.10 (±48.97) b	751.85 (±59.22) a	8.25	2	<0.001
Myrcene	3.67 (±0.27) b	2.89 (±0.27) b	4.75(±0.29) a	11.43	2	<0.001
Limonene	2.51 (±0.24) b	3.07 (±0.27) b	3.90(±0.26) a	8.41	2	<0.001
β-pinene	24.55 (±5.49)	50.26 (±5.82)	54.43 (±11.12)	2.58	2	0.083
3-carene	(2.69±0.28)	(2.58±0.23)	2.89(±0.17)	0.47	2	0.626
Total mono	(537.46±57.05) b	(565.69±49.28) b	(825.21±61.97) a	7.87	2	<0.001



Suppl. Fig. 2.1 Mean phloem nitrogen concentration ±SE (% of dry weight) in phloem tissues of *Pinus banksiana* trees sampled at sites in Lac la Biche, Alberta. Sites were categorized as Low, Medium and High AWHC (available water holding capacity). Different letters indicate significant differences between means at α=0.05 in one-way ANOVA.

Chapter 3

Soil available water holding capacity can alter the reproductive performance of mountain pine beetle (Coleoptera: Curculionidae) in jack pine (Pinales: Pinaceae) through phloem nitrogen concentration

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3.1 Abstract

Mountain pine beetle (*Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae, Scolytidae) has recently invaded novel jack pine (*Pinus banksiana* Lamb., Pinales: Pinaceae) forests in western Canada. Jack pine seems to be a suitable host for mountain pine beetle, but how growing conditions influence jack pine's quality as a host, and hence, its susceptibility for mountain pine beetle, is unknown. Specifically, how soil nutrient concentrations and available water holding capacity (AWHC) affect jack pine quality should be investigated. Host plant quality is an important determinant of mountain pine beetle host colonization and reproduction and is usually assessed by primary (nutrients) and secondary (defense chemistry) constituents of host phloem. I evaluated mountain pine beetle host acceptance and brood production by

recording the percentage of female mountain pine beetle that entered the phloem and oviposited in 30 jack pine bolts from two sites that differed in soil nutrient concentrations and AWHC. I also compared the concentrations of phloem nutrients and defense monoterpenes among the selected trees and found that trees at the Low AWHC site had higher amounts of nitrogen, phosphorus, and potassium. Monoterpene concentrations did not differ among trees at the two sites. Host acceptance by and brood production of mountain pine beetle were greater in bolts from the Low AWHC site. I conclude that AWHC of the soil may influence mountain pine beetle host acceptance and brood production through altering host plant quality, particularly nitrogen in the phloem, and will potentially influence any further range expansion of the beetle in eastern North America

Keywords: *Dendroctonus ponderosae*, *Pinus banksiana*, drought, reproductive success, macro- and micronutrients.

3.2 Introduction

The recent range expansion by mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae, Scolytidae) into the boreal plains ecoregion in western North America has led to a direct interaction with a novel host, jack pine (*Pinus banksiana* Lamb) (Cullingham et al. 2011; Erbilgin et al. 2014; Erbilgin 2019). Successful colonization of a host by a bark beetle is a combination of three continuous processes: host selection, host acceptance, and brood development and emergence (Raffa et al. 1993; Safranyik et al. 2010; Lindgren and Raffa 2013; Erbilgin et al. 2017a). Of these, MPB has a bimodal host selection behaviour, depending on its population density. At the endemic low densities, bark beetle attacks are limited to trees that are stressed due to biotic (e.g., insects and diseases) or abiotic (e.g., drought) agents.

In contrast, at the epidemic densities, bark beetles are not limited by the host conditions, and host availability and climatic suitability become more critical aspects driving the insect host colonization. By comparing these two population densities, MPB is more likely to be affected by host suitability at the endemic density (Safranyik et al. 2010); however, the attack behaviour and brood production of MPB in endemic phase are poorly understood (Safranyik and Carroll 2006; Boone et al. 2011; Bleiker et al. 2014).

Like most conifer species, jack pine has a unique ecophysiological niche across North America and dominates the boreal regions that offer the specific temperature, precipitation, and soil resource regimes (Raffa et al. 2016). Unlike mountain pine beetle's historical host, lodgepole pine (*Pinus contorta* Douglas), jack pine may be better adapted to abiotic stress factors due to its water-conserving nature and its ability to grow on well-drained, nutrient-poor boreal soils (Moore et al. 2000). Moreover, jack pine covers a large geographic area with known spatial heterogeneities in soil types and nutrient availability (Cayford and McRae 1983; Critchfield 1985; Visser 1995). I lack critical information to understand how such variations in jack pine growing conditions could affect host selection behavior and reproductive performance of MPB in the novel host.

Host tree quality is of critical influence when assessing bark beetles' fitness, and is usually characterized by measurements of nutrient and defense chemical concentrations of host phloem tissue. For example, nitrogen is required by insect herbivores in all metabolic processes, cellular structure, and genetic coding (Mattson 1980). The lack of nitrogen in host phloem significantly affects the growth and reproduction of bark beetles (Mattson 1980; Ayres et al. 2000; Goodsman et al. 2012). Similarly, phosphorus is needed for ATP production and the

synthesis of RNA and DNA, and thus proteins in insect herbivores (Sterner and Elser 2002; Plassard 2018). To be biologically active, ATP must be bound to a magnesium ion (Mg.ATP). Magnesium and zinc are also used in the production of various enzymes (Dow 2017). The latter is also essential for DNA-binding proteins (Richards and Burke 2016; Dow 2017). Potassium and sodium are the two most dynamic and plentiful ions in cells and biological fluids and are crucial for physiological functions such as maintaining the internal homeostasis and neuronal transmission (Cano et al. 2017; Dow 2017). Likewise, iron is indispensable for life as it senses and transports oxygen (Papanikolaou and Pantopoulos 2005), and is a cofactor in numerous enzymes (Dow 2017). Host terpenes, mainly monoterpenes, are utilized by MPB as aggregation and anti-aggregation pheromone precursors; in host location; or they may act as attractants, anti-attractants, or antifeedants (Seybold et al. 2006; Erbilgin et al. 2017a; Erbilgin 2019).

There has been extensive research on the interactions among jack pine, MPB, and its fungal associates (e.g., Lusebrink et al. 2011; Erbilgin et al. 2014, 2017a, b; Arango-Velez et al. 2016; Roth et al. 2018; Erbilgin 2019). However, less attention has been paid to the large geoclimatic variations and geospatial patterns present in the distribution of jack pine in the expanded boreal range (Critchfield 1985; Bone et al. 2013; Taft et al. 2015a, b). These differences could potentially impact jack pine's susceptibility to MPB (Taft et al. 2015a, b; Ishangulyyeva et al. 2016; Raffa et al. 2016; Erbilgin 2019). The distribution and abundance of jack pine in some of the most resource deficient and stressed stands could provide a foothold for the beetles to sustain their critical attack threshold in the boreal forest (Safranyik et al. 2010). Therefore, our goal was to understand whether host acceptance by and brood production success of MPB vary with site conditions. I assessed two aspects of site conditions (nutrient concentrations and available water holding capacity (AWHC) of the soil). I used AWHC as a

proxy to test the effects of water availability on plant responses because AWHC is more relevant to predict the long-term soil characteristics, including water availability, influencing vegetation growth and development (De Jong and Shields 1988; Reynolds et al. 2007; Piedallu et al. 2011). I also investigated the effect of site conditions on the host plant quality, nutrient and monoterpene concentrations of jack pine phloem. Finally, I evaluated the impacts of site conditions and host quality on MPB host selection behaviour and reproductive performance. I focused on phloem chemistry because that is where MPB feed, reproduce, and complete their development. I hypothesized that AWHC of soil can be used as a proxy to assess host quality, which can then affect MPB host acceptance and brood production. Results from this study would help us better understand the role of site quality in host and range expansion of MPB.

3.3 Material and methods

3.3.1 Experimental design and sampling

I preselected two sites with jack pine trees located in Lac la Biche, AB, Canada, that potentially differed in AWHC after visually inspecting their soils, vegetation types, and then assessing their soil moisture content by using an electromagnetic sensor (ML3 Theta Probe, Delta-T Devices, Cambridge, United Kingdom) at multiple points on each site. Both sites were separated by 45 km. The site with the lowest soil moisture (mean 8.61 ± 0.43% SE) was located at (55°04′13.8″N, 111°59′48.2″W) and the site with the highest soil moisture (mean 22.48 ± 2.61% SE) at (54°55′10.2″N, 111°28′05.6″W). The High AWHC site was dominated by black spruce (*Picea mariana*) and had a thick moss layer on the forest floor. The site with Low AWHC had a thin to no organic matter layer, and the mineral sandy soil was clearly visible. On 14 August 2016, phloem tissue samples (3 cm × 3 cm) from 15 apparently healthy trees (i.e., no signs or

symptoms of any insect or pathogen activity) were taken at each site at 1.3 m height at north and south cardinal directions (i.e., two samples per tree). Diameter at breast height (mean ± SE) of sampled trees was 27.7 ± 0.84 cm. Both tissue samples from each tree were pooled and kept in liquid nitrogen in the field before storing them at −40°C in the laboratory. I also collected soil samples from each site for nutrient analysis and AWHC assessment. Briefly, I established a 15 m × 30 m rectangular grid at each site near the experimental trees and four sampling points evenly distributed in each grid were selected. After removing the litter layer, I used a soil auger to collect a soil sample to a depth of 25 cm at every sampling point. Soil samples were immediately transferred into Ziploc bags and kept on dry ice in the field before storing them at −40°C in the laboratory. Each soil sample weighed approximately 250 g. I felled all 30 trees on the same day (14 August 2016) and cut one bolt (35 cm long) from each tree at 1.3 m height. All bolts were waxed at both ends and stored at 4°C in a walk-in cooler be- fore inoculating them with live MPB.

3.3.2 Inoculation of bolts with live mountain pine beetles

Host acceptance by MPB in the laboratory was assessed by successful female inoculation into three equidistant entrance holes around the bottom of each bolt during the first 24 h between 28 and 29 September 2016. Phloem surfaces were exposed before inoculations by using a cork borer with a diameter of 0.6 cm. Female beetles were placed in 0.5 ml microcentrifuge tubes that were inserted into the boreholes with their open ends and taped securely. To ensure mating and oviposition, every successful female beetle entrance was followed by the introduction of a male beetle within 12 h in the same way as female beetles. All beetles used in this experiment were newly emerged (<2 d old) from MPB infested lodgepole pine bolts that were harvested on 29

August 2016 in Jasper National Park, AB, Canada (52°46′25.6″N, 118°01′45.9″W). To simulate overwintering, the inoculated bolts were placed in rearing boxes and held in cold storage at 4°C for 2 mo (4 November 2016 to 4 January 2017). On 5 January 2017, the bolts were transferred to another room at ambient temperature to let the beetles complete their development (Erbilgin et al. 2014; Lusebrink et al. 2016). Emerging offspring were collected daily for 5 wk. After all beetles had emerged, the outer bark of the bolts was removed, and the length of each maternal gallery was measured. Since the presence of larval galleries indicates mated pairs accepted the bolt and laid fertile eggs, I also assessed brood size by counting the number of larval galleries emerging from each maternal oviposition gallery.

3.3.3 Nutrient analysis

Phloem and soil total manganese, sulfur, phosphorus, magnesium, potassium, calcium, copper, sodium, iron, and zinc were analyzed at NRAL at the University of Alberta (http://nral.ualberta.ca). The analysis followed a similar protocol described in Müller et al. (2014). Briefly, all phloem (n = 15 for each site) and soil (n = 4 for each site) samples were oven-dried at 40°C for 24 h before grinding them with a Wiley Mini Mill (Wiley, Thomas Scientific, Swedesboro, NJ). Samples were analyzed in an inductively coupled plasma-optical emission spectrometer (ICP-OES) on an iCAP 6300 DUO (North America) apparatus (Thermo Fisher Corp., Cambridge, United Kingdom). Randomly selected samples from each group (i.e., phloem and soil samples) were run twice to validate the results. Total phloem and soil nitrogen concentrations were determined by the Dumas combustion method by using a Costech Model EA 4010 Elemental Analyzer (Costech International Strumatzione, Florence, Italy) (Gaster et al.

2015). Total phloem nutrients are reported as percentage of dry weight of tissues, whereas total soil nutrients are reported as g kg^{-1} of dry weight of soil samples.

Plant available soil nutrients were also analyzed at the same facility as soil and phloem nutrients. Chloride and sulfate were extracted from 10 g of field moist soil with 20 ml of Milli-Q water. Available potassium and phosphate were extracted from 5 g of oven-dried soil with 50 ml of Modified Kelowna (Frankenberger et al. 1996). Ammonium and nitrate were extracted from 5 g oven-dried soil with 2 M KCl. All samples were filtered through Q2 filter paper. The available nutrients were analyzed by using Dionex Ion Chromatography DX 600 (Dionex, Sunnyvale, CA) and the nutrients are reported in mg kg^{-1} of dry weight of soil samples.

3.3.4 Assessment of plant available water holding capacity

The AWHC of a soil is the difference between field capacity or the maximum amount of water the soil can hold, and permanent wilting point where the plant can no longer extract any water from the soil (Kirkham 2014). Water available to trees is generally held by energy forces that range from 100 to 1,500 kPa, and theoretically, plants cannot recover once the permanent wilting point is exceeded (Fowells and Means 1990). I used pressure plate chambers (Extractor 1500F2, Soil Moisture Equipment Corp., Santa Barbara, CA) as described by Dane and Hopmans (2002) to assess the AWHC of soil samples from the two experimental sites. Briefly, soil samples (n = 4 for each site) were sieved with a 2 mm round-hole sieve and separate 25 g subsamples were placed in the retaining rings to measure their field capacities and permanent wilting points. To saturate the samples with water, they were placed in the chambers and water was poured gently to slightly cover them. After 24 h, excess water was removed, and the chambers were pressurized (100 kPa for field capacity and 1,500 kPa for permanent wilting point). After 48 h, soil samples

were removed from the chambers and weighed immediately. Finally, the samples were oven-dried at 105°C for 24 h and weighed again for the differences. I repeated these steps four times for each soil sample to validate the results, and used the mean readings in the statistical analysis. The AWHC of soil samples was calculated as the difference between field capacity and permanent wilting point. Results are reported volumetrically in cm^3 of water per cm^3 of soil (Reynolds et al. 2007; Liu et al. 2016).

3.3.5 Monoterpene analysis

Phloem tissues collected from 30 trees were cleaned by removing their outer bark, ground with a cryogrinder (SPEX Sample Prep Freezer Mill 6770, Metuchen, NJ, USA), and then stored at −40°C. Sample extraction and analysis followed a similar protocol as described by Erbilgin et al. (2014). Briefly, using 1.5 mL microcentrifuge tubes, monoterpenes were extracted twice from 100 mg (±2) of ground fresh tissues in 0.5 mL pentane with 0.004% tridecane added as an internal standard. Extracts were vortexed for 30 s at 3,000 rpm, sonicated for 10 min, centrifuged at 13,000 rpm for 15 min at 4°C, and placed in a −40°C freezer for at least 2 h. Extracts were transferred into gas chromatography (GC) vials and 1 µL was injected at a split ratio of 10:1 in a coupled GC-Mass Spectrometer (GC-MS; 7890A-5975C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP Innowax column (ID 0.25 mm; length 30 m; Agilent Tech.). Helium was used as the carrier gas at a flow rate of 1.1 ml min^{-1}. The temperature program started at 55°C, held for 1 min and then ramped by 40°C min^{-1} to 65°C (held for 1 min), then 40°C min^{-1} to 75°C (held for 0.5 min), then 7°C min^{-1} to 130°C, and then 20°C min^{-1} to 250°C (held for 0.5 min). Peaks were identified by using the following standards: α-pinene, β-pinene, 3-carene, myrcene, limonene, *p*-cymene, camphor, 4-allyanisole, borneol (Fluka, Sigma-Aldrich, Buchs,

Switzerland; chemical purity >90%), γ-terpinene, α-terpinene, pulegone, terpineol (Sigma-Aldrich, St. Louis, MO, USA; >90%), ocimene, terpinolene, bornyl acetate (>90%), camphene (SAFC Supply Solutions, St. Louis, MO, USA; >80%), and β-phellandrene (Erbilgin laboratory; >90%). Monoterpenes were identified by comparing their retention times and mass spectra with those of the commercial standards and quantified through calibration curves with the standards generated from analyses of a serial of dilution of known quantities of standards (20 μg ml^{-1}, 2 μg ml^{-1}, 0.2 μg ml^{-1}), and calculated and reported hereafter as ng mg^{-1} of fresh tissue.

3.3.6 Statistical analyses

All statistical analyses were performed in R version 3.4.3 (R Core Team 2017). Trees (n = 15 for each site) were compared for differences in phloem nutrient concentrations, and their cut bolts (n = 15 for each site) for host acceptance (% in first 24 h), and the number of larval galleries (broods) by using permutational multi- variate analysis of variance (PERMANOVA, permutations = 10,000, method = Gower). Differences between the two sites were reflected by using principal coordinates analysis (PCoA) which is an ordination method that allows for clustering of the test groups based on their similarities or dissimilarities (Gower 1971). The PERMANOVA was performed by using the *adonis* procedure and PCoA was performed with the default functions provided in R package *vegan* version 2.4-4 (Oksanen et al. 2017). Differences for each numeric variable between the two sites were evaluated for statistical significance by using two-sample t-tests. I used a chi-square test to compare host acceptance in the first 24 h by female MPB between bolts from the two experimental sites. The relationship between the number of larval galleries and potential explanatory variables was investigated by using a linear mixed model in the lme4 package with the *lmer* function (Bates et al. 2015). I used AWHC of

the soil samples and phloem nutrient concentrations as fixed effects and bolts as a random effect, and selected the most parsimonious model by first performing a maximum likelihood test and then choosing a model with the lowest AIC value. To achieve normality and to meet model assumptions, variables (total soil potassium, copper, calcium, magnesium, and total soil nutrients) were log(x + 1) transformed. The number of larval galleries (broods) was cube root, and sulfate concentration was BoxCox transformed. All other variables were normally distributed and did not require transformation to meet the assumptions of the analysis. The significance of the fixed effects in the mixed model was determined by Wald chi-square tests from the *car* package in R (Fox and Weisberg 2018). I used an alpha level of 0.05 for all statistical tests. Raw, non-transformed data were used to construct graphs, unless otherwise stated. Means (±SE) are presented in the text.

3.4 Results

Soil from the High AWHC site had a significantly higher volume of available water holding capacity [$t(6) = -2.93$, $P=0.02$], (0.080 ± 0.011 cm^3 cm^{-3}) than the Low AWHC site (0.059 ± 0.004 cm^3 cm^{-3}). Concentrations of total nutrients in the soil samples from the two sites also differed and the High AWHC site had significantly higher concentrations of total copper, sulfur, sodium, nitrogen, calcium, potassium, magnesium, and iron (Table 3.1). Total manganese and zinc did not differ ($P>0.05$). Among the available nutrients, soil samples from the High AWHC site had a higher concentration of sulfate [$t(6) = -2.48$, $P<0.05$], and the samples from the Low AWHC site had a higher concentration of phosphate [$t(6) = 4.62$, $P=0.003$, Fig. 3.1]. Concentrations of available nitrogen, potassium, chloride, ammonium, and nitrate did not differ ($P>0.05$).

Phloem tissues collected from trees on the Low AWHC site had higher concentrations of nitrogen, phosphorus, and potassium, whereas phloem tissues collected from trees on the High AWHC site had a higher concentration of iron (Table 3.2). Other nutrients did not differ between phloem tissues from trees on the two sites (P>0.05).

I also compared the concentrations of individual and total monoterpenes in phloem tissues sampled from trees on the two sites, and none of the monoterpenes showed any statistically significant difference (P>0.05). However, trees on the Low AWHC site had relatively higher concentrations of α-pinene (1.26X), camphene (1.08X), β-pinene (1.25X), 3-carene (2.35X), limonene (1.15X), β-phellandrene (18.89X), γ-terpinene (1.5X), terpineol (6.83X), bornyl acetate (1.22X), and total monoterpenes (1.26X). Trees on the High AWHC site had a higher relative concentration of myrcene (1.6X).

Host acceptance (% in first 24 h) and number of larval galleries (broods) differed among bolts from the two sites (PERMANOVA $F_{1,89}$= 12.88, P<0.001). Principal Coordinates Analysis reflected the separation among bolts from the two sites (Fig. 3.2). The percentage of female beetles accepting bolts from Low AWHC site in the first 24 h was higher than those accepting the bolts from the High AWHC site [$\chi^2(1, N=90) = 4.60$, P<0.05, Fig. 3.3a]. Mated pairs in bolts from the Low AWHC site also produced larger broods (measured by counting the number of larval galleries) relative to bolts from the High AWHC site [$t(56.92) = 2.05$, P<0.05, Fig. 3.3b]. The lengths of the maternal galleries in bolts did not differ between the two sites (P=0.7). I did not conduct any statistical analysis for the number of beetles emerging from the bolts as there was only one beetle that emerged from the bolts from the High AWHC site and 26 beetles from the bolts from the Low AWHC site. Overall, the 26 beetles from the bolts from the Low AWHC

site emerged from 73.3% of the bolts (11 out of 15 bolts). Both AWHC and phloem nitrogen significantly correlated with the number of larval galleries ($P<0.05$ in both instances; Table 3.3). There was a strong interaction effect on the number of larval galleries between AWHC of the soil samples and the concentration of nitrogen in the phloem tissues of trees sampled on the two sites ($P=0.007$; Table 3.3, Fig. 3.4).

3.5 Discussion

Host acceptance behavior and brood production by MPB varied, depending on the phloem nitrogen and AWHC of the sampled soils. A higher number of female beetles entered and oviposited in the bolts from the Low AWHC site, which also had higher nutrient concentrations. The mated pairs also produced more broods in these bolts compared to those from the High AWHC site, which had lower nutrient concentrations. I found a significant interaction effect of AWHC of the soil samples and nitrogen concentration of phloem on MPB brood production. Monoterpene concentrations of bolts did not vary between the two sites. These results are highly pertinent for understanding the roles of soil characteristics, particularly AWHC of soils, and host quality, phloem nutrient concentrations, in the range expansion of MPB in the boreal forest. First, I found differences in the AWHC of the soils in jack pine forests. Although I did not investigate any possible mechanisms underlying the differences in AWHC of soils, the literature suggests that soil texture likely determines the AWHC of soils (De Jong and Shields 1988). Soils with high silt and clay-sized particles are likely to have a higher AWHC, compared to soils containing more sand-sized particles and less silt and clay particles. Considering the large geographical range of jack pine in North America, such differences in soils are expected. Parallel to the AWHC of soils, nutrient concentrations of soil samples from the two sites also varied. The

High AWHC site had higher concentrations of total soil nutrients, and some other micro and macro nutrients including nitrogen, calcium, and potassium. Earlier studies have also found similar interactions between soil water levels and soil nutrients (Lieffers 1988; Jurgensen et al. 1997; Laiho et al. 2003; Deluca and Boisvenue 2012).

Second, jack pine phloem chemistry also differed between the two sites. In particular, the phloem tissues collected from trees on the site with Low AWHC had higher concentrations of nitrogen, phosphorus, and potassium, whereas phloem tissues collected from trees on the High AWHC site had a higher concentration of iron, supporting earlier studies (Laiho et al. 2003). These results suggest that soil conditions can influence the nutritional quality of host phloem tissues where MPB larvae feed extensively. Our earlier studies with jack pine found similar results (Lusebrink et al. 2011, 2016; Erbilgin et al. 2017b). In contrast to phloem nutrients, AWHC of the soils did not correlate with the monoterpene concentrations of jack pine trees. Similarly, Lusebrink et al. (2016) found no effect of water treatments on monoterpene concentrations of mature jack pine trees in another jack pine stand in the same area. However, trees from the Low AWHC site had relatively higher proportions of some of monoterpenes (albeit not statistically significant), which may indicate that these trees were potentially drought-stressed relative to the trees on the High AWHC site. Conifers tend to respond with higher concentrations of monoterpenes when there are biotic or abiotic stresses (Lusebrink et al. 2011). Monoterpenes are some of the principal defense compounds in pines that are deployed when faced with biotic and abiotic threats (Seybold et al. 2006; Boone et al. 2011; Cale et al. 2017; Erbilgin et al. 2017b; Erbilgin 2019).

Finally, soil and tree level differences likely influenced beetle performance, as measured by host acceptance and brood production in the current study. The beetles performed better on bolts cut from trees in the Low AWHC site. Furthermore, I found a significant interaction effect of AWHC of the soils and phloem nitrogen concentration on MPB brood production, suggesting that the effect of the former on brood production is dependent on the effect of the latter. In general, reproductive success in bark beetles has been reported to be correlated positively with phloem nitrogen concentration (Kirkendall 1983; Ayres et al. 2000; Goodsman et al. 2012). For example, Bleiker and Six (2007) demonstrated that MPB responded to dietary nitrogen in lodgepole pine phloem through increased body size. In my study, apparently, the relatively higher concentrations of monoterpenes did not deter successful host acceptance and higher reproduction in bolts from the Low AWHC site. This result indicates the importance of the nutritional value of the host tree phloem, e.g., the limited dietary nitrogen of plants can affect growth and reproduction in herbivores, and insects tend to select for qualities that can help them better exploit the available resources, such as better host selection, and enhanced feeding and reproductive behaviours (Mattson 1980; Kirkendall 1983; Ayres et al. 2000; Sterner and Elser 2002; Colinet et al. 2015; Dow 2017; Plassard 2018). These results are in consistence with Lusebrink et al. (2016) who found that female MPB brood beetles emerged with higher fat content from jack pine bolts despite shorter maternal galleries and a lower phloem nitrogen concentration compared to the bolts from the historical host, lodgepole pine. According to the AWHC maps of the three Prairie Provinces (Alberta, Saskatchewan, and Manitoba) in Canada, low AWHC is widely spread across the range of jack pine forests (De Jong and Shields 1988). This may be a major concern for the spread of MPB in the boreal forest because jack pine trees

growing in such environments may favour beetle performance either due to low AWHC of soil, higher phloem nitrogen concentration, or through their interaction, as in this study.

In the current study, I used bolts cut from trees to test beetle performance, which is a frequently used technique to study the impact of host plant quality on beetle biology and pheromone production (e.g., Erbilgin et al. 2014; Erbilgin 2019). However, a recent study by Guevara-Rozo et al. (2019) reported that cutting and long-term storage (3–6 mo) may affect both nutrient and monoterpene concentrations of bolts, which may differentially affect beetle performance by influencing pheromone production by mature bark beetles or larval development (Goodsman et al. 2012; Erbilgin et al. 2017a). Additional studies may be needed to further substantiate my findings. Nevertheless, researchers should consider understanding the role of soil and tree resources while studying plant–insect interactions, and insect range expansion in novel habitats.

Table 3.1 Total soil nutrient concentrations at the field sites (*N*=2) in Lac la Biche, Alberta, where jack pine (*Pinus banksiana*) trees (*n*=15 for each site) were sampled and felled. The available water holding capacity (AWHC) of soil samples (*n*=4 for each site) were assessed and the sites were categorized as 'Low AWHC' or 'High AWHC'. Independent two sample *t*-tests were conducted to compare nutrient concentrations in soil samples (*n* = 4 for each site).

Element	Mean (±SE) concentrations (g kg^{-1})		$t_{(df)}$[1,2]	*P*-value
	Low AWHC	High AWHC		
Copper	0.001 (±0.00001)	0.004 (±0.001)	$-4.25_{(6.0)}$	0.005
Sulfur	0.022 (±0.001)	0.068 (±0.008)	$-5.56_{(3.3)}$	0.008
Sodium	0.026 (±0.003)	0.089 (±0.012)	$-5.01_{(3.4)}$	0.010
Nitrogen	0.198 (±0.019)	0.368 (±0.022)	$-5.67_{(6.0)}$	<0.001
Calcium	0.197 (±0.023)	1.226 (±0.344)	$-3.97_{(6.0)}$	0.007
Potassium	0.188 (±0.021)	1.699 (±0.492)	$-5.02_{(6.0)}$	0.002
Magnesium	0.211 (±0.017)	2.753 (±0.657)	$-7.11_{(6.0)}$	<0.001
Iron	3.506 (±0.111)	14.93 (±2.084)	$-5.47_{(3.1)}$	0.011
Total soil nutrients	4.870 (±0.205)	21.53 (±3.582)	$-7.42_{(6.0)}$	<0.001

[1] Welch tests (when the variances were not equal) conducted for sulfur, sodium, and iron.

[2] –/+sign denotes the direction of the relationship.

Table 3.2 Total phloem nutrient concentrations (% of dry weight) at the field sites (*N*=2) in Lac la Biche, Alberta, where jack pine (*Pinus banksiana*) trees (*n*=15 for each site) were sampled and felled. The available water holding capacities (AWHC) of soil samples (*n*=4 for each site) were assessed and the sites were categorized as 'Low AWHC' or 'High AWHC'. Independent two sample *t*-tests were conducted to compare nutrient concentrations in the phloem tissues collected from trees at both sites (*n* =15 for each site).

Element	Mean (±SE) concentrations (%)		$t_{(df)}$[1,2]	*P*-value
	Low AWHC	High AWHC		
Iron	0.002 (±0.0001)	0.003 (±0.0003)	$-2.13_{(17.14)}$	<0.05
Phosphorus	0.060 (±0.0023)	0.048 (±0.0020)	$+3.25_{(24)}$	0.003
Nitrogen	0.288 (±0.0109)	0.223 (±0.012)	$+3.25_{(24)}$	0.003
Potassium	0.296 (±0.0147)	0.237 (±0.011)	$+2.50_{(24)}$	0.019

[1] Welch test (when the variances were not equal) used for iron.

[2] −/+sign denotes the direction of the relationship.

Table 3.3 Analysis of deviance (type III Wald Chi-square test) table showing the effect of available water holding capacity (AWHC) of the soil samples (n=4 for each site), phloem nitrogen (N) concentration (% of dry weight) in the tissues sampled from the experimental trees (n=15 for each site), and interaction of the two factors on the number of mountain pine beetle (*Dendroctonus ponderosae*) larval galleries (broods) in jack pine (*Pinus banksiana*) bolts (n=15 for each site) collected from sites (N=2) with 'Low AWHC' and 'High AWHC' located in Lac la Biche, Alberta.

Variables	Coefficients	$\chi^2_{(df)}$	P-value
AWHC	−2.80	$4.96_{(1)}$	0.025
N	−6.01	$3.93_{(1)}$	0.047
AWHC * N	12.88	$7.05_{(1)}$	0.007

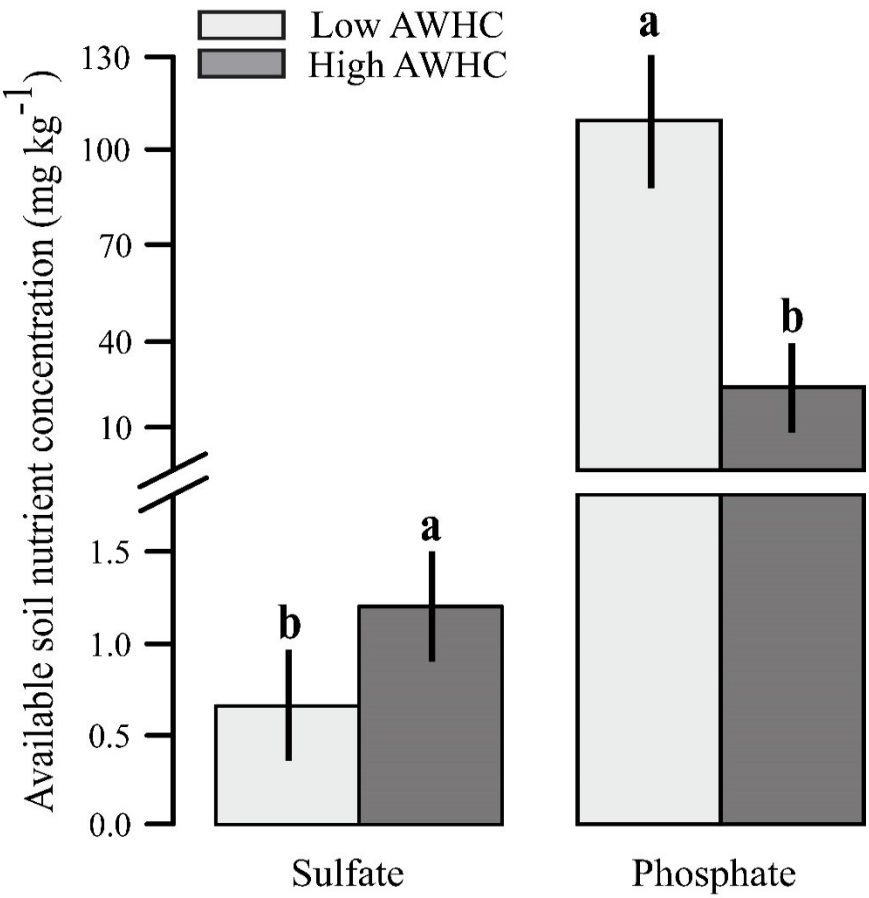

Fig. 3.1 Available sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}) concentrations (mean ± SE) in soil samples from the field sites (*N*=2) in Lac la Biche, Alberta where jack pine (*Pinus banksiana*) trees (*n*=15 for each site) were sampled and felled. Two sample *t*-tests were conducted to compare the mean concentrations of each nutrient among the soil samples (*n*=4 for each site). Sites were categorized as 'Low AWHC' and 'High AWHC' after comparing the available water holding capacities (AWHC) of their soil samples (*n*=4 for each site).

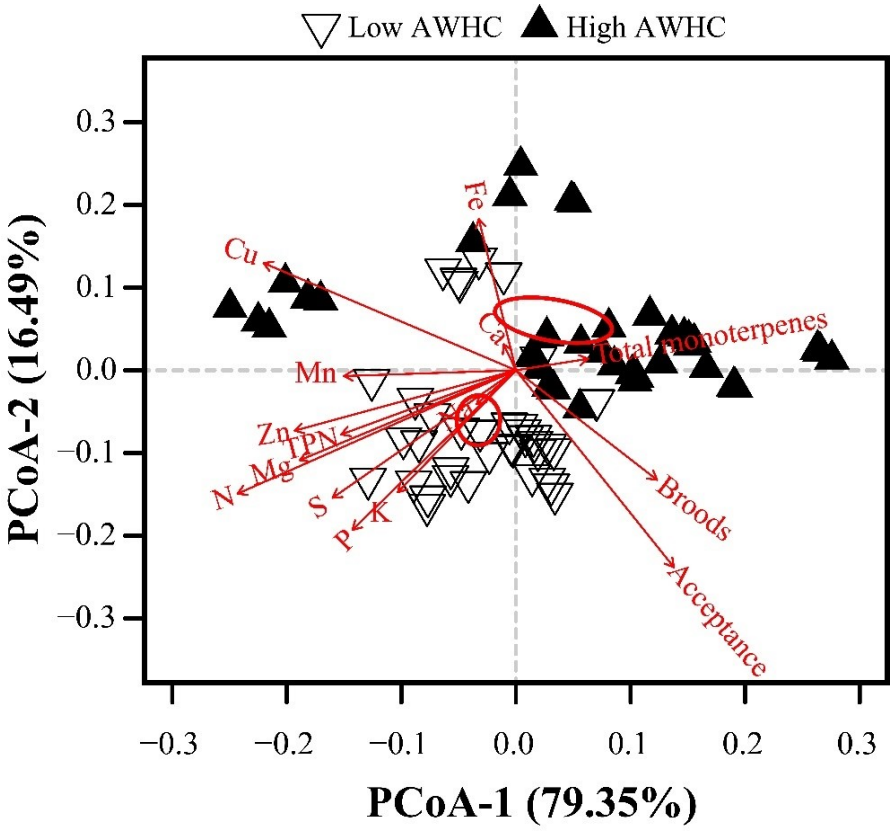

Fig. 3.2 Principal Coordinates Analysis (PCoA) showing the separation between jack pine (*Pinus banksiana*) bolts (*n*=15 for each site) collected from the field sites (*N*=2) in Lac la Biche, Alberta. Sites were categorized as 'Low AWHC' and 'High AWHC' by comparing the available water holding capacities (AWHC) of their soil samples (*n*=4 for each site). Separation is based on total monoterpene concentrations (ng mg^{-1} fresh weight) in the phloem tissue samples from each experimental tree (*n*=15 for each site), phloem nutrient concentrations (% of dry weight, *n*=15 for each site), mountain pine beetle (*Dendroctonus ponderosae*) host acceptance (%) in the first 24 h by using bolts from the sampled trees, and the number of mountain pine beetle larval galleries (broods) in the inoculated bolts. Ellipses represent the 95% confidence intervals around group centroids using model standard errors. Arrows indicate the contribution of individual variables to the PCoA axes. TPN = Total phloem nutrients.

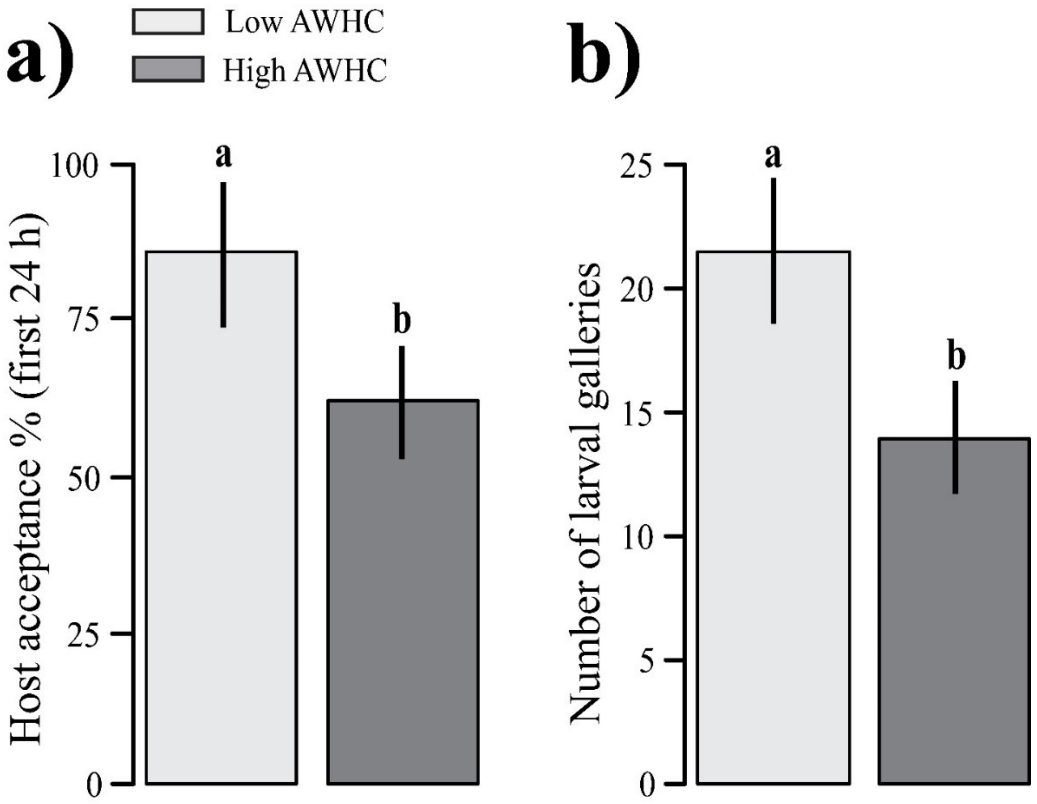

Fig. 3.3 Host acceptance (%) in first 24 h and reproductive performance (number of larval galleries) by mountain pine beetle (*Dendroctonus ponderosae*) in jack pine (*Pinus banksiana*) bolts (n=15 for each site) from the field sites (N=2) in Lac la Biche, Alberta. The available water holding capacities (AWHC) of soil samples (n=4 for each site) were assessed for each site and the sites were categorized as 'Low AWHC' and 'High AWHC'. a) Percentage of female beetles accepting the bolts in the first 24 h from the two sites with 'Low AWHC' and 'High AWHC'. Chi-squared test was conducted to compare host acceptance (%) among bolts from the two sites inoculated into three equidistant entrance holes around the bottom of each bolt during the first 24 h (n=45 for each site). Error bars represent the 95% confidence intervals. b) Number of larval galleries (mean ± SE) per mated pair in bolts from the two sites with 'Low AWHC' and 'High AWHC'. Two-sample t-test was conducted to determine the statistical differences among bolts from the wo sites (n=45 for each site).

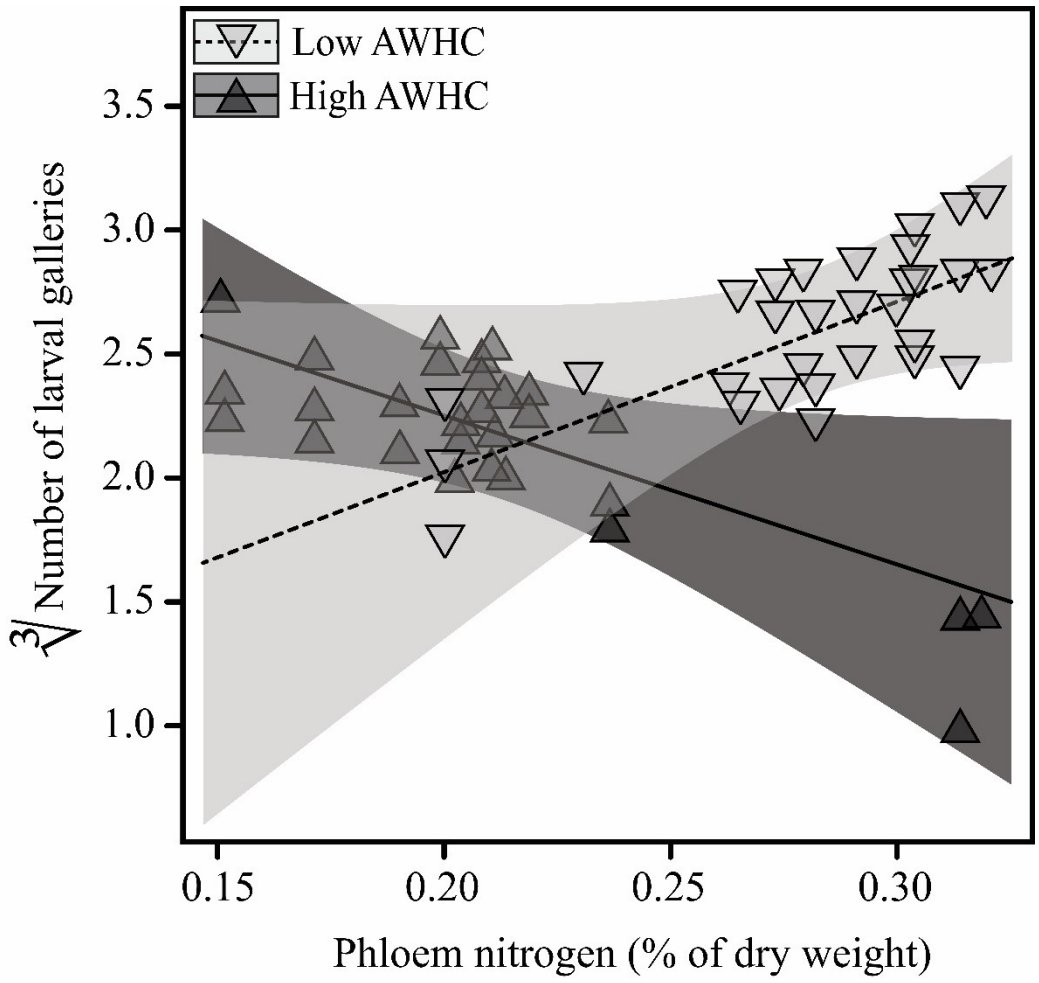

Fig. 3.4 Results of the linear mixed model showing the interactions among phloem nitrogen concentration (% of dry weight), available water holding capacities (AWHC), and the cube root of the number of larval galleries (broods). Sites (N=2) were categorized as 'Low AWHC' and 'High AWHC' after comparing the AWHC of their soil samples (n=4 for each site). The effect of AWHC on the number of larval galleries is dependent on the concentration of phloem nitrogen, and vice versa. The shaded regions represent 95% confidence intervals based on the linear mixed model standard errors.

Chapter 4

Spatial characteristics of volatile communication in lodgepole pine (*Pinus contorta*) trees: evidence of kin recognition and intra-species support

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4.1 Abstract

Plant interactions using volatile organic compounds, particularly in the context of kin recognition have received considerable attention in recent years, but several discrepancies and conflicting results have restricted our understanding. I propose that some of these discrepancies in literature are in part due to integral spatial characteristics of sites, and plant attributes. Chemotypic plasticity is commonly used to characterize kin, particularly in conifers. I studied constitutive and induced monoterpene chemotypes of non-attacked lodgepole pine trees within 30 m radii of pine trees attacked by mountain pine beetle (*Dendroctonus ponderosae* Hopkins). I tested the effects of volatile compounds emitted from the attacked trees on the non-attacked trees by challenge inoculations with a mountain pine beetle associated fungus (*Grosmannia clavigera*). I found no relationship between constitutive monoterpene concentrations of the non- attacked trees and distance or direction from the attacked trees or site aspects. In contrast, the effects of volatile

compounds were evident after inoculations, depending on distance from the attacked trees and site aspects. However, these interactions only emerged among chemotypically related trees. These results suggest that plants discriminate between chemical cues from kin and strangers, and the emitters likely aid only chemotypically related plants by emitting specific blends of volatiles that can only be deciphered by the receiving kin. These results further demonstrate the importance of incorporating spatial characteristics of sites and plant attributes in studies aimed at investigating intra-species interactions using volatile organic compounds

Keywords: *Dendroctonus ponderosae*; *Grosmannia clavigera*; kin facilitation; phenotypic plasticity; plant communication; talking trees.

4.2 Introduction

Plants mediate aboveground intra- and inter-specific interactions, and respond to environmental stimuli by releasing volatile organic com- pounds (VOC) (Kessler and Baldwin 2002; Crepy and Casal 2015; Kollist et al. 2018). Most studies have focused on VOC-mediated mutualistic ecological interactions, such as pollination and dispersal (Heil and Karban, 2009; Troncoso et al. 2010; Lemaitre et al. 2012), however, little attention has been paid to the role of VOC on antagonistic interactions with herbivores and pathogens (D'Alessandro and Turlings 2006; Biere and Bennett 2013). The VOC-mediated interactions require an 'emitter', a 'receiver' and a 'field' where the exchange of information occurs (Baldwin and Schultz 1983; Kollist et al. 2018). Little is known about the role of heterogeneous field conditions in VOC-mediated interactions despite they affect the dispersal and concentrations of VOC plumes (Thistle et al. 2011; Lowman and Schowalter 2012; Zitouna-Chebbi et al. 2015).

Plant VOC-mediated responses are moderated by gene expression to elicit induced defenses (Kessler and Baldwin 2002), a phenomenon which involves the de novo expression of chemical traits at greater concentrations to control tissue damage (Yi et al. 2009; Karban and Maron 2011; Karban et al. 2014a). However, antagonists can spread to the nearby undamaged plant organs, initiating induced responses to be expressed systemically (Heil and Ton 2008; Yi et al. 2009). VOC emitted in response to antagonists may be transmitted either internally or externally by getting airborne as part of the indirect systemic induced defense (Baldwin and Schultz 1983; Dolch and Tscharntke 2000; Heil and Karban 2009; Yi et al. 2009; Karban and Maron 2011; Karban et al. 2014b). Since VOC move spontaneously in the air, they may also influence neighbouring non-attacked conspecifics mainly through stomatal uptake (Oikawa and Lerdau 2013). Thus, VOC-mediated communication involves volatile signaling by a plant that causes a response in the same or a different individual that receives the cue (Karban et al. 2014b). Studies focusing on VOC-mediated intra-species plant interactions have mainly compared such communications among strangers and kin (e.g., Karban et al. 2013) or explored the overall existence of kin support among genetically identical plants (e.g., Karban and Shiojiri 2009).

Presently, I lack evidence of VOC-mediated plant-plant interactions in forest trees. A major barrier to assessing such interactions in trees arises from the complex forest conditions (e.g., density, slope, aspect) as well as tree attributes (e.g., age, size) that prevent us from detecting differences in tree responses. In addition, because airborne VOC are carried by the wind, their dispersal, and thus, their concentrations depend on the distance between the interacting plants, as well as wind direction and speed (Barbosa et al. 2009; Song et al. 2010). The exact concentrations (Baldwin et al. 2006; Kessler et al. 2006) or distance (Dolch and

Tscharntke 2000; Karban 2001; Song et al. 2010) at which attacked plants ultimately regulate a non-attacked plant's defensive response remain, largely unknown. However, to verify the ecological relevance of such interactions among trees, it is imperative to validate them in their natural growing conditions (Baldwin et al. 2006).

Since closely related plant species are more likely to host common antagonists, further research has exposed the complex, yet cooperative nature of chemical interactions among plants (Baldwin and Schultz 1983; Dudley and File 2007; Barbosa et al. 2009; Heil and Karban 2009; Karban and Shiojiri 2009; Crepy and Casal 2015). Nevertheless, these interactions depend on the physiologically active VOC concentrations. However, in a chemotypically diverse community, neighbouring plants may respond differentially even if exposed to VOC cues of equal concentrations (Bruin and Dicke 2001; Heil and Karban 2009). Therefore, while assessing population-wide variations in VOC-mediated plant responses, signature patterns may emerge when chemotypic plasticity exhibited by conspecific plants is brought into context, which from an evolutionary perspective functions to counter adaptations by herbivores and pathogens (Heil and Karban 2009; Karban et al. 2014a; Taft et al. 2015).

The VOC emissions of some plants have been reported to cluster into chemotypes, defined as chemically distinct but morphologically similar individuals of a species within a population (Keefover-Ring et al. 2009; Pieruschka and Schurr 2019). The complex ecological relationship between host chemistry and antagonists suggests the relevance of understanding the phytochemical aspect of multiple chemotypes to interpret VOC-mediated plant communication (Karban and Shiojiri 2009; Keefover-Ring et al. 2009; Karban et al. 2014a; Taft et al. 2015; Pieruschka and Schurr 2019). If kin facilitation occurs, individual plants may respectively

increase their survival through improving their defense responses prior to the arrival of the expected antagonists (Axelrod and Hamilton 1981; Waldman 1988; Dudley and File 2007; Karban et al. 2013; Crepy and Casal 2015). However, to our knowledge, no studies have yet tested VOC-mediated communication, kin recognition or support in pines against bark beetles.

The recent unprecedented range expansion by mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae) in western North America (Erbilgin 2019) motivated me to study the roles of VOC in influencing the induced defenses of mature lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) trees. Across its natural range, lodgepole pine monoterpenes are known to persist in different chemotypes at constitutive level, including β-phellandrene, β-pinene and five rare chemotypes (Forrest 1981). Spatial variations in monoterpene concentrations in response to antagonists (i.e., induced defenses) in lodgepole pine are also known to exist (Clark et al. 2014). However, how these chemotypic variations at the constitutive and induced levels and spatial characteristics of attacked and non-attacked trees affect VOC-mediated communication in lodgepole pine, is unknown.

Beetles locate and land on suitable hosts, followed by boring through the outer tree bark, and inoculation of the phloem and xylem with symbiotic fungi, including *G. clavigera* (Six 2003). The trees confront the MPB attack with their constitutive defenses in the phloem (Erbilgin 2019). However, as the MPB colonization intensifies, the trees respond by producing induced defense compounds, followed by the formation of resin-filled necrotic lesions which comprises of local autolysis of parenchyma cells, and a further increase in the secretion of defense compounds, intended to render the phloem no longer suitable for larval or fungal development

(Keeling and Bohlmann 2006). Therefore, comparing monoterpene concentrations is pertinent as they are the most abundant and vastly volatile organic defense compounds in the oleoresins of conifers (Trapp and Croteau 2001; Keeling and Bohlmann 2006) and biologically the most important groups against MPB (Erbilgin 2019). Similarly, lesion length in the attacked trees is considered a good predictor of resistance to a pathogen, and smaller lesions are reported to indicate more efficient defenses (Goodsman et al. 2013; Erbilgin 2019).

I conducted a field survey of trees to retrospectively deduce whether VOC emitted from central trees attacked and killed by MPB have affected the neighbouring non-attacked trees across heterogeneous forest conditions by comparing their constitutive and induced chemistry in relation to the distance and direction to the central trees in the same stands. I pursued these research questions. (1) How stable are lodgepole pine chemotypes at constitutive level? (2) Is herbivory informing communication general in lodgepole pine, or only effective in individuals of related chemotypes? (3) Can site aspects and distance between the attacked and non-attacked trees influence such communication? I hypothesized that lodgepole pine trees will exhibit further chemotypic plasticity when challenged and that VOC-mediated interactions will be more pronounced in chemotypically related trees; however, the overall response will depend on the integral spatial characteristics of sites, and tree attributes. As a proxy to MPB and to simulate induced tree chemical defenses, I inoculated the non-attacked trees with live *G. clavigera*, and as evidence of direct communication in lodgepole pines, I compared lesion lengths formed as a result of subsequent fungal infections (Goodsman et al. 2013).

4.3 Material and methods

4.3.1 Experimental design and sampling

On 13-14 June 2016, I selected non-attacked mature lodgepole pine trees (*N*=201) on five sites within 30 m radii of individual trees (*N*=39) that were attacked by MPB in 2015 in Jasper National Park, Alberta (Suppl. Table 4.1). On each site, I established sub-sites by measuring the distances and directions of all non-attacked trees (focal trees hereafter) from their corresponding nearest attacked tree (central tree hereafter). I conducted this field experiment just before MPB emergence from the central trees, allowing the neighbouring focal trees to be exposed to the VOC from the central trees for at least a year. I identified MPB attacked trees by the presence of pitch tubes and verified successful beetle attacks on the selected trees by the presence of larval galleries, pupal chambers, and fungal staining around the oviposition galleries (Erbilgin et al. 2017a). The non-attacked trees were free of any biotic stress based on external aboveground visual signs and symptoms.

The sites either had an east or west aspect, with slopes roughly 20-25% and elevations ranging from 1,209 m to 1,461 m (Suppl. Table 4.1). The mean diameter at 1.3 m of the central and focal trees were 27.12 cm (±0.71 SE) and 24.34 cm (±0.32 SE), respectively. Because VOC-mediated plant interactions have been reported to occur over relatively shorter distances (Dolch and Tscharntke 2000; Karban et al. 2006), I categorized my focal trees in three concentric circles (0-10 m, 10-20 m, and 20-30 m) to detect any spatially distinguishable tree responses. Similarly, VOC plume dispersal is a random process that shows an outward expansion due to factors like wind direction and speed (Song et al. 2010; Thistle et al. 2011), therefore, I also categorized the focal trees in four intercardinal directional groups (NE, SE, SW, and NW). This also enabled me

to account for the non-uniform and sparse distribution of the focal trees at finer spatial or directional scales.

I collected four 1 cm diameter phloem tissue samples (two from north and two from south aspects) from the focal trees at 1.3 m stem heights. I inoculated the focal trees with a single isolate of *G. clavigera* (EL033) on the north aspect by placing 0.9 cm diameter circular fungus-agar plugs in the two bore holes created during tissue collection with the mycelia facing the sapwood (Goodsman et al. 2013). The fungus had been originally isolated from blue-stain sapwood between MPB larval galleries in mature lodgepole pine trees.

Six weeks later (25-26 July 2016), I exposed the lesions induced by the subsequent fungal infection by removing the outer tree bark, measured their lengths, and collected one (1 cm x 2 cm) sample from each of the two lesions to study the local induced chemical defenses. At the same time, I also collected two samples (1 cm diameter) from non-necrotic phloem tissues adjacent to the edges of fungal inoculation bore marks (about 4 cm away from the lesions). I pooled samples together from each tree by tissue type (i.e., phloem or lesion) and sampling round (constitutive, induced), wrapped them in aluminum foils, and flash froze them in liquid nitrogen before storing them at –40°C in the laboratory.

4.3.2 Monoterpene analysis

I ground the combined lesion, and phloem samples from each tree in liquid nitrogen with a cryogrinder (SPEX Sample Prep Freezer Mill 6770, Metuchen, NJ, USA), and then stored at −40°C. I extracted monoterpenes from 100 mg (±2) of ground tissue twice with 0.5 ml dichloromethane (Sigma-Aldrich, St Louis, MO, USA) with 0.004% tridecane (Sigma-Aldrich) as surrogate standard at room temperature, as described in (Erbilgin et al. 2014). Briefly, I

vortexed samples for 30 s at 3,000 rpm, sonicated for 10 min, and centrifuged for 15 min at 0°C and 13,000 rpm, and kept at −40°C for at least 2 h. I transferred the extracts to 2 ml gas chromatograph (GC) vials and stored at −40°C until analysis.

For the analysis, I injected 1 µl of extracts with a 10:1 split ratio into a GC fitted with an enantioselective column (HP Chiral 20β; ID 0.25mm, length 30m; Agilent Tech. Santa Clara, CA, USA) and coupled to a Mass Spectrometer (GC-MS; GC: 7890A, MS: 5975C, Agilent Tech.). I used helium as the carrier gas at a flow rate of 1.1 ml min^{-1}, and the temperature program included four ramps, starting at 50°C (held for 5 min), then 75°C min^{-1} to 75°C (held for 3 min), then 1.5°C min^{-1} to 100°C (held for 30 s), then 60°C min^{-1} to 200°C (held for 0 min), and then 25°C min^{-1} to 250°C (held for 0 min). I identified the peaks by using the following standards: (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, (−)-camphene, (+)-camphene, myrcene, (S)-(−)-limonene, (R)-(+)-limonene, 3-carene, terpineol (chemical purity >90%), γ-terpinene (>97%), (+)-cymene, sabinene, β-thujone (enantiomeric ratio of 92.5/ 7.5), pulegone (>97%), terpinolene (>90%), borneol, α-terpinene (>95%) (Sigma-Aldrich), *cis*-ocimene (>90%, SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (>90%, Erbilgin laboratory). I identified compounds by comparing their retention times and mass spectra with those of the standards and quantified their concentrations through calibration curves generated from analyses of a serial of four dilutions of known quantities of standards and calculated as µg of compound per mg of wet weight (WW) of tissue.

4.3.3 Data analysis

I used R v3.4.4 (R Core Team 2017) for all statistical analyses. I first calculated descriptive statistics, and then checked data for the assumptions of homoscedasticity and normality by using

Levene's and Shapiro–Wilk tests, respectively, and where necessary, I transformed data prior to analyses. I performed separate tests for defense compounds at constitutive, induced-phloem, and induced-lesion levels, and lesion lengths. My statistical models included Permutational Multivariate Analyses of Variance (PERMANOVA, permutations = 9,999, method = Gower) for multivariate analyses (Oksanen et al. 2017), followed by univariate analyses using either ANOVAs or *t*-tests, and mixed models for lesion lengths.

For the identification of different chemotypes based on the constitutive, induced-phloem, and induced-lesion monoterpene concentrations, I used the *pamk* function of R package *fpc* to determine the optimal number of clusters (Hennig 2018), followed by proportion tests to compare percent representation of each chemotype in each cluster. I also compared the means of monoterpene concentrations between the test groups (chemotypes) using two-sample *t*-tests or one-way ANOVA to confirm differences between chemotypes.

Because of the circular nature of my sampling scheme, I constructed bivariate polar plots in the R package *openair* to visualize statistically different results (Carslaw and Ropkins 2012). I performed PERMANOVAs with the *adonis* function in the R package *vegan* (Oksanen et al. 2017) and used linear mixed models with the *lmer* function in the R package *lme4* (Bates et al. 2015). I constructed separate mixed models for each chemotype identified at constitutive level, and used lesion chemotypes, total monoterpene concentrations, site aspects, and distance and direction of the focal trees from the corresponding central tree as fixed effects, and sites as a random effect in which the constitutive chemotype for that model was nested. I conducted Tukey's HSD tests to examine pair-wise differences for significant main effects or interactions. I

used an alpha level of 0.05 for all statistical tests and constructed all graphs by using raw and non-transformed data.

4.4 Results

4.4.1 Chemotypes and spatial characteristics of focal trees before fungal infection

Constitutive monoterpene concentrations of the focal trees clustered in Low and High β-phellandrene chemotypes that represented 66.66% and 33.33% of the focal trees, respectively [proportion test, $P<0.001$] (Fig. 4.1). I found no correlations among monoterpene concentrations, site aspects, direction or distance of the focal trees from their central trees, or any variations among sites for any of the two chemotypes (Suppl. Table 4.2).

4.4.2 Chemotypes and spatial characteristics of focal trees after fungal infection

Induced monoterpene concentrations in the phloem tissues of the focal trees in the High β-phellandrene chemotype at constitutive level further clustered in two distinct myrcene chemotypes (Fig. 4.2a). The Low and High myrcene chemotype represented 80.60% and 19.40% of the focal trees, respectively [proportion test, $P<0.001$]. Similarly, induced monoterpene concentrations in the phloem tissues of the focal trees in the Low β-phellandrene chemotype at constitutive level further clustered in two distinct 3-carene chemotypes (Fig. 4.2b). The Low and High 3-carene chemotypes represented 60.45% and 39.55% of the focal trees, respectively [proportion test, $P<0.001$]. However, I found no statistical correlations among induced monoterpene concentrations, site aspects, direction or distance of the focal trees from their central trees, or any variations among sites for any of the four induced chemotypes (Suppl. Table 4.3).

4.4.3 Lesion monoterpene chemotypes, spatial characteristics, and lesion lengths

Lesion monoterpene concentrations in the lesion samples of the focal trees in the High β-phellandrene chemotype at constitutive level further clustered in High and Low 3-carene chemotypes (Fig. 4.3a). The Low and High 3-carene chemotypes represented 70.15% and 29.85% of the focal trees, respectively [proportion test, $P<0.001$]. Similarly, lesion monoterpene concentrations in the lesion samples of the focal trees in the Low β-phellandrene chemotype at constitutive level further clustered in (−)-β-pinene, myrcene, and 3-carene chemotypes. The (−)-β-pinene chemotype represented 45.53%, the myrcene chemotype represented 33.58%, and the 3-carene chemotype represented 20.89% of the focal trees respectively (Fig. 4.3b).

I found no significant correlations between lesion monoterpene concentrations, direction or distance of focal trees from their central trees, or any variations among sites for the Low and High 3-carene chemotypes in the High β-phellandrene chemotype, or 3-carene chemotype in the Lowβ-phellandrene chemotype (Suppl. Table 4.4). However, I found significant correlations between lesion monoterpene concentrations and site aspects in the lesion myrcene chemotype, and distance from the central trees and site aspects in the lesion (−)-β-pinene chemotype (Suppl. Table 4.4).

For the lesion myrcene chemotype, I found significantly higher concentrations of myrcene, (−)-α-pinene, (−)-camphene, (+)-camphene, and total monoterpenes in the lesion samples collected from the focal trees on the west-facing sites (Fig. 4.4, Table 4.1). For the lesion (−)-β-pinene chemotype, the concentrations of β-phellandrene, myrcene, (+)-limonene, (−)-α-pinene, (−)-camphene, and total monoterpenes decreased with an increase in the distance from the central trees, whereas the concentration of (−)-β-pinene increased (Fig. 4.5, Table 4.2).

I did not find any differences in the lesion lengths for any of the lesion chemotypes, or their correlation with total lesion monoterpene concentration, site aspects, direction or distance of the focal trees from their green attack trees, or variations among sites (Suppl. Table 4.5). Chemotypic expression and their percent representation in constitutive phloem, induced phloem, and lesion tissue samples have been summarized in (Suppl. Fig. 4.1).

4.5 Discussion

I identified two distinct monoterpene chemotypes in lodgepole pine at constitutive level, characterized by low or high concentrations of β-phellandrene, in agreement with Forrest (1981). I anticipated such variations because an environmental change within the range of a plant species decreases the likelihood of a single chemotype to persist and show equal resilience under an environment of predictable challenges (Via et al. 1995; Pieruschka and Schurr 2019). The coexistence of chemically heterogeneous forests is also a strong indication of genetic influence on chemotypic expression in lodgepole pine, supporting earlier conclusions that pine monoterpenes are in part genetically controlled (Hanover 1971; Forrest 1981; Clark et al. 2014; Taft et al. 2015).

I found an intensification of chemotypic plasticity in my focal trees in response to the fungal infection at induced levels. These results highlight the co-evolutionary roles of biotic pressures across the heterogeneous environments, driving intraspecific differentiation of the defensive metabolome in time and space (Via et al. 1995; Keefover-Ring et al. 2009; Karban et al. 2014a; Pieruschka and Schurr 2019). Therefore, as the defense compounds of plants can differentially influence the attacks by diverse antagonists, plants may have evolved to fine-tune their defenses against specific threats by further optimizing their chemotypes (Heil and Karban

2009; Hansen et al. 2012; Karban et al. 2014a; Taft et al. 2015; Erbilgin 2019; Zhao et al. 2019). The observed refined differentiation of chemotypic expression with the severity of fungal threat also suggests that pines potentially evolved by facing an array of selective pressures, enabling them to favor one chemotype over the other under specific conditions in their dynamic life-long environments. Such a chemical polymorphism in plants is critical for reciprocal organismal natural selection (Via et al. 1995, 1998; Agrawal 2011; Mithöfer and Boland 2012; Taft et al. 2015; Bamba et al. 2019).

Interestingly, I also found significant correlations of monoterpene concentrations with distance from the central trees and site aspects in two chemotypes identified in induced-lesion monoterpenes. In the (−)-β-pinene chemotype, concentrations of total and some individual monoterpenes, such as β-phellandrene, (−)-α-pinene, myrcene, and (+)-limonene decreased, whereas the concentration of (−)-β-pinene increased with distance from the central trees. These results suggest that VOC-mediated communications in pines can occur, but the mechanisms are likely spatially constrained, and therefore, may be very fine-grained. My results are consistent with the findings of Dolch and Tscharntke (2000) who found alder (*Alnus glutinosa*) defoliation induced defenses only in the nearby plants, but the response was greatly concentrated within a few meters of the damaged tree.

Although the roles of site aspects or distance from central trees in the VOC-mediated communication are not fully understood, VOC plume dispersal in plants is known to be multidimensional and a complex process which heavily depends on the ambient environment (Baldwin et al. 2006; Thistle et al. 2011; Lowman and Schowalter 2012). Therefore, factors such as wind speed or direction, and the intricate mosaic of solar insolation due to topographic and

surface aerodynamic properties may potentially influence VOC plume dispersal (Barbosa et al. 2009; Thistle et al. 2011; Zitouna-Chebbi et al. 2015). In fact, these landscape features can be linked to the diurnal and nocturnal variations observed in VOC concentrations in angiosperms (e.g., De Moraes et al. 2001; Loughrin et al. 2006); the downwind enhanced induced resistance in neighbouring plants (Karban 2001) or greater VOC dispersal in tall plants (Lowman and Schowalter 2012). In the current study, I only observed these interactions in chemotypically identical focal lodgepole pine trees, suggesting kin facilitated VOC communication.

As chemotypes are heritable, they are reasonably a reliable way to predict relatedness in plants (Hanover 1971; Axelrod and Hamilton 1981; Karban et al. 2014a). Therefore, the patterns observed in the responses of chemotypically related trees in my study may highlight an important mechanism in pines, i.e., to recognize and support kin by keeping the VOC-mediated communication very discrete within the family (Baldwin and Schultz 1983; Waldman 1988; Dudley and File 2007; Barbosa et al. 2009; Heil and Karban 2009; Karban and Shiojiri 2009; Karban et al. 2014a).

The ability of kin recognition in order to cooperate is prevalent across all taxa (Lizé et al. 2006; Waldman 1988; Karban et al. 2013; Crepy and Casal 2015). Surprisingly, most studies focusing on kin recognition and support in plants have looked at belowground responses in environments of competitive interactions and niche partitioning. For example low competition for resources in *Cakile edentula* when planted with siblings (Dudley and File 2007); interspecific genetic material exchange in plants via mycorrhizal connections (Giovannetti et al. 2004); greater mycorrhiza-mediated carbon sharing in roots of Douglas-fir siblings (Pickles et al. 2017), and conspecific facilitation of younger trees by older trees (Beiler et al. 2010). However, my

results show that pines may also have the ability of kin recognition and cooperation by using the aboveground VOC cues.

Since I did not sample the central trees prior to MPB colonization, I cannot speculate that the central and focal trees were chemotypically similar or hence within the same kinship, this may limit our interpretation of my results. Nevertheless, consistent results across sites suggest the importance of incorporating spatial characteristics of sites and tree attributes in studies aimed at investigating intra-species interactions using volatile organic compounds.

Whether to term the differences observed in my study 'communication' or 'kin recognition and support' is currently lacking consensus in the literature (Dudley and File 2007; Scott-Phillips 2008; Crepy and Casal 2015). Nevertheless, an interactive neighbourhood could reduce potential losses due to antagonists (Baldwin and Schultz 1983; Barbosa et al. 2009; Heil and Karban 2009; Karban and Shiojiri 2009). Although it is not clear which VOC elicit induced responses, it is commonly thought that the interacting kin have similar VOC profiles, thereby the high chemical incompatibility exhibited by strangers makes it difficult for them to decipher the critical airborne information (Karban et al. 2013). These abilities in interacting individuals have been linked to evolution and speciation potential through natural selection (Platt and Bever 2009; Gardner and West 2010). Additional studies with spatially-explicit models and genetic markers are needed to further substantiate my findings.

Table 4.1 Mean concentrations and enantiomeric ratios of defense compounds in lesion samples of myrcene chemotype (*N*=45) characterized in the Low β-phellandrene chemotype (at constitutive level) of *Pinus contorta* trees sampled at five sites in Jasper National Park. Non-attacked trees were categorized in East (*n*=10) or West (*n*=35) facing aspects around their corresponding *Dendroctonus ponderosae* attacked central trees (*N*=22). *P*-values significant at α=0.05.

Compounds	Mean (SE) concentration (μg mg^{-1} FW)			
	East-facing	West-facing	$t_{(df)}$ †	*P-value*
β-phellandrene	37.68 (3.72)	42.77 (1.60)	$1.40_{(43)}$	0.165
Myrcene	9.09 (0.93) b	12.42 (0.42) a	$3.56_{(43)}$	<0.001
3-Carene	8.91 (2.77)	14.29 (1.82)	$1.31_{(43)}$	0.194
(–)-limonene	1.53 (0.13)	2.97 (0.61)	$1.26_{(43)}$	0.213
(+)-limonene	0.34 (0.04)	0.71 (0.03)	$1.77_{(43)}$	0.083
(–)-β-pinene	9.67 (1.44)	14.05 (1.74)	$0.88_{(43)}$	0.383
(–)-α-pinene	2.50 (0.27) b	3.47 (0.22) a	$2.57_{(43)}$	0.013
(+)-α-pinene	1.44 (0.19)	3.11 (0.53)	$1.58_{(43)}$	0.121
4-allylanisole	0.56 (0.27)	0.50 (0.06)	$0.64_{(43)}$	0.524
Terpinolene	0.68 (0.18)	1.04 (0.12)	$1.49_{(43)}$	0.142
(–)-camphene	0.24 (0.02) b	0.33 (0.01) a	$2.89_{(43)}$	0.005
(+)-camphene	0.09 (0.02) b	0.16 (0.01) a	$2.84_{(43)}$	0.006
γ-terpinene	0.10 (0.02)	0.13 (0.01)	$1.02_{(43)}$	0.310
p-cymene	0.12 (0.01)	0.14 (0.01)	$1.81_{(27.99)}$ †	0.081
Total monoterpenes	72.97 (6.70) b	95.80 (3.14) a	$3.32_{(43)}$	0.001

(–):(+)-α-pinene	81.02 (11.19)	90.01 (19.80)	0.04 $_{(36.03)}$ †	0.963
(–):(+)-limonene	368.25 (37.01)	638.96 (152.43)	0.92 $_{(28.62)}$ †	0.363

†Welch's *t*-test (when homogeneity of variance was not equal).

Table 4.2 Mean concentrations and enantiomeric ratios of defense compounds in lesion samples of the (–)-β-pinene chemotype of *Pinus contorta* trees (*N*=61) sampled at five sites in Jasper National Park. Non-attacked focal trees were categorized in 0-10 m, 10-20 m, 20-30 m distances from their corresponding *Dendroctonus ponderosae* attacked central trees (*N*=26). *P*-values significant at α=0.05. df=2.

Compounds	Mean (SE) concentration (μg mg^{-1} FW)			ANOVA	
	0-10 m	10-20 m	20-30 m	*F*	*P-value*
β- phellandrene	79.85 (3.02) a	69.09 (1.63) b	69.03 (2.57) b	6.178	0.003
Myrcene	18.91 (0.55) a	15.97 (0.35) b	17.92 (0.59) ab	10.95	<0.001
3-carene	8.22 (1.19)	7.14 (0.87)	8.77 (2.08)	0.11	0.895
(–)-limonene	4.93 (1.03)	4.42 (0.76)	6.49 (1.98)	0.80	0.453
(+)-limonene	0.80 (0.03) a	0.63 (0.02) b	0.68 (0.04) ab	12.12	<0.001
(–)-β-pinene	19.89 (1.85) ab	14.78 (1.85) b	24.38 (4.52) a	3.42	0.039
(–)-α-pinene	5.59(0.26) a	4.49 (0.20) b	6.03 (0.63) a	7.15	0.001
(+)-α-pinene	3.53 (0.33)	2.55 (0.21)	2.88 (0.44)	2.51	0.089
4-allylanisole	1.07 (0.17)	0.62 (0.08)	0.95 (0.29)	1.65	0.201
Terpinolene	0.79 (0.09)	0.60 (0.05)	0.87 (0.16)	1.49	0.234
(–)-camphene	0.57 (0.02) a	0.47 (0.01) b	0.53 (0.03) ab	11.81	<0.001
(+)-camphene	0.23 (0.01)	0.19 (0.01)	0.23 (0.04)	2.04	0.139
γ-terpinene	0.12 (0.01)	0.09 (0.01)	0.11 (0.02)	1.55	0.221
p-cymene	0.19 (0.01)	0.19 (0.01)	0.16 (0.02)	0.64	0.527
Total monoterpenes	144.75 (4.01) a	121.25 (2.12) b	139.03 (4.26) a	14.73	<0.001
(–):(+)-α-pinene	89.28 (16.56)	97.30 (13.89)	144.90 (39.02)	1.46	0.239

(–):(+)-limonene	537.74 (134.16)	691.29 (179.24)	838.32 (266.78)	1.05	0.355

Suppl. Table 4.1 Details of site characteristics of lodgepole pine (*Pinus contorta* var. *latifolia*) forests selected for the study.

Site	Aspect	Elevation (m)	Central (ID)	Focal (*n*)	Location
1	East	1280	A	8	52.766533, -118.025617
			B	2	
			C	7	
			D	9	
			E	5	
			F	2	
			G	3	
			H	4	
			I	2	
2	East	1209	A	2	52.774233, -118.029467
			B	2	
			C	4	
			D	2	
			E	2	
			F	4	
			G	7	
			H	6	
3	West	1461	A	7	52.838967, -117.718467
			B	9	
			C	2	
			D	8	
			E	8	
			F	3	
			G	2	
			H	7	
			I	6	
4	West	1261	A	2	52.918918, -118.090646
			B	2	
			C	4	
			D	8	
5	West	1246	A	7	52.916625, -118.084935
			B	4	
			C	9	
			D	9	
			E	7	
			F	6	
			G	7	
			H	7	
			I	6	

Suppl. Table 4.2 Results of the four-way PERMANOVAs comparing differences in the concentrations of constitutive monoterpenes in the Low (*N*=134) and High (*N*=67) β-phellandrene chemotypes of *Pinus contorta* trees on field sites (*N*=5). *P*-values are significant at α=0.05. Subscript numbers indicate the numbers of residuals of each *F* test.

Chemotypes	Factors	df	SS	MS	R^2	*F-value*	*P- value*
	$Aspects_{124}$	1	0.01	0.01	0.01	1.42	0.197
Low β-	$Direction_{124}$	3	0.01	0.00	0.01	0.56	0.891
phellandrene	$Distance_{124}$	2	0.01	0.00	0.00	0.58	0.812
	$Sites_{124}$	3	0.05	0.01	0.03	1.70	0.071
	$Aspects_{57}$	1	0.00	0.00	0.00	0.37	0.897
High β-	$Direction_{57}$	3	0.07	0.02	0.05	1.23	0.231
phellandrene	$Distance_{57}$	2	0.02	0.01	0.02	0.68	0.749
	$Sites_{57}$	3	0.06	0.02	0.04	1.08	0.358

Suppl. Table 4.3 Results of the PERMANOVAs comparing differences in the concentrations of induced monoterpenes and site aspects, direction and distance of the non-attacked *Pinus contorta* trees from their corresponding mountain pine beetle (*Dendroctonus ponderosae*) attacked trees, and variations among sites. *P*-values are significant at α=0.05. Subscript numbers indicate the numbers of residuals of each *F* test.

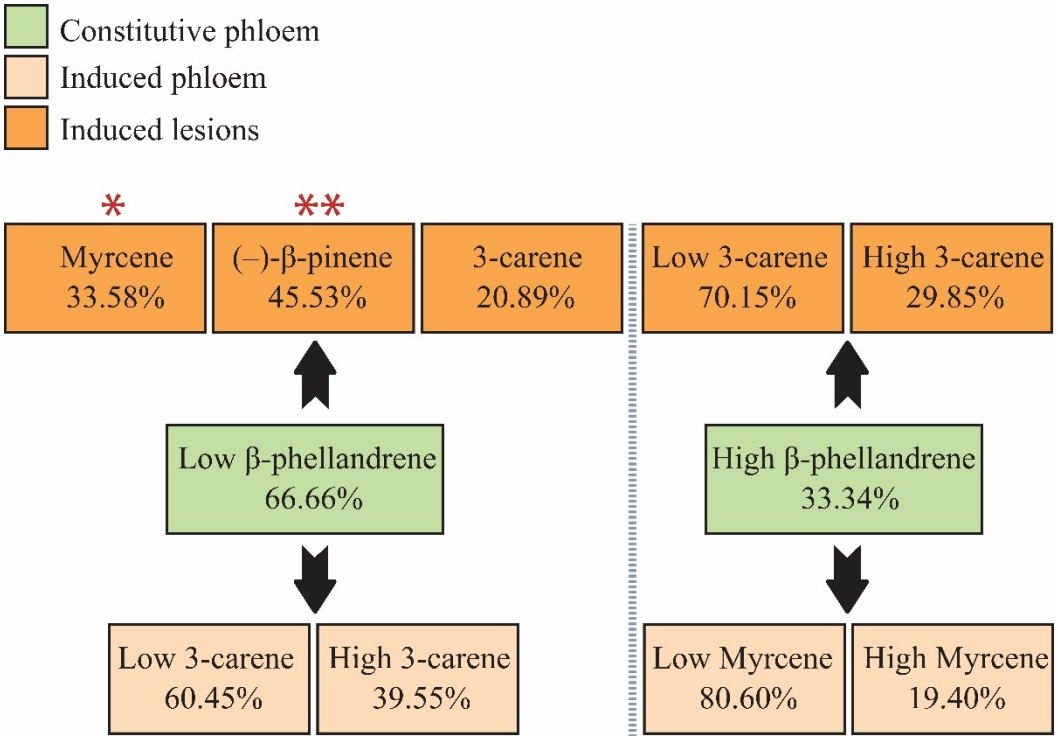
Chemotype (Constitutive)	Chemotype (Induced)	Factors	df	SS	MS	R^2	*F*	*P*
High β-phellandrene (*N=67*)	Low Myrcene	$Aspects_{44}$	1	0.02	0.02	0.02	1.06	0.369
		$Direction_{44}$	3	0.06	0.02	0.06	1.06	0.378
		$Distance_{44}$	2	0.02	0.01	0.02	0.61	0.798
		$Sites_{44}$	3	0.06	0.01	1.01	0.05	0.432
	High Myrcene	$Aspects_{3}$	1	0.02	0.02	0.03	0.90	0.480
		$Direction_{3}$	3	0.37	0.12	0.56	2.16	0.061
		$Distance_{3}$	2	0.08	0.04	0.12	1.47	0.267
		$Sites_{3}$	3	0.09	0.03	0.14	1.20	0.384
Low β-phellandrene (*N=134*)	Low 3-Carene	$Aspects_{71}$	1	0.02	0.02	0.01	1.25	0.255
		$Direction_{71}$	3	0.06	0.02	0.03	1.06	0.371
		$Distance_{71}$	2	0.02	0.01	0.01	0.71	0.729
		$Sites_{71}$	3	0.06	0.02	0.03	1.03	0.405
	High 3-Carene	$Aspects_{43}$	1	0.02	0.02	0.02	1.30	0.245
		$Direction_{43}$	3	0.05	0.01	0.06	1.10	0.319
		$Distance_{43}$	2	0.01	0.00	0.02	0.51	0.810
		$Sites_{43}$	3	0.04	0.01	0.04	0.84	0.460

Suppl. Table 4.4 Results of the PERMANOVAs comparing differences in the monoterpene concentrations in lesions and site aspects, direction and distance of the non-attacked *Pinus contorta* trees from their corresponding central trees, and variations among sites. *P*-values are significant at α=0.05. Subscript numbers indicate the numbers of residuals of each *F* test.

Chemotypes (Constitutive)	Chemotypes (Lesion)	Factors	df	SS	MS	R^2	*F*	*P*
High β-phellandrene (*N=67*)	Low 3-carene	$Aspects_{37}$	1	0.04	0.04	0.03	1.90	0.109
		$Direction_{37}$	3	0.11	0.03	0.09	1.48	0.130
		$Distance_{37}$	2	0.02	0.01	0.02	0.51	0.855
		$Sites_{37}$	3	0.11	0.03	0.08	1.43	0.159
	High 3-carene	$Aspects_{13}$	1	0.02	0.02	0.02	0.45	0.745
		$Direction_{13}$	3	0.12	0.04	0.12	0.66	0.726
		$Distance_{13}$	1	0.04	0.04	0.04	0.70	0.561
		$Sites_{13}$	1	0.04	0.00	0.00	0.07	0.985
Low β-phellandrene (*N=134*)	Myrcene	$Aspects_{35}$	1	0.12	0.12	0.08	4.22	0.003
		$Direction_{35}$	3	0.06	0.02	0.04	0.75	0.725
		$Distance_{35}$	2	0.10	0.05	0.06	1.60	0.089
		$Sites_{35}$	3	0.11	0.03	0.08	1.32	0.196
	3-carene	$Aspects_{18}$	1	0.09	0.09	0.10	2.71	0.061
		$Direction_{18}$	3	0.11	0.03	0.11	1.02	0.408
		$Distance_{18}$	2	0.06	0.03	0.06	0.89	0.485
		$Sites_{18}$	3	0.03	0.01	0.03	0.27	0.989
	(−)-β-pinene	$Aspects_{51}$	1	0.05	0.05	0.03	2.27	0.048
		$Direction_{51}$	3	0.11	0.03	0.06	1.51	0.104
		$Distance_{51}$	2	0.22	0.11	0.12	4.41	<0.001
		$Sites_{51}$	3	0.07	0.02	0.04	1.03	0.443

Suppl. Table 4.5 Results of the linear mixed model analyses comparing mean lesion lengths (cm) and their correlation with the concentration of total monoterpenes (µg mg^{-1} WW), site aspects (East or West), direction (NE, SE, SW and NW) and distance (0-10, 10-20 and 20-30 m) of the non-attacked focal trees (*N*=201) from their corresponding mountain pine beetle attacked central trees (*N*=39) in two lesion chemotypes originating from the High (*N=67*), and three originating from the Low (*N=134*) β-phellandrene chemotypes (at constitutive level) in *Pinus contorta* trees. *P*-values are significant at α=0.05.

Chemotype (constitutive)	Fixed effect	Wald χ^2 (*2*)	*P*
High β-phellandrene	Total monoterpenes	0.49	0.480
	Lesion chemotypes	2.67	0.101
	Aspects	1.91	0.166
	Direction	4.20	0.239
	Distance	3.39	0.182
Low β-phellandrene	Total monoterpenes	0.50	0.477
	Lesion chemotypes	2.89	0.234
	Aspects	0.30	0.582
	Direction	4.68	0.196
	Distance	1.85	0.395



Suppl. Fig. 4.1 Chemotypic expression of lodgepole pine trees and their percent representation in constitutive and induced phloem, and induced lesion samples. Chemotypes indicated with '*' and '**' correlated with site aspects, and distance from the central trees and site aspects, respectively. None of the other chemotypes correlated with any of these two variables.

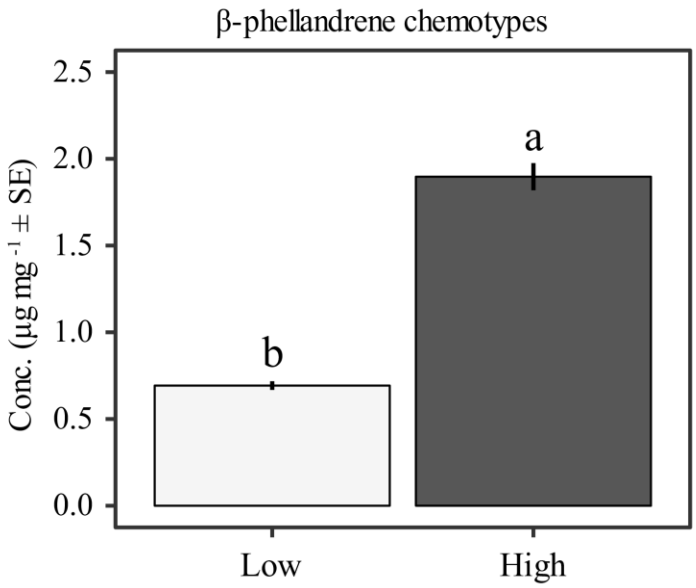


Fig. 4.1 Concentrations of β-phellandrene in the Low (*N*=134) and High (*N*=67) β-phellandrene chemotypes characterized in *Pinus contorta* trees on five field sites around central trees (*N*=39) in 30 m radii in Jasper National Park. Different letters indicate significant differences at α=0.05 in two-sample *t*-test, *P*<0.001.

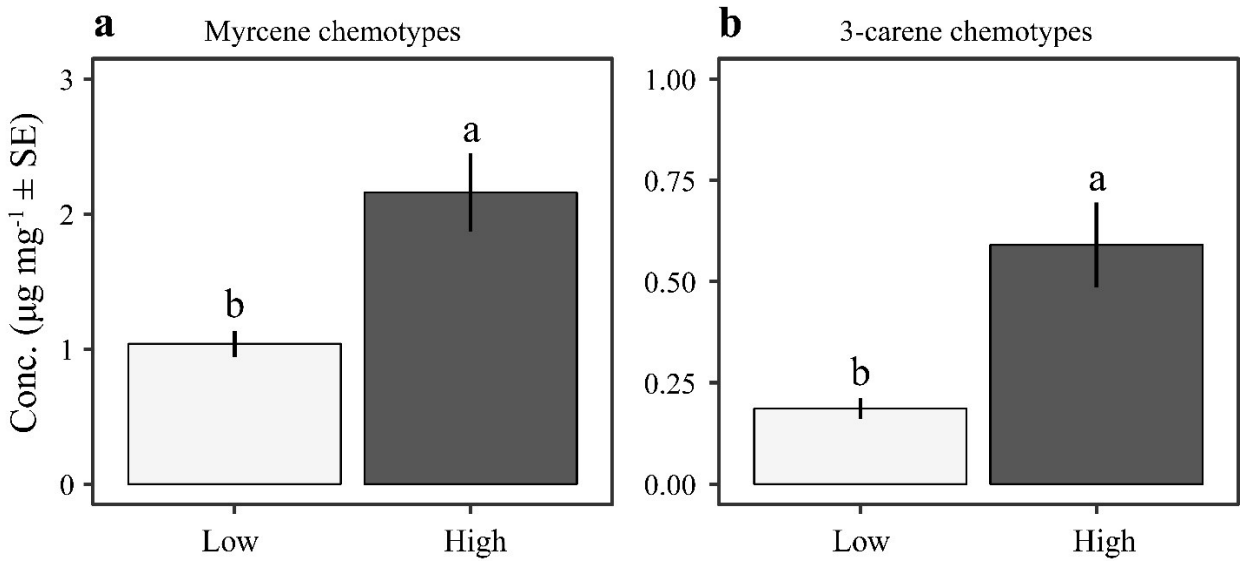


Fig. 4.2 Concentrations of induced myrcene and 3-carene chemotypes characterized in *Pinus contorta* trees on field sites around central trees in 30 m radii in Jasper National Park. a) The myrcene chemotype characterized in the High β-phellandrene chemotype (at constitutive level) had Low and High myrcene chemotypes (n=54 and 13 respectively). b) The 3-carene chemotype characterized in the Low β-phellandrene chemotype (at constitutive level) had Low and High 3-carene chemotypes (n=81 and 53 respectively). Different letters indicate significant differences at α=0.05 in two-sample t-tests, P<0.001 in both instances.

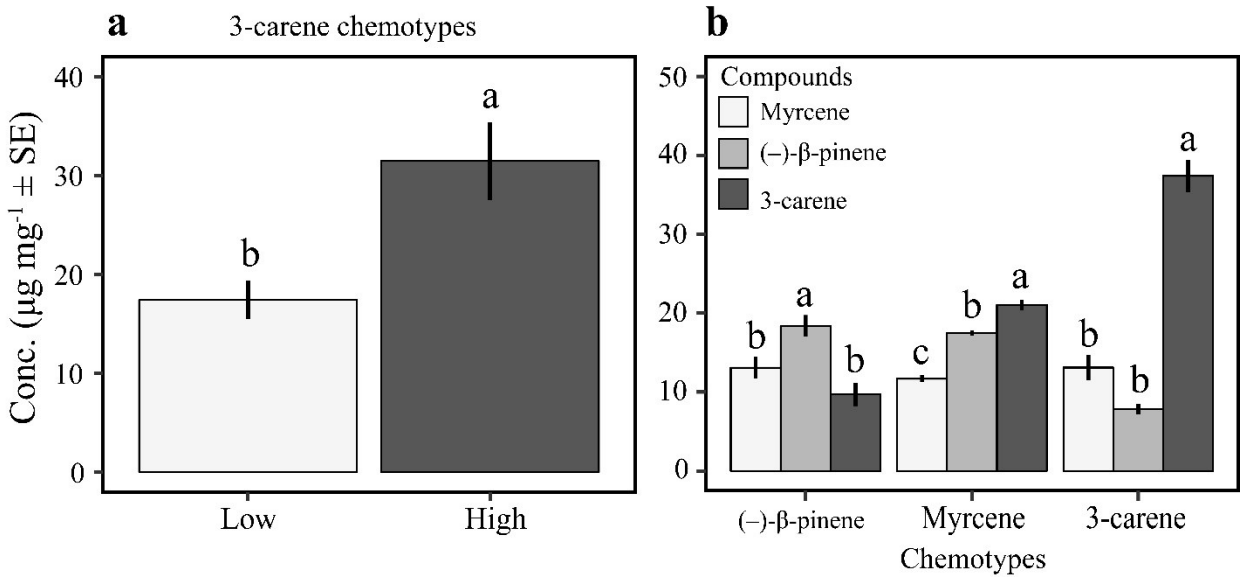


Fig. 4.3 Concentrations of 3-carene, (–)-β-pinene, and myrcene characterized in lesion samples of *Pinus contorta* in their respective chemotypes on field sites around central trees in 30 m radii in Jasper National Park. a) The 3-carene chemotype characterized in the High β-phellandrene chemotype (at constitutive level) had Low and High 3-carene chemotypes (n=47 and 20 respectively). b) The (–)-β-pinene chemotype (n=61), myrcene chemotype (n=45) and 3-carene chemotype (n=28) characterized in the Low β-phellandrene chemotype (at constitutive level). Different letters indicate significant differences at α=0.05, P<0.001 in all instances.

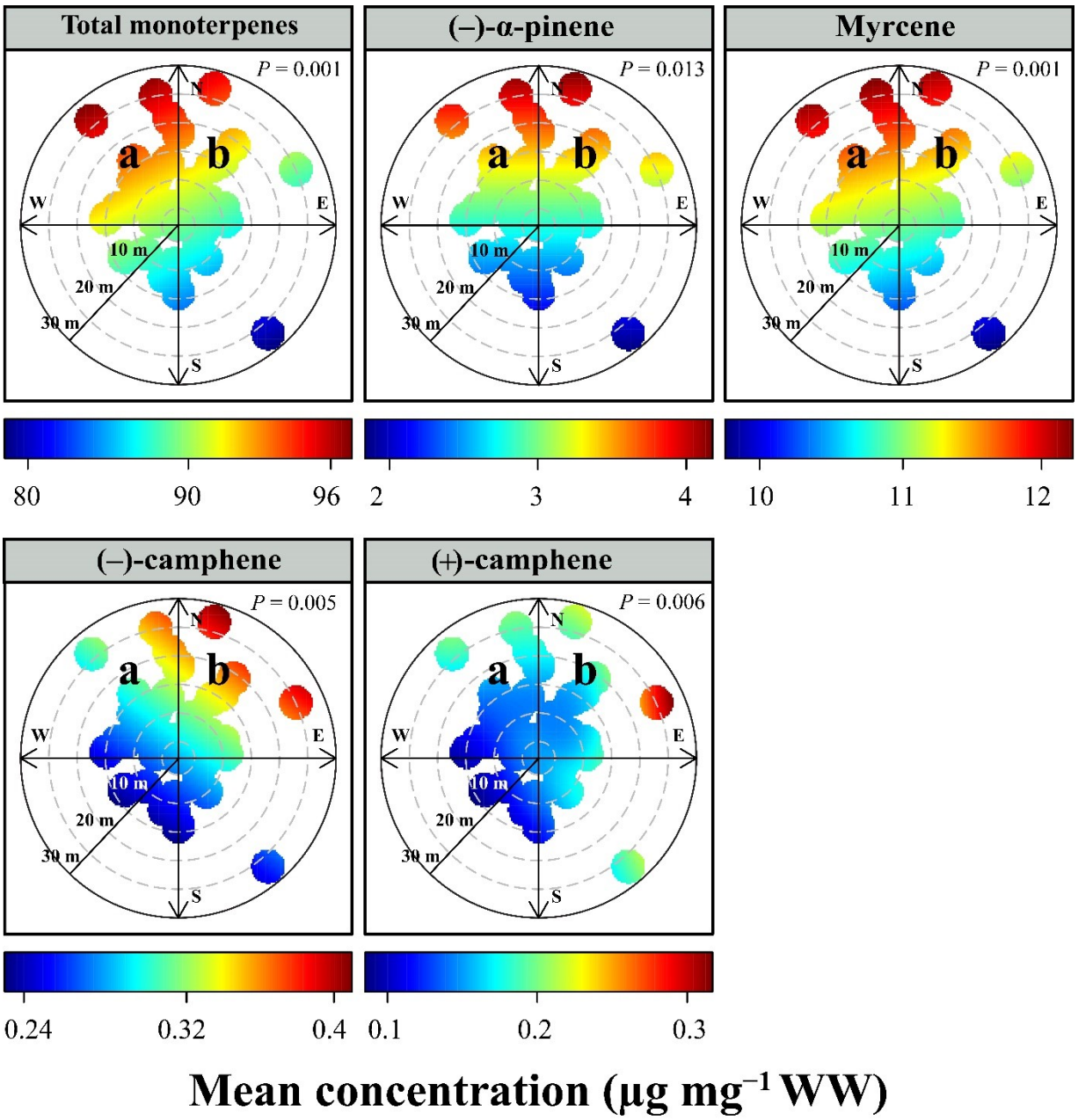


Fig. 4.4 Mean monoterpene concentrations (µg mg^{-1} WW) of focal *Pinus contorta* trees on east (*n*=10) and west (*n*=35) site aspects in the myrcene chemotype characterized in the Low β-phellandrene chemotype (at constitutive level) in Jasper National Park. Different letters indicate significant differences at α=0.05 in two sample *t*-tests, *P*<0.05 in all instances (Table 4.1).

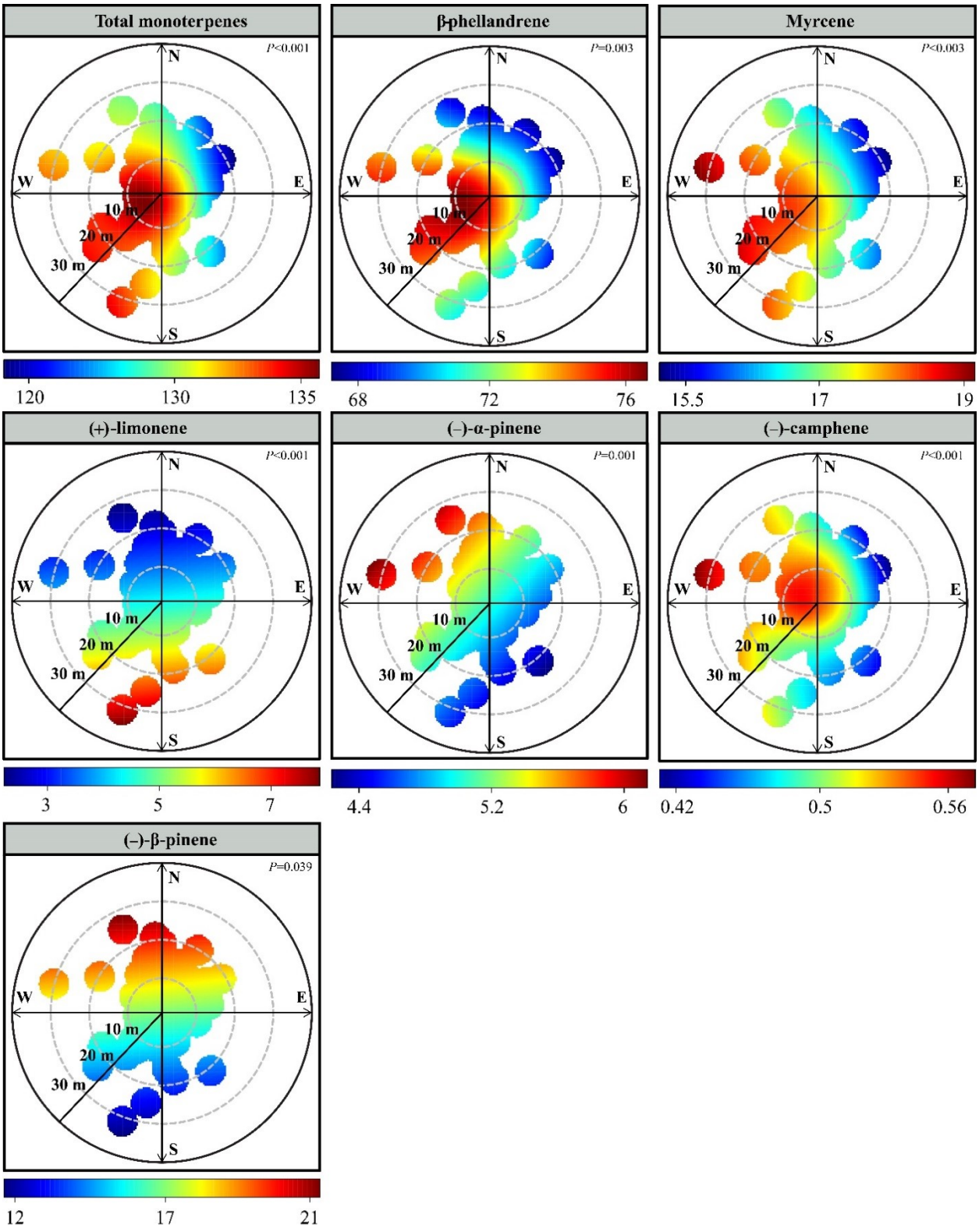

Total monoterpenes
P<0.001
N
W
E
S
10 m
20 m
30 m
120
130
135
β-phellandrene
P=0.003
68
72
76
Myrcene
P<0.003
15.5
17
19
(+)-limonene
P<0.001
3
5
7
(–)-α-pinene
P=0.001
4.4
5.2
6
(–)-camphene
P<0.001
0.42
0.5
0.56
(–)-β-pinene
P=0.039
12
17
21
Mean concentration (µg mg⁻¹ WW)

Fig. 4.5 Variations in mean lesion monoterpene concentrations (μg mg^{-1} WW) of focal *Pinus contorta* trees (*N*=45) and their correlation with distance from their corresponding central trees (*N*=22) and site aspects on east (*n*=10) and west (*n*=35) in the (–)-β-pinene chemotype characterized in the Low β-phellandrene chemotype (at constitutive level) at field sites in Jasper National Park. Different letters indicate significant differences at α=0.05 in one-way ANOVAs, *P*<0.05 in all instances (Table 4.2).

Chapter 5

Thesis Discussion

5.1 Major findings

My thesis demonstrates that soil water availability can influence susceptibility to biotic agents in jack pine (*Pinus banksiana*) by altering carbon mobilization and allocation to chemical defenses. In response to a mountain pine beetle (*Dendroctonus ponderosae*; MPB) associated phytopathogenic fungus (*Grosmannia clavigera*), jack pine trees develop smaller necrotic lesions on sites with higher water availability. These trees also mobilize their local non-structural carbohydrates (NSC) to the site of attack, resulting in higher concentrations of induced monoterpenes. I also show that jack pine trees do not distinguish between low and high fungal inoculation densities, as a proxy to low and high beetle attack densities respectively. Furthermore, my research highlights the role of soil water availability in altering host quality by influencing the nutritional value of the tree phloem: MPB host acceptance and brood production are greater in bolts from the site with lower water availability. I also demonstrate that lodgepole pine trees can interact with neighbouring conspecifics by using volatile organic compounds (VOC). I further show the variability in these interactions with the distance separating the interacting pines, and site aspects. Since these interactions only occur among pines that are chemotypically related, my findings provide evidence of VOC-mediated kin recognition and support.

5.2 Defense in jack pine is soil water dependent

Water is indispensable for plants due to its role in plant growth, cellular structure, and solute transportation, for example, nutrients. Water also serves as a raw material for various chemical processes, including photosynthesis and defense (Grote et al. 2009; Lusebrink et al. 2011, 2016; Arango-velez et al. 2016). In Chapter 2, jack pine trees respond to variations in soil water availability by mobilizing local NSC resources to induced defenses under potentially less water stressed conditions. This was supported by the observed reduced ability of the phloem to mobilize NSC from near the canopy (5 m) to the fungal inoculations at the lower stem (1.3 m) under low soil water conditions. These two results were further verified by the larger lesions formed by *G. clavigera* in the potentially water stressed trees. My results are consistent with the findings of Goodsman et al. (2013) who also reported similar relationships between chemical defenses and NSC in pines. Likewise, larger lesions indicate higher susceptibility in trees to fungal infections (Bonello et al. 2006; Krokene et al. 2008; Goodsman et al. 2013; Klutsch et al. 2017b). This relationship between soil water availability and defense suggests that jack pine may be more susceptible to *G. clavigera* and potentially MPB under water deficient conditions.

5.3 Jack pine trees may not distinguish different attack densities of MPB

My results demonstrate that *G. clavigera* inoculation density (4 vs 16) does not correlate with lesion areas, or influence concentrations of the induced defense compounds differentially under the tested growing conditions. Plants emit VOC upon attacks by herbivores and pathogens (Wood 1982; Blomquist et al. 2010; Erbilgin et al. 2014; Erbilgin 2019). Generally, the total amounts of VOC emitted by plants increase with the intensity and coordination of the attack, thereby informing the predators about the abundance of the herbivore (Shiojiri et al. 2010). Some insects may even sequester these VOC for their own benefit, for example, MPB biosynthesize

some of its aggregation and anti-aggregation pheromones from host monoterpene, α-pinene (Wood 1982; Blomquist et al. 2010).

In lodgepole pine, the historic host of MPB, the initial induction rate of the defensive monoterpenes (VOC) likely determines the successful colonization of MPB at low attack densities (Raffa and Berryman 1983; Raffa et al. 2008; Boone et al. 2011; Cale et al. 2017). However, the composition of herbivore-induced VOC shows little change with the number of the herbivores (Turlings et al. 1995; Horiuchi et al. 2003), suggesting that signal quantity may play an important role in the colonization behaviour of MPB. Therefore, factors affecting VOC concentrations, such as soil water availability may influence tree responses to fungal inoculation densities.

Moreover, phloem is a limited tree resource and overcrowding of the invading beetles may result in intraspecific competition which is also disadvantageous to the survival of the brood. Thus, the aggregation of additional beetles stops by the production of higher concentrations of the *exo*-brevicomin and frontalin pheromones by males upon achieving the maximum attack density (Raffa and Berryman 1983; Erbilgin et al. 2014; Taft et al. 2015) and verbenone by intestinal and gallery-inhabiting microbes in both sexes (Leufvén et al. 1984; Hunt and Borden 1989, 1990). I anticipated that the overall response to the fungal inoculation densities will differ in trees in different growing conditions and would be reflected by tree responses (i.e., defense compounds and lesion areas) to different fungal inoculation densities. However, my results suggest that jack pine lacks the capacity to discriminate between low and high intensity pressures from at least certain novel biotic agents and allocates resources and defenses equally, supporting earlier studies (Cale et al. 2017, 2019; Klutsch and Erbilgin 2018).

5.4 Soil water availability alters reproductive performance of mountain pine beetle

I found that soil water availability can alter the reproductive performance of MPB by influencing host acceptance and brood production in cut bolts of jack pine. This was expected as variation in soil water availability in space and time affect plant development and defense (Laiho et al. 2003; Deluca and Boisvenue 2012, Chapter 3). This variation may differentially influence the ability of plant roots to take up the nutrients available in the soil. For example, phloem nitrogen concentration varied in trees on sites with different soil water availability in my study (Chapter 3). In fact, a large body of evidence suggests that water related stresses in plants are a major underlying cause of herbivorous insect and pathogen outbreaks (Lieffers 1988; Jurgensen et al. 1997; Laiho et al. 2003; Deluca and Boisvenue 2012). For instance, bark beetles are mostly limited to stressed trees at endemic population densities (Safranyik et al. 2010). This can have serious consequences for the current MPB outbreak in the boreal jack pine forest due to the widespread water deficient growing conditions (De Jong and Shields 1988; Reynolds et al. 2007; Piedallu et al. 2011; Price et al. 2013). Beetles may rely on trees on these water deficient patches across the boreal forest to maintain endemic populations and continue spreading eastward.

Currently, studies are underway about the attack behavior and brood production of MPB in the endemic phase (Safranyik and Carroll 2006; Boone et al. 2011; Bleiker et al. 2014). I further show that an interaction between soil water availability and phloem nitrogen concentrations can influence MPB brood production. Nitrogen is essential for metabolic processes, cellular structure, and genetic coding in insect herbivores (Mattson 1980). Indeed, a lack of nitrogen severely affects growth and reproduction of bark beetles (Mattson 1980; Ayres et al. 2000; Goodsman et al. 2012).

5.5 Pines may recognize and support kin by using VOC to control MPB dispersal

My thesis demonstrates that pines recognize and support chemotypically related kin, potentially to curb insect dispersal and that these interactions are dependent on the integral spatial characteristics of sites, and plant attributes. These results may have important implications for understanding MPB dispersal at stand level. Mountain pine beetle dispersal occurs over short or very long ranges (Safranyik and Carroll 2006). Nevertheless, MPB dispersal ecology is the least understood mechanism (Chen and Walton 2011).

In my thesis, I focus on understanding factors influencing short range interactions among pines that can potentially influence MPB dispersal. It is well documented that insect dispersal over shorter ranges is mediated by host tree VOC emitted as a result of herbivory or pathogen attacks (Baldwin and Schultz 1983; Dolch and Tscharntke 2000; Heil and Karban 2009; Yi et al. 2009; Karban and Maron 2011; Karban et al. 2014b). However, as VOC disperse spontaneously in the air, they may also stimulate induced responses in the neighbouring non-attacked conspecifics (Baldwin and Schultz 1983; Heil and Karban 2009). In general, VOC-mediated plant-plant interactions are dependent on the composition and strength of the signal, the ability of the receiver to decipher the signal, and the ambient environment of the field that mediates the signal to the receiver (Baldwin and Schultz 1983; Sampaio et al. 2016; Kollist et al. 2018). Of these, signal strength and its composition are important traits to consider while understanding VOC-mediated interactions in pines.

Conifers tend to exist as different chemotypes (Keefover-Ring et al. 2009; Pieruschka and Schurr 2019). Therefore, in my study, I was expecting chemotypic distinctiveness in pine-pine interactions. Moreover, chemotypes are heritable traits, so they can also represent relatedness of

the pines (Hanover 1971; Axelrod and Hamilton 1981; Karban et al. 2014a). Therefore, the interaction observed in chemotypically related pines in my research (Chapter 4) may indicate kin recognition and support (Baldwin and Schultz 1983; Waldman 1988; Dudley and File 2007; Barbosa et al. 2009; Heil and Karban 2009; Karban and Shiojiri 2009; Karban et al. 2014a).

Similarly, VOC-mediated plant-plant interactions are known to vary with the distance between the interacting plants and spatial characteristics of the site, as observed in my study (Chapter 4). Since VOC interactions are dose dependent in the ambient environment (Bruin and Dicke 2001; Heil and Karban 2009), VOC strength may weaken with the outward expansion due to wind dispersal (Song et al. 2010; Thistle et al. 2011). Topographic and surface aerodynamic properties of the site may further influence VOC-mediated interactions by affecting solar insolation and energy balances at the landscape level (Barbosa et al. 2009; Thistle et al. 2011; Zitouna-Chebbi et al. 2015).

5.6 Limitations of the study systems

This thesis is a combination of several quantitative field studies. Hence, there are several important caveats and limitations that should be considered with respect to the conclusions drawn from the study systems presented here. First, jack pine cut bolts were used to study host-MPB interactions, which is a common method to investigate the role of host plant quality in determining beetle biology and ecology (e.g., Safranyik and Linton 1982; Nevill and Safranyik 1996; Erbilgin et al. 2014). However, unlike live trees, insects do not experience induced tree responses in cut bolts. This may have influenced my results in Chapter 3 as I stored the bolts at 4°C for one month before and two months after MPB inoculation. In another related study by Guevara-Rozo et al. (2019), we have demonstrated that nutrient and monoterpene concentrations

significantly vary with log-term storage (i.e., 3-6 months). These variations can potentially impact MPB larval development and pheromone production by mature beetles (Goodsman et al. 2012; Erbilgin et al. 2017a). It is worth mentioning here that all my studies were designed to comply with the provincial and federal legislations. Currently, MPB and jack pine do not readily co-occur. Therefore, experiments involving the two species need to be restricted to cut bolts or simulations by MPB associated fungi. Similar limitations have been reported by other studies that also focused on MPB and jack pine interactions (e.g., Lusebrink et al. 2013; Erbilgin et al. 2014; Klutsch et al. 2017a, b).

Second, all my experimental sites in the studies presented in Chapters 2 and 3 were selected in Lac la Biche, Alberta. In order to better understand beetle performance across jack pine's extremely diverse range, I would design sampling scheme to select trees from across the range of jack pine, representing greater variations in the edaphic conditions.

Third, I used *G. clavigera* as a proxy for MPB in jack pine. However, *G. clavigera* and jack pine likely lack any prior co-evolutionary encounters (Tsui et al. 2012; Cale et al. 2019; Erbilgin 2019). Therefore, the assumptions of larger lesions in trees on potentially water stressed sites or higher tree susceptibility due to lower concentrations of defense compounds need to be further validated.

5.6 Management implications

As the MPB invasion of jack pine forest continues, my research addresses several important issues central to the sustainability of forest resources in Canada. These include understanding the dynamics and resilience of jack pine forests, and the magnitude of potential consequences of an MPB invasion. Although studies have shown that MPB has already successfully invaded jack

pine stands, our understanding of factors contributing to the invasion dynamics is far from complete. This research contributes to the field of invasion biology by (1) identifying potentially susceptible stands that could be targeted by managers to mitigate the risk of MPB invasion in order to control or avoid socioeconomic and ecological losses, (2) improving our understanding of the dynamics of primary and secondary plant metabolites in pines and their effects on MPB biology, thus contributing to many interdisciplinary fields of science, (3) understanding the attack dynamics of MPB and its associated fungi with respect to the defense capacity of host plants at landscape level, and (4) investigating the defense related interactions among pine trees at community level. The information gathered through my research clarifies the invasiveness of MPB and the role of resources in determining the susceptibility of jack pine stands.

5.7 Future research recommendations

My research focused on lesion areas or lengths, monoterpenes, diterpene resin acids, and non-structural carbohydrates, however there are many other factors that can be considered for future research. These include anatomical evidence of these interactions (Franceschi et al. 2005; Zhao et al. 2019), fungal spread within the phloem, xylem penetration by the fungus, and responses mediated by other groups of compounds and organisms.

In my research, kinship and relatedness was restricted to the chemical similarities exhibited by the signal receiving pines. However, in order to better understand kin recognition and support in pines, future research needs to focus both on the emitter and the receiver. Since the emitting trees in my research were already dead, they could not be compared to the signal receiving kin for chemical profiling. Therefore, considering kinship based on genotypical resemblance is recommended.

As defense compounds are funded by primary metabolites, kin support can further be verified by understanding the non-structural carbohydrate dynamics of the interacting and not interacting pines.

Finally, the VOC-mediated communication and kin recognition in pines presented in Chapter 4 requires more stringent verification. Due to the volatile nature of the compounds quantified in the study, other environmental factors need to be controlled. These include determining exact wind direction and speed, degree days, relative humidity and precipitation, temperature variations, atmospheric pressure and several other factors influencing these variables (Thistle et al. 2011; Lowman and Schowalter 2012; Zitouna-Chebbi et al. 2015). As a final note, scrutinizing my findings in other coniferous species could be helpful, especially in jack pine and other pine species in eastern North America.

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